

The Role of Erythropoietin and its Receptor in the Immune, Central Nervous and Cardiovascular Systems

Katarzyna Aleksandra Lisowska

Katarzyna Aleksandra Lisowska, Department of Pathophysiology, Medical University of Gdańsk, Dębinki 7, 80-211 Gdańsk, Poland
Correspondence to: Katarzyna Aleksandra Lisowska, Department of Pathophysiology, Medical University of Gdańsk, Dębinki 7, 80-211 Gdańsk, Poland
Email: katlis@gumed.edu.pl
Telephone: +48583491510
Received: March 5, 2015 Revised: April 16, 2015
Accepted: April 21, 2015
Published online: July 6, 2015

ABSTRACT

Primary role of erythropoietin (EPO) is to protect red blood cell progenitors from apoptosis and to stimulate their growth and differentiation by binding to its receptor (EPOR). However, the expression of EPOR has been identified on other human cells, such as granulocytes, monocytes, lymphocytes, endothelial cells and neurons. Its presence on these cells suggests that beyond erythropoietic function EPO might have some other properties. Various studies show that recombinant human erythropoietin (rhEPO) used in the treatment of anemia associated with chronic kidney disease (CKD) has potentially beneficial influence on cellular processes such as proliferation and apoptosis of cells other than erythrocyte progenitors. It can modulate the activity of immune cells, protect neurons from apoptosis and even stimulate angiogenesis, but also it has a pro-thrombotic properties. This review presents current knowledge on the role of EPO and its receptor in the immune, central nervous and cardiovascular systems in various pathophysiological situations.

© 2015 ACT. All rights reserved.

Key words: Erythropoietin, Erythropoietin Receptor, Lymphocytes, Neurons, Endothelial Cells, Apoptosis, Neuroprotection

Lisowska KA. The Role of Erythropoietin and its Receptor in the

Immune, Central Nervous and Cardiovascular Systems. *International Journal of Hematology Research* 2015; 1(2): 42-51 Available from: URL: <http://www.ghrnet.org/index.php/ijhr/article/view/1099>

INTRODUCTION

Interest in erythropoietin (EPO) reaches late 80s, when its recombinant analogue has been approved by the US Food and Drug Administration for the treatment for anemia in patients suffering from chronic kidney disease (CKD). In healthy people, EPO is produced in peritubular capillary epithelial kidney cells under hypoxic conditions^[1]. It is not stored in kidneys and therefore requires activation of gene expression. The sequence preceding the EPO gene promoter is characterized by the presence of DNA sequences which determine its tissue specificity (KIE – kidney inducible element, NRE – negative regulatory element)^[2]. Enhancer sequence responsible for the hypoxia-dependent expression of EPO gene is located at its 3' end where HIF-1 (hypoxia inducible factor 1) and HNF-4 (hepatocyte nuclear factor 4) bind^[2]. HIF-1 is a heterodimer composed of two subunits: HIF-1 α and HIF-1 β ^[3], activity of which is regulated posttranscriptionally^[4]. Under normal oxygen concentration, HIF-1 α subunit is modified by an oxidative α -prolyl hydroxylase, which allows attachment of ubiquitin ligase complex, degradation of HIF-1 α and thereby inhibition of the synthesis of EPO. In hypoxic conditions, the enzyme is inactive, which allows to create complex of HIF-1 α and HIF-1 β that binds to the enhancer sequence and initiates EPO gene transcription^[3,5]. EPO produced in the kidney passes to the blood plasma and then to the bone marrow, where it protects erythroid progenitors from apoptosis and promotes their proliferation and differentiation by binding to its receptor (EPOR) on cells at the BFU-E (erythroid burst-forming unit) and CFU-E (erythroid colony-forming unit) stages of erythropoiesis^[6]. EPO binding to EPOR results in receptor homodimerization followed by an activation of JAK (Janus kinase) family members^[7] and phosphorylation of several signaling proteins, including signal transducer and activator of transcription 5 (STAT5)^[8], phosphatidylinositol 3-kinase (PI-3K)^[9], protein kinase C

(PKC)^[10] and mitogen-activated protein kinase (MAPK)^[11] (Figure 1).

Erythropoietin was isolated from urine of patients with pernicious anemia in 1977^[12]. This step allowed for the cloning of the gene encoding EPO, which was achieved by Lin and Jacobs in 1985^[13,14]. Two years later Eschbach *et al*^[15] presented the results of phase I and II of clinical trials with recombinant human erythropoietin (rhEPO), which showed an increase in hematocrit in patients with end-stage renal disease (ESRD). Principles of treatment of anemia with rhEPO in ESRD patients were defined for the first time in 2000^[16] and, currently, specific recommendations for the use of rhEPO are updated by ERBP (European Renal Best Practice) and NKF DOQI (National Kidney Foundation Dialysis Outcomes Quality Initiative). Since the introduction of the drug, many articles describing other, beyond correction of anemia, positive effects of rhEPO therapy have been published. This was related to, among other things, the discovery of receptor expression in cells other than erythroid progenitors, including polymorphonuclear leukocytes (PMNLs)^[17], monocytes and lymphocytes^[18], endothelial cells^[19,20], neurons^[21,22] and some tumors cells^[23]. Multiple studies suggest that rhEPO/EPO can protect brain tissue from hypoxic damage or prevent ischemia-reperfusion myocardial injury, stimulate angiogenesis and modulate immune responses. However, its exact effect on these cells is not as clear as its role in erythropoiesis.

ROLE OF ERYTHROPOIETIN AND ITS RECEPTOR IN MODULATING IMMUNE RESPONSES

Anemia in ESRD patients is mainly a consequence of kidney damage, although several other factors, including chronic blood loss, iron deficiency and presence of pro-inflammatory cytokines also affects its development. In addition to the disturbances in water and electrolyte balance, acid-base balance, calcium-phosphate or hormonal balance, these patients also present defects in both innate and acquired immunity resulting in an increased susceptibility to infections, which are a frequent cause of death, especially among hemodialyzed (HD) patients^[24]. Studies have shown that the complement system components and immune cells, especially neutrophils and macrophages, are activated when blood flows through the dialyzer, which leads to the release of large amounts of pro-inflammatory cytokines, mainly interleukin (IL-) 1, IL-6 and tumor necrosis factor alpha (TNF- α)^[25]. Moreover, CD4⁺ T lymphocytes from HD patients are characterized by a reduced expression of key surface antigens (especially co-stimulatory CD28 and activation markers: CD69 and CD25), impaired proliferation parameters (decreased percentage of proliferating cells and number of divisions, long period of time required to enter the first phase of cell cycle) and a decreased production of a key cytokine IL-2 in response to stimulation with mitogen^[26,27]. Since the function of B cells largely depends on the activation of CD4 lymphocytes, their impaired activity can support the progression of immune deficiency manifested as the reduced response of HD patients to vaccination against hepatitis B^[28] and frequent infections^[24]. In the late 90's, first publications describing the effect of treatment with rhEPO on immunological parameters in patients on dialysis have been published. Studies showed an increase of IL-2 concentration after stimulation of whole blood cells cultures of HD patients with phytohemagglutinin (PHA) as well as with rhEPO in the same concentration as the physiological concentration of EPO in blood, which is 0.05 U/mL^[29,30]. After few months of rhEPO therapy decreased production of pro-inflammatory cytokines (IL-6 and TNF- α) and elevated secretion of anti-inflammatory IL-10 in whole blood cell cultures stimulated with PHA were also

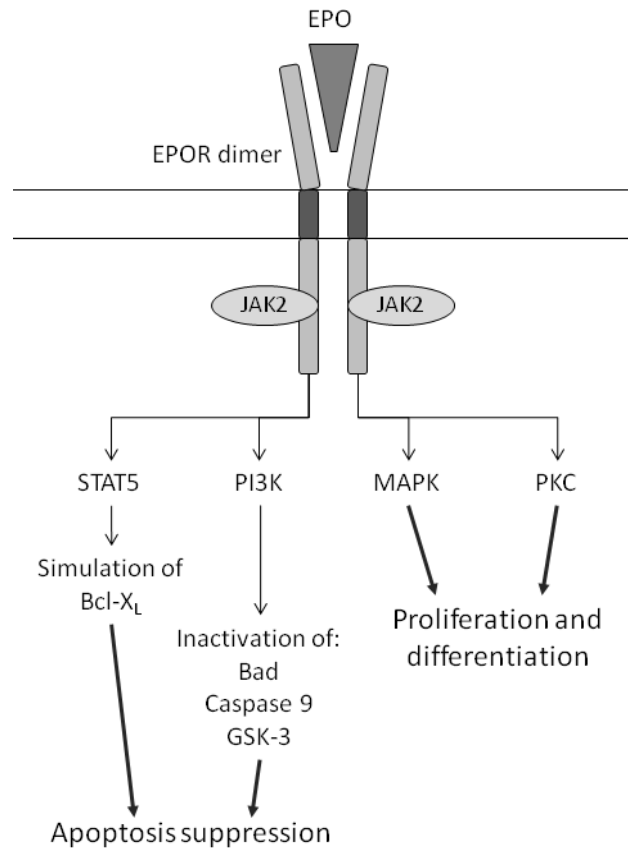


Figure 1 Signaling pathways activated by the binding of EPO to its receptor.

observed^[30,31]. There were also studies showing higher concentration of TNF- α in serum of HD patients receiving rhEPO^[32] but its level was determined after the dialysis procedure, during which the release of large amount of pro-inflammatory cytokines occurs^[25]. Bryl *et al*^[29,31] and Trzonkowski *et al*^[30] demonstrated that rhEPO acts directly on production of cytokines by whole blood cells *in vitro*. It has also been demonstrated that rhEPO directly enhances B lymphocytes antibody (IgG, IgM and IgA) production in serum-free medium^[33]. However, at that time some authors suggested that rhEPO-induced immunomodulation is mainly dependent on general improvement of patients' condition after few months of rhEPO treatment because an elevation of hematological parameters is accompanied by a simultaneous increase in the number naive T lymphocytes^[34].

Expression of EPOR on the surface of granulocytes was first described more than a decade ago pointing towards a possible role of EPO and its receptor in the immune responses. Sela *et al*^[17] and Kristal *et al*^[35] showed that there is a strong positive correlation between the percentage of EPOR-positive cells and the level of EPO in the serum which suggested that presence of hormone up-regulates the expression of receptor. Moreover, following few weeks of rhEPO treatment, in HD patients, the rate of superoxide release from granulocytes fell significantly when compared with the pretreatment values. Authors concluded that rhEPO reduces the extent of inflammation by interaction with granulocytes through the receptor present on their cell surface. Similar results were obtained in patients on continuous ambulatory peritoneal dialysis (CAPD)^[36] and ESRD patients not treated with renal replacement therapy^[37], *in vitro*, incubation of granulocytes from CAPD patients with increasing concentration of rhEPO also resulted in a significant reduction superoxide release.

EPOR belongs to the same family of type I cytokine receptors as IL-2, IL-4, IL-6, IL-7, IL-12, IL-13 or GM-CSF (Figure 2), which transmit signals through JAK-STAT pathway using common signaling chain^[38]. It has been demonstrated that IL-2 as well as rhEPO stimulate STAT5 by inducing phosphorylation of the same tyrosine residue and that these IL-2- and EPO-induced STATs have an identical DNA binding specificity and immunoreactivity^[39]. Some other studies have revealed activation of specific pathway containing STAT5 in EPOR-transfected lymphoid cell line by rhEPO which suggested that lymphocytes could contain a machinery necessary for signal transduction from this receptor^[40]. Therefore, using molecular methods and quantitative flow cytometry, few years ago we tested whether peripheral blood mononuclear cells (PBMCs) express EPOR. Our studies have shown that every PBMC type tested *ex vivo* (CD4⁺ and CD8⁺ T lymphocytes, B lymphocytes and monocytes) demonstrate some basic level of EPOR expression with the CD4⁺ cells presenting the lowest (about 50-100) number of receptor molecules per cell on their surface^[18]. At the same time we observed low percentage of lymphocytes expressing very high number of EPOR reaching about 1500 molecules per cell^[18]. Our further studies have shown that, although the level of EPOR on the surface of CD4⁺ cells is very low, stimulation with an immobilized monoclonal anti-CD3 antibody results in its increase to a value as high as 1000 molecules per cell^[18,41] which is reported to be typical for the most immature erythroid cells^[42]. Our findings were also confirmed by other authors; Spaan *et al.*^[43] showed that human monocytes not only express EPOR mRNA, but also are responsive to rhEPO in a cell culture. *In vitro*, exposure of PBMC from individuals to rhEPO in the presence of lipopolysaccharide (LPS) showed a significant reduction of monocytes producing pro-inflammatory cytokines^[43]. Last year, Cravedi *et al.*^[44] have published results which confirmed that resting CD4⁺ and CD8⁺ cells express low level of EPOR on their surface but its expression is up-regulated after few days of stimulation with anti-CD3 and anti-CD28 antibodies. EPOR up-regulation on T lymphocytes is probably, at least in part, a consequence of an increased EPOR gene expression^[44,45] which seems to be dependent on the expression of specific transcription factors^[45].

This raises the question about the role of the receptor for erythropoiesis-stimulating hormone in the cells of the immune system. Various studies show that the presence of rhEPO in the

culture of mononuclear cells modulates the levels of pro- and anti-inflammatory cytokines produced by either monocytes or lymphocytes^[29,31,46]. It seems that rhEPO itself does not influence T lymphocytes proliferation, at least not directly^[34]. However, according to Cravedi *et al.*^[44], rhEPO inhibits expansion and proliferation of T lymphocytes by uncoupling IL-2R signaling *in vitro*. In their studies, rhEPO inhibited phosphorylation of PI-3K-AKT signaling pathway without affecting STAT5, while our studies have shown that rhEPO in low doses stimulates phosphorylation of STAT5 depending on stimulation through T cell receptor (TCR)/CD3 complex^[41]. Different results seem to be largely dependent on the doses of rhEPO used *in vitro* – we used a concentrations similar to the physiological concentration of EPO in blood serum (0.1, 0.5 U/mL), while Cravedi *et al.* used a very high doses (1,000-2,000 U/mL). This shows how the experimental design can affect the results of research. On the other hand, observation that rhEPO is able to inhibit expansion of T lymphocytes in response to polyclonal stimulation would explain its protective effects in kidney transplant recipients^[47]. This may be one of the factors, in addition to correction of hemoglobin levels during rhEPO therapy, that support prolonged graft survival and slow down the progression of chronic graft nephropathy. Experiments performed using animal model showed that treatment with rhEPO, not the normalization of post-transplant hemoglobin levels by blood transfusions, prevents chronic renal allograft injury^[48].

In HD patients, rhEPO therapy lasting for at least several months not only corrects the anemia state but also normalizes activation phenotype of CD4⁺ T lymphocytes and their proliferative capacity^[26]. It is associated, among other things, with the increased expression of CD28 and CD69 antigens on lymphocytes' surface^[26]. Surface antigens not only are involved in maintaining contact between T lymphocytes and other cells, including B cells (for example, through CD40L antigen) or antigen presenting cells (APCs), but also influence proliferation and cytokine production. CD28, for example, regulates IL-2 production on the transcriptional level^[49]; its lower expression on CD4⁺ cells would explain reduced levels of IL-2 in response to mitogenic stimulation in HD patients^[29,30]. At the same time, expression of antigens depends on many factors, including the level of various cytokines secreted by lymphocytes themselves and other cells in their area. A good example is the relationship between the level of CD28 expression on T lymphocytes and the level of

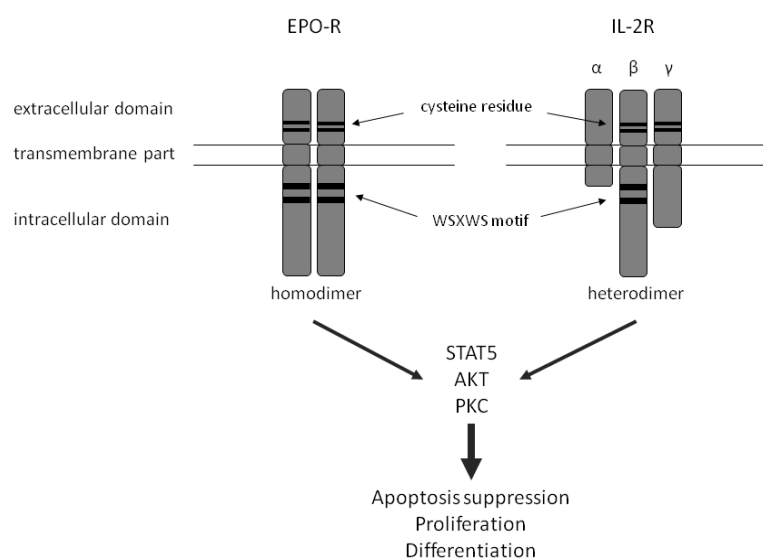


Figure 2 Structure of the EPO and IL-2 receptors.

TNF- α , which was shown to regulate transcription of CD28 gene directly^[50]. Chronically high levels of TNF in HD patients could therefore reduce the level of CD28 on CD4⁺ cells, which in turn would lead to a reduction in the concentration of IL-2. Our studies demonstrated that *in vitro* incubation of activated PBMCs of HD patients with rhEPO in a concentration similar to the physiological (0.1 U/mL) decreased the level of TNF- α while increasing the levels of IFN- γ and IL-10^[46]. However, similar experiment carried out in a group of healthy people showed no changes in Th1 or Th2 cytokine levels when rhEPO was added to cell culture. It means that effect of rhEPO is dependent on the initial concentration of cytokines in an individual; the level of TNF- α was decreased while the level of IL-10 and IFN- γ was increased in healthy people compared to HD patients^[46]. In our opinion, long-term rhEPO administration could regulate the functioning of immune cells of ESRD patients on chronic dialysis (Figure 3). Although the mechanism of action of rhEPO on T lymphocytes is still unclear, it seems that their improved (normalized) phenotype and proliferation capacity in HD patients depends mainly on changes in the concentrations of pro- and anti-inflammatory cytokines, which can be regulated by the presence of relevant concentrations of EPO in the serum. Our own studies^[18,39] and results of Cravedi *et al*^[44] have shown that the presence of rhEPO may affect the activity of various signaling molecules (e.g., JAK2, AKT or STAT5) in human T lymphocytes stimulated through the CD3/TCR complex in a dose dependent manner and its role in the modulation of T cell activity is probably also related to the number of EPOR molecules on their surface. However, further studies are required in order to explore the mechanisms of the impact of EPO on T lymphocytes.

ERYTHROPOIETIN AND ITS RECEPTOR IN THE CARDIOVASCULAR SYSTEM

Much more is known about potential role of erythropoietin in human cardiovascular system. Many studies show that administration of rhEPO may have an important impact on the development of hypertension by increasing viscosity, vasoconstriction and enhancing vascular reactivity. However, at the same time it seems that EPO/rhEPO can protect the myocardium against ischemia-induced damage.

The relationship between the endothelium and hematopoiesis has been known since the 90s, when it was shown that endothelial cells produce proteins which support growth and differentiation of BFU-E and CFU-E colonies^[51]. In 1990, Anagnostou *et al*^[20] showed that endothelial cells have on their surface about 30000 molecules of EPOR per cell. Studies of EPOR expression on erythroid progenitors and activated T lymphocytes have shown only a small number of surface receptors (usually about 1,000 molecules per cell). However, different authors speculated that receptors present on erythroid progenitor cells have high affinity for EPO which is sufficient for its differentiating effect on these cells despite the small number of receptor molecules on their surface^[52,53]. Anagnostou *et al*^[20] showed that endothelial cells express a high number of relatively low-affinity receptors and demonstrated that rhEPO at the concentration of 5 to 20 U/mL enhances the migration of endothelial cells and stimulates their proliferation (Table 1).

Studies performed in patients with advanced renal failure and in healthy individuals have also shown that rhEPO can regulate proliferation and differentiation of endothelial progenitor cells (EPCs)^[54,55]. These authors demonstrated that the number of circulating peripheral blood hematopoietic progenitor cells increases,

especially at the beginning of rhEPO treatment with a standard therapeutic dose^[54]. *In vitro*, the effect of rhEPO on EPCs was dose-dependent and led to the activation of the AKT signaling pathway and enhanced formation of tube-like structures^[54,55]. George *et al*^[56] partly confirmed their observations examining the effect of chronic treatment with rhEPO on the numbers and functional properties of EPCs in patients with congestive heart failure (CHF). Although they did not demonstrate changes in the number of circulating EPCs, they showed that adhesive and proliferative properties of EPCs are enhanced during rhEPO treatment. Also, the presence of rhEPO in cell culture increases proliferation of EPCs in a dose-dependent manner^[56]. These effects suggested that EPO or its recombinant analogue can regulate the process of vascular repair and neoangiogenesis by mobilizing EPCs. At the same time, it has been shown that rhEPO stimulates the production of endothelin 1 (ET-1)^[57] and plasminogen activator inhibitor-1 (PAI-1)^[58], both involved in the increasing of vascular resistance, which is one of the factors contributing to the development of hypertension in CKD patients. Moreover, rhEPO also directly suppresses activity of endothelial nitric oxide synthase (eNOS), a primary controller of smooth muscle tone^[59]. It is very important information, since the hematocrit value increased during rhEPO therapy is not itself associated with an increased risk for hypertension as long as endothelial NOS production and the level of nitric oxide (NO) are adequate.

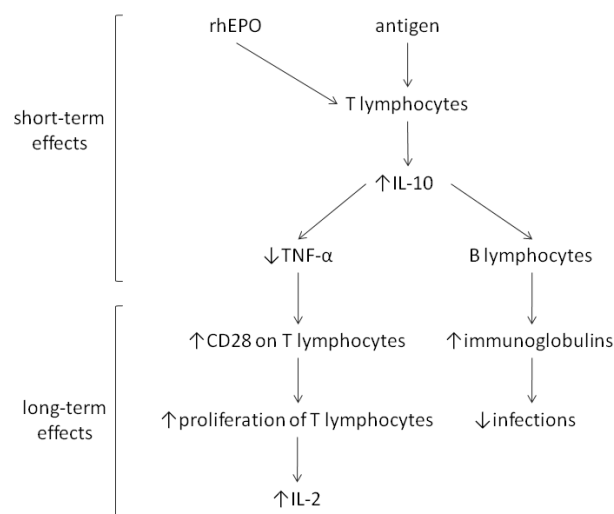


Figure 3 Influence of rhEPO treatment on immune responses.

Table 1 The effects of EPO on cellular components of the cardiovascular system.

Cells	Effects
Endothelial cells	Increased number of endothelial progenitor cells (EPCs)
	Increased endothelin-1 (ET-1) and plasminogen activator inhibitor-1 (PAI-1) production
	Decreased nitric oxide synthase (NOS) production
	Mitogenic, chemotactic and angiogenic stimulation
Smooth muscle cells	Increased cytosolic Ca ²⁺ concentration
	Increased response to norepinephrine, angiotensin II and ET-1
	Mitogenic effect
	Stimulation of Na ⁺ K ⁺ ATPase activity
Cardiomyocytes	Increased vascular endothelial growth factor (VEGF) production
	Mitogenic effect

So far, at least eight genes has been shown to be up-regulated by rhEPO in endothelial cells which included proteins implicated in the regulation of vascular functions (thrombospondin-1 and myosin regulatory light chain), gene products involved in gene transcription and/or translation (c-myc purine-binding transcription factor PuF, tryptophanyl-tRNA synthetase and S19 ribosomal protein), subunits of mitochondrial enzymes related to energy transfer (NADH dehydrogenase subunit 6 and cytochrome C oxidase subunit 1) and regulators of signal transduction (protein tyrosine phosphatase G1)^[60].

EPO has also an impact on vascular smooth muscle cells, as demonstrated for animal models. It has been shown that rhEPO alone or in the company of norepinephrine, angiotensin II and ET-1 raises the cytosolic concentration of calcium by stimulating PKC and MAPK signaling pathways^[61,62]. However, studies in renal failure patients confirmed that rhEPO therapy enhances vascular responsiveness to norepinephrine, which explains development of hypertension during treatment^[63]. Although there are no studies confirming the presence of EPOR on human smooth muscle cells, its presence has been demonstrated in human skeletal muscle cells^[64]. Rundqvist *et al*^[64] have shown that both satellite cells and skeletal muscle fibers express the receptor. Expression of EPOR in these cells increases after exercises simultaneously with the expression of vascular endothelial growth factor (VEGF) A, which would support the thesis that EPO could influence process of angiogenesis^[54,55,64]. Authors also suggested that another reason for EPOR activation in skeletal muscle would be protection against apoptosis induced by reversible ischemia.

As in the case of smooth muscle cells, numerous articles described the effect of EPO/rhEPO on cardiac myocytes using animal models. *In vitro* studies demonstrated activation of multiple signaling pathways in rhEPO-induced cardioprotection, including JAK1/2, STAT3 and STAT5A, PI-3K and their downstream kinases, AKT, PKC and MAP^[65,66]. Moreover, Shi *et al*^[66] showed that rhEPO mediates resistance to myocardial ischemia not only by PKC and MAP kinases, but also by activation of two potassium (KATP) channels and the calcium-activated potassium (KCa) channel. Studies of Parsa *et al*^[67] revealed that rhEPO treatment can protect the ischemic and infarcted heart by inhibition of apoptosis of cardiac myocytes and augmented cardiac contractility and relaxation, even when rhEPO was given at the time of myocardial infarction. Westenbrink *et al*^[68] demonstrated that rhEPO promotes VEGF expression predominantly in cardiomyocytes through STAT3 pathway, which in turn stimulates myocardial endothelial proliferation and incorporation of EPCs. According to their results, the direct effects of rhEPO on endothelial sprouting is VEGF independent. Cardioprotective effects of rhEPO have also been demonstrated in experimental autoimmune myocarditis (EAM)^[69]. The expression of EPOR was up-regulated in the myocardium of EAM and rhEPO reduced the level of pro-inflammatory cytokines (IL-6 and TNF- α) and apoptotic cardiomyocytes. These studies show that rhEPO can reduce myocardial inflammation and heart failure, at least in part, by modulating the inflammatory cytokine profiles and protecting myocytes from apoptosis.

Anemia is a risk factor for increased mortality in patients with congestive heart failure. Tissue hypoxia worsens the natural history of CHF by causing systemic vasodilatation and reduction of arterial and perfusion process, which leads to reflex vasoconstriction and tachycardia through activation of sympathetic nervous system and activation of renin-angiotensin system (RAS) leading to sodium and water retention. The increased plasma volume contributes to peripheral edema and increases cardiac load resulting in progressive

heart remodeling and left ventricular hypertrophy (LVH). In dialysis patients, anemia is also associated with increased frequency of LVH, mainly due to activation of RAS. In both groups of patients rhEPO treatment not only increases hemoglobin level, but also enhances cardiac output, reduces sympathetic activation, improves coronary circulation and induces LVH regression^[70-73]. Silverberg *et al*^[74] draws attention to the need for close cooperation between nephrologists, cardiologists and internists in recognizing and treating the anemia, which would allow prevent the deterioration of the CHF, chronic kidney disease and the anemia itself. At the same time, one must be aware of the other effects of the use of rhEPO, mainly an increased blood viscosity as a result of increased red blood cell mass but also intensified production of ET-1^[57] and PAI-1^[58] in endothelial cells and raised calcium concentrations in vascular smooth muscle cells^[61-63], since all these mechanisms promote an increased risk of thrombotic events.

ERYTHROPOIETIN AND ITS RECEPTOR AS POTENTIAL NEUROPROTECTORS

In 1997, the EPO has been detected in ventricular cerebrospinal fluid (CSF) of patients with traumatic brain injuries^[75]. These studies also revealed that EPO does not cross the intact blood-brain barrier (BBB), implying that it is produced in the brain itself, probably in an oxygen-dependent manner. Springborg *et al*^[76] not only have confirmed these observations in patients with subarachnoid haemorrhage 5 years later, but also established the EPO concentration in CSF which was about 1 mU/mL.

Both EPO and EPOR has been identified in undifferentiated neuroepithelial cells in human embryos; their expression pattern showed a specific distribution changing during fetal development^[77]. In adults, EPOR expression is found mainly in neurons, astrocytes and microglia, while EPO is produced only by astrocytes^[78]. Furthermore, presence of pro-inflammatory cytokines (IL-1, IL-6 and TNF- α) decreased expression of EPO and its receptor in astrocytes^[78]. In the same way pro-inflammatory cytokines suppress renal EPO production and thereby erythropoiesis in patients with cancer or chronic inflammatory diseases, which leads to development of pro-inflammatory cytokine-mediated anemia^[79]. It has also been shown that TNF- α down-regulates EPOR expression in erythroid cell lines probably through decreasing expression and binding activity of GATA1^[80], which is known to be one of the main transcription factors regulating the expression of the EPOR gene in erythroid cells^[81]. Pro-inflammatory cytokines have been shown to be involved very early in the course of cerebral palsy (CP); they may cause brain damage through promoting toxic substances, like free radicals, increasing activity and aggregation of platelet and activating blood coagulation, or destroying BBB^[82-84]. It has been demonstrated that the amount of EPO was decreased while IL-6 and TNF- α was simultaneously increased in CP children^[85]. According to Tao *et al*^[85], although CP children have BBB injury, the long-time secretion of EPO in CNS mostly depends on brain tissue. Authors hypothesized that early in CP HIF-1 activated by inflammatory stimulation enhances the secretion of EPO in the brain. However, deepening of hypoxia or increased severity of infection induces the release of many pro-inflammatory cytokines which compete with EPO through the JAK/STAT pathway and consequently abolish its neuroprotective effect^[85].

As in the kidneys, the expression of EPO mRNA and the production of EPO in the central nervous system (CNS) are stimulated by hypoxia and ischemia^[86,87]. According to Chin *et al*^[88], the neuroprotective effect of EPO and its receptor requires the

induction of EPO production by hypoxia and an increase in EPOR expression in neurons resulting in increased sensitivity to EPO, which is in contrast to erythropoiesis, where erythroid progenitor cells already exhibit high levels of EPOR and are directly responsive to EPO stimulation in hypoxic conditions. Their studies have also shown that hypoxia induces EPOR expression, which appears to be a consequence of increased EPOR gene transcription from the upstream region and proximal promoter^[88].

As in the case of vascular smooth muscle cells or cardiac myocytes, knowledge of the potential functions of EPO in the brain is based on animal models and the available data on the action of the hormone on erythropoiesis progenitors. Several direct-acting mechanisms, by which EPO may protect neurons, has been proposed, including modulation of synaptic transmission, protection from oxidative stress, apoptosis and inflammatory factors or stimulation of angiogenesis (Figure 4). It is generally believed that EPO in nervous cells activates exactly the same signaling pathways as in the erythroid progenitors. Various experimental models have demonstrated that EPO regulates many neural cell functions, including regulation of the flow of calcium, membrane depolarization or neurotransmitter synthesis. Koshimura *et al*^[89] showed that rhEPO increases uptake and intracellular calcium concentration in a dose-related manner, induces membrane depolarization, activates MAPK and stimulates dopamine release from neurons. Although rhEPO had no effect on maximal activity of NOS, the increase in the level of NO was also observed. Since NO is reported to stimulate release of different neurotransmitters^[90], authors suggested that EPO stimulates dopamine release, partly, by increasing NO production. Other studies have shown that rhEPO inhibits calcium-dependent glutamate release from neurons and at the same time it increases desensitization of glutamate receptors thereby protecting cells from ischemic damage^[91-93]. However, Yamasaki *et al*^[93] determined, by comparing the effects of rhEPO and brain-derived neurotrophic factor (BDNF), that the rhEPO itself is not sufficient to promote cell survival, although it is useful in protecting neurons from glutamate- or NO-induced toxicity. On the other hand, studies of Chong *et al*^[94] showed that, although endogenous EPO is not sufficient to maintain neuron survival during free radical injury, administration of rhEPO at the concentration of 1 U/mL prevents neuronal cell death by activation of AKT with subsequent phosphorylation of the Bcl-2 family pro-apoptotic

member Bad. Additionally, the presence of rhEPO in neuronal cell culture protects neurons from DNA fragmentation and membrane phosphatidylserine (PS) exposure through the modulation of cysteine protease activities. Authors also demonstrated that rhEPO prevents the induction of apoptotic protease-activating factor-1 (Apaf-1) and enhances the expression of anti-apoptotic Bcl-xL^[95]. Anti-apoptotic properties of erythropoietin in nervous central system has also been demonstrated by Um *et al*^[96] who showed that rhEPO prevents apoptosis of differentiated human neuroblastoma cells expressing small numbers of high-affinity EPOR by activating the STAT5, AKT and MAPK signaling pathways.

As mentioned above, pro-inflammatory cytokines decrease EPO and its receptor expression in nervous tissue, specifically in astrocytes^[78]. However, EPO itself can also interfere with the inflammatory process by reducing the expression of pro-inflammatory cytokines, such as IL-6 and TNF- α in neuronal injury. Villa *et al*^[97] have demonstrated that rhEPO markedly reduces the recruitment of leukocytes and microglia to the nerve tissue deprived of oxygen supply. The production of the TNF- α , IL-6 and monocyte chemoattractant protein (MCP) was decreased by more than 50% after rhEPO administration. Similar results were obtained in the experimental autoimmune encephalomyelitis (EAE); rhEPO not only diminished inflammation by reducing the level of IL-6, and glial activation and proliferation in the spinal cord, but also delayed the onset of EAE^[98].

Indirectly, EPO may act in neuroprotective manner by stimulating angiogenesis which increases the transport capability of oxygen-carrying blood, provides extra oxygen to brain and counteracts the effect of ischemia on neurons^[99]. This is related to the above mentioned presence of EPOR on endothelial cells. Brain endothelial cells not only express receptor but also proliferate, migrate and differentiate in the presence of rhEPO^[99,100]. Sirén *et al*^[87] demonstrated that hypoxic brain damage is associated with vascular EPOR expression accompanied by increased expression of EPO and its receptor in astrocytes. Authors showed that administration of rhEPO dramatically reduces the volume of infarction through the inhibition of apoptosis of cells in the tissue directly adjacent to the site damaged by cerebral occlusion causing hypoxic injury^[101]. Other studies have shown that rhEPO antagonizes VEGF-induced permeability of the BBB by restoring changes in the distribution

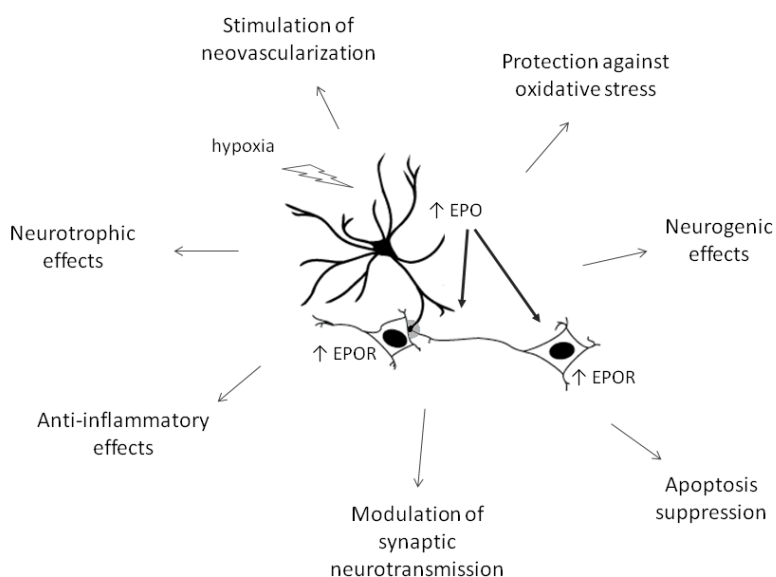


Figure 4 The possible EPO-dependent mechanisms of neuroprotection.

pattern of inter-endothelial junction proteins and decreases the levels of eNOS^[102].

The above-described effects of EPO on brain tissue suggest a potential use of rhEPO as a neuroprotective agent in a variety of disorders of the central nervous system, especially in chronic neurodegenerative disorders like Alzheimer disease (AD), Parkinson's disease (PD) or multiple sclerosis. Epidemiological studies in USA have shown that AD is less common in states with higher geographic altitude suggesting that individual level of EPO can be of importance in the development of the disease^[103]. Indeed, Assaraf *et al*^[104] showed that the expression of EPOR is significantly increased in human AD and mild cognitive impairment temporal cortex compared to no cognitive impairment brain tissue. The number of EPOR-positive astrocytes in temporal and hippocampal regions was correlated with clinical, neuropsychologic, and neuropathologic indices^[104]. In the authors' opinion, the high expression of the receptor in astrocytes is probably an adaptive response to oxidative stress that reaches its maximum intensity in preclinical and the very earliest stages of sporadic AD. In 2012, Zhi-Kun *et al*^[105] demonstrated that rhEPO can protect cells from apoptosis induced by neurotoxic decapeptide A β 25–35 which was associated with the activation of PI-3K-AKT signaling pathway. On the other hand, studies of Jang *et al*^[106] revealed that rhEPO administration for 12 months has beneficial effects on non-motor symptoms in PD patients according to the NMS Scale (NMSS) and the 39-Item Parkinson's Disease Questionnaire (PDQ-39). Since oxidative stress, apoptosis, excitotoxicity, protein mishandling, inflammation and microglial activation are part of the processes that eventually result in neurodegeneration, it is possible that rhEPO could provide a useful therapeutic strategy for these diseases.

CONCLUSIONS

The use of rhEPO significantly improved the health and quality of life of ESRD patients. This is related not only to the improvement of parameters of red blood cells, but probably also the potential impact of rhEPO on other cells and tissues, especially monocytes, lymphocytes, endothelial cells and neurons. Studies show that it has potentially beneficial influence on cellular processes such as proliferation or angiogenesis. However, one must carefully draw conclusions from the research results that can be found in the scientific literature because not all of the observations have been confirmed in human material. While the effects of EPO on the immune system in many cases have been reported in human immune cells and have been conducted mainly in patients treated with rhEPO, knowledge of hormone action on the central nervous or cardiovascular system is based largely on animal models. In 2012, Elliott *et al*^[107] demonstrated that only erythroid cells have high levels of EPOR mRNA and protein while in nonhematopoietic tissues (like brain or heart) and other hematopoietic cell types its expression is undetectable. In addition, they have failed to demonstrate the effect of rhEPO on angiogenesis or survival of different cell types^[107]. The authors drew attention to the fact that the cytoprotective or angiogenic properties of rhEPO observed in animal studies does not necessarily translate into the clinic. In fact, the promising results from some experiments conducted in animal models of stroke led to clinical trials that had failed in phase II/III because patients demonstrated many different complications, including intercerebral hemorrhage, brain edema or thrombotic events^[108]. Also, a higher death rate was observed in patients receiving EPO, particularly in those who were pretreated with thrombolysis^[108]. Similarly concerns

apply to studies on the effect of rhEPO/EPO in the immune system; significant differences between mouse and human immunology have been described for years^[109]. However, in light of so many studies, the effect of EPO on cells other than erythroid progenitors cannot be underestimated. But surely, the potential role of EPO beyond erythropoiesis requires a well-planned experiments on human material. Until then, rhEPO will be still indicated for treatment of anemia in patients with chronic kidney disease.

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

REFERENCES

- Schuster SJ, Badiavas EV, Costa-Giomi P, Weinmann R, Erslev AJ, Caro J. Stimulation of erythropoietin gene transcription during hypoxia and cobalt exposure. *Blood* 1998; 73: 13-16.
- Lacombe C, Mayeux P. Biology of erythropoietin. *Haematologica* 1998; 83: 724-732.
- Wang GL, Semenza GL. Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J Biol Chem* 1993; 268: 21513-21518.
- Kallio PJ, Pongratz I, Gradin K, McGuire J, Poellinger L. Activation of hypoxia-inducible factor 1 alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc Natl Acad Sci USA* 1997; 94: 5667-5672.
- Salceda S, Caro J. Hypoxia-inducible factor 1 alpha (HIF-1 alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 1997; 272: 22642-22647.
- Fisher JW. Erythropoietin: physiologic and pharmacologic update. *Exp Biol Med* 2003; 228:1-14.
- Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* 1993; 74: 227-236.
- Wakao H, Harada N, Kitamura T, Mui AL, Miyajima A. Interleukin 2 and erythropoietin activate STAT5/MGF via distinct pathways. *EMBO J* 1995; 14: 2527-2535.
- Damen JE, Mui ALF, Puil L, Pawson T, Krystal G. Phosphatidylinositol 3-kinase associates, via its Src homology 2 domains, with the activated erythropoietin receptor. *Blood* 1993; 81: 3204-3210.
- Myklebust JH, Smeland EB, Josefsen D, Sioud M. Protein kinase C-alpha isoform is involved in erythropoietin-induced erythroid differentiation of CD34(+) progenitor cells from human bone marrow. *Blood* 2000; 95: 510-518.
- Sui X, Krantz SB, You M, Zhao Z. Synergistic activation of MAP kinase (ERK1/2) by erythropoietin and stem cell factor is essential for expanded erythropoiesis. *Blood* 1998; 92: 1142-1149.
- Miyake T, Kung CKH, Goldwasser E. Purification of human erythropoietin. *J Biol Chem* 1977; 252: 5558-5564.
- Jacobs K, Shoemaker C, Rudersdorf R, Neill SD, Kaufman RJ, Mufson A, Seehra J, Jones SS, Hewick R, Fritsch EF. Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature* 1985; 313: 806-810.
- Lin FK, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, Chen KK, Fox GM, Martin F, Stabinsky Z, Badrawi SM, Lai PH, Goldwasser E. Cloning and expression of the human erythropoietin gene. *Proc Natl Acad Sci USA* 1985; 82: 7580-7584.
- Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. *N Engl J Med* 1987; 316: 73-78.
- Eschbach JW. Current concepts of anemia management in chronic renal failure: impact of NKF-DOQI. *Semin Nephrol* 2000; 20:

- 320-329.
17. Sela S, Shurtz-Swirski R, Sharon R, Manaster J, Chezar J, Shkolnik G, Shapiro G, Shasha SM, Merchav S, Kristal B. The polymorphonuclear leukocyte – a new target for erythropoietin. *Nephron* 2001; 88: 205-210.
 18. Lisowska KA, Debska-Slizien A, Bryl E, Rutkowski B, Witkowski JM. Erythropoietin receptor is expressed on human peripheral blood T and B lymphocytes and monocytes and is modulated by recombinant human erythropoietin treatment. *Artif Organs* 2010; 348: 654-662.
 19. Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, Kesseimian N, Noguchi CT. Erythropoietin receptor mRNA expression in human endothelial cells. *Proc Natl Acad Sci USA* 1994; 91: 3974-3978.
 20. Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M. Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. *Proc Natl Acad Sci USA* 1990; 87: 5978-5982.
 21. Chin K, Yu X, Beleslin-Cokic B, Liu C, Shen K, Mohrenweiser HW, Noguchi CT. Production and processing of erythropoietin receptor transcripts in brain. *Mol Brain Res* 2000; 81: 29-42.
 22. Nagai A, Nakagawa E, Choi HB, Hatori K, Kobayashi S, Kim SU. Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture. *J Neuropathol Exp Neurol* 2001; 60: 386-392.
 23. Farrell F, Lee A. The erythropoietin receptor and its expression in tumor cells and other tissues. *The Oncologist* 2004; 9: 18-30.
 24. Berman SJ. Infections in patients with end-stage renal disease. An overview. *Infect Dis Clin North Am* 2001; 15: 709-720.
 25. Rysz J, Banach M, Cialkowska-Rysz A, Stolarek R, Barylski M, Drozd J, Okonski P. Blood serum levels of IL-2, IL-6, IL-8, TNF-alpha and IL-1beta in patients on maintenance hemodialysis. *Cell Mol Immunol* 2006; 3: 151-154.
 26. Lisowska KA, Dębska-Ślizien A, Radzka M, Witkowski JM, Rutkowski B, Bryl E. Recombinant human erythropoietin treatment of chronic renal failure patients normalizes altered phenotype and proliferation of CD4-positive T lymphocytes. *Artif Organs* 2010; 34: e77-e84.
 27. Lisowska KA, Dębska-Ślizien A, Jasiulewicz A, Heleniak Z, Bryl E, Witkowski JM. Hemodialysis affects phenotype and proliferation of CD4-positive T lymphocytes. *J Clin Immunol* 2012; 1: 189-200.
 28. Allegra V, Vasile A, Maschio M, Mengozzi G. Immune response after vaccination with recombinant hepatitis surface antigen in maintenance hemodialysis patients and healthy controls. *Nephron* 1992; 61: 339-340.
 29. Bryl E, Myśliwska J, Dębska-Ślizien A, Trzonkowski P, Rachoń D, Bułło B, Zdrojewski Z, Myśliwski A, Rutkowski B. Recombinant human erythropoietin stimulates production of interleukin 2 by whole blood cell cultures of hemodialysis patients. *Artif Organs* 1999; 23: 809-816.
 30. Trzonkowski P, Myśliwska J, Dębska-Ślizien A, Bryl E, Rachoń D, Myśliwski A, Rutkowski B. Long-term therapy with recombinant human erythropoietin decreases percentage of CD152(+) lymphocytes in primary glomerulonephritis haemodialysis patients. *Nephrol Dial Transplant* 2002; 17: 1070-1080.
 31. Bryl E, Myśliwska J, Dębska-Ślizien A, Rachoń D, Bułło B, Lizakowski S, Myśliwski A, Rutkowski B. The influence of recombinant human erythropoietin on tumor necrosis factor α and interleukin 10 production by whole blood cell cultures of hemodialysis patients. *Artif Organs* 1998; 22: 177-184.
 32. William J, Saad N, Salib M, Riad H, Mahran KS, Iskander I, Cozma G, Barsoum R. The acute effect of intravenously administered recombinant human erythropoietin on the immune response of uremic patients maintained on regular hemodialysis. *Artif Organs* 1998; 22: 192-196.
 33. Kimata H, Yoshida A, Ishioka C, Masuda S, Sasaki R, Mikawa H. Human recombinant erythropoietin directly stimulates B cells immunoglobulin production and proliferation in serum-free medium. *Clin Exp Immunol* 1991; 85: 151-156.
 34. Ueki Y, Nagata M, Miyake S, Tominaga Y. Lymphocyte subsets in hemodialysis patients treated with recombinant human erythropoietin. *J Clin Immunol* 1993; 13: 279-287.
 35. Kristal B, Shurtz-Swirski R, Shasha SM, Manaster J, Shapiro G, Furmanov M, Hassan K, Weissman I, Sela S. Interaction between erythropoietin and peripheral polymorphonuclear leukocytes in hemodialysis patients. *Nephron* 1999; 81: 406-413.
 36. Shurtz-Swirski R, Kristal B, Shasha SM, Shapiro G, Geron R, Sela S. Interaction between erythropoietin and peripheral polymorphonuclear leukocytes in continuous ambulatory dialysis patients. *Nephron* 2002; 91: 759-761.
 37. Kristal B, Shurtz-Swirski R, Tanhilevski O, Shapiro G, Shkolnik G, Chezar J, Snitkovsky T, Cohen-Mazor M, Sela S. Epoetin-alpha: preserving kidney function via attenuation of polymorphonuclear leukocyte priming. *Isr Med Assoc J* 2008; 10: 266-272.
 38. Lee S, Margolin K. Cytokines in cancer immunotherapy. *Cancers* 2011; 3: 3856-3893.
 39. Wakao H, Harada N, Kitamura T, Mui AL, Miyajima A. Interleukin 2 and erythropoietin activate STAT5/MGF via distinct pathways. *EMBO J* 1995; 14: 2527-2535.
 40. Pallard C, Goulleux F, Charon M, Groner B, Gisselbrecht S, Dusanter-Fourt I. Interleukin-3, erythropoietin and prolactin activate a STAT-5-like factor in lymphoid cells. *J Biol Chem* 1995; 270: 15942-15945.
 41. Lisowska KA, Debska-Slizien A, Jasiulewicz A, Józwick A, Rutkowski B, Bryl E, Witkowski JM. Flow cytometric analysis of STAT5 phosphorylation and CD95 expression in CD4+ T lymphocytes treated with recombinant human erythropoietin. *J Recept Signal Transduct Res* 2011; 31: 241-246.
 42. Shinjo K, Takeshita A, Higuchi M, Ohnishi K, Ohno R. Erythropoietin receptor expression on human bone marrow erythroid precursor cells by a newly-devised quantitative flow-cytometric assay. *Br J Haematol* 1997; 96: 551-558.
 43. Spaan M, Groothuismink ZM, Koning L, Roomer R, Janssen HL, De Knegt RJ, Boonstra A. Erythropoietin administration suppresses human monocyte function *in vitro* and during therapy-induced anemia in HCV patients. *Antiviral Res* 2013; 98: 469-475.
 44. Cravedi P, Manrique J, Hanlon KE, Reid-Adam J, Brody J, Prathuangasuk P, Mehrotra A, Heeger PS. Immunosuppressive effects of erythropoietin on human alloreactive T cells. *J Am Soc Nephrol* 2014; 25: 2003-2015.
 45. Lisowska KA, Frackowiak JE, Mikosik A, Witkowski JM: Changes in the expression of transcription factors involved in modulating the expression of EPO-R in activated human CD4-positive lymphocytes. *PLoS One* 2013; 8: e60326.
 46. Lisowska KA, Debska-Slizien A, Jasiulewicz A, Daca A, Bryl E, Witkowski JM. The influence of recombinant human erythropoietin on apoptosis and cytokine production of CD4+ lymphocytes from hemodialyzed patients. *J Clin Immunol* 2013; 33: 661-665.
 47. Choukroun G, Kamar N, Dussol B, Etienne I, Cassuto-Viguier E, Toupance O, Glowacki F, Moulin B, Lebranchu Y, Touchard G, Jaureguy M, Pallet N, Le Meur Y, Rostaing L, Martinez F, CAPRIT Study Investigators. Correction of postkidney transplant anemia reduces progression of allograft nephropathy. *J Am Soc Nephrol* 2012; 23: 360-368.
 48. Cassis P, Gallon L, Benigni A, Mister M, Pezzotta A, Solini S, Gagliardini E, Cugini D, Abbate M, Aiello S, Rocchetta F, Scudeletti P, Perico N, Noris M, Remuzzi G. Erythropoietin, but not the correction of anemia alone, protects from chronic kidney allograft injury. *Kidney Int* 2012; 81: 903-918.
 49. Shapiro VS, Truitt K, Imboden JB, Weiss A. CD28 mediates transcriptional upregulation of the interleukin-2 (IL-2) promoter through a composite element containing the CD28RE and NF-IL-2B AP-1 sites. *Mol Cell Biol* 1997; 17: 4501-4508.
 50. Bryl E, Vallejo AN, Weyand CM, Goronzy JJ. Down-regulation of CD28 expression by TNF-alpha. *J Immunol* 2001; 167: 3231-

- 3238.
51. Ascensao JL, Vercellotti GM, Jacob HS, Zanjani ED. Role of endothelial cells in human hematopoiesis: modulation of mixed colony growth *in vitro*. *Blood* 1984; 63: 553-558.
 52. Graber SE, Krantz SB. Erythropoietin: biology and clinical use. *Hematol Oncol Clin North Am* 1989; 3: 369-400.
 53. Sawyer ST, Krantz SB, Sawada K. Receptors for erythropoietin in mouse and human erythroid cells and placenta. *Blood* 1989; 74: 103-109.
 54. Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T, Boehm SM, Menne J, Haller H, Fliser D. Erythropoietin regulates endothelial progenitor cells. *Blood* 2004; 103: 921-926.
 55. Bahlmann FH, DeGroot K, Duckert T, Niemczyk E, Bahlmann E, Boehm SM, Haller H, Fliser D. Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin. *Kidney Int* 2003; 64: 1648-1652.
 56. George J, Goldstein E, Abashidze A, Wexler D, Hamed S, Shmilovich H, Deutsch V, Miller H, Keren G, Roth A. Erythropoietin promotes endothelial progenitor cell proliferative and adhesive properties in a PI 3-kinase-dependent manner. *Cardiovasc Res* 2005; 68: 299-306.
 57. Carlini RG, Gupta A, Liapis H, Rothstein M. Endothelin-1 release by erythropoietin involves calcium signaling in endothelial cells. *J Cardiovasc Pharmacol* 1995; 26: 889-892.
 58. Nagai T, Akizawa T, Kohjiro S, Koiwa F, Nabeshima K, Niikura K, Kino K, Kanamori N, Kinugasa E, Ideura T. rHuEPO enhances the production of plasminogen activator inhibitor-1 in cultured endothelial cells. *Kidney Int* 1996; 50: 102-107.
 59. Wang XQ, Vaziri ND. Erythropoietin depresses nitric oxide synthase expression by human endothelial cells. *Hypertension* 1999; 33: 894-899.
 60. Födinger M, Fritsche-Polanz R, Buchmayer H, Skoupy S, Sengoele G, Hörl WH, Sunder-Plassmann G. Erythropoietin-inducible immediate-early genes in human vascular endothelial cells. *J Investig Med* 2000; 48: 137-149.
 61. Akimoto T, Kusano E, Fujita N, Okada K, Saito O, Ono S, Ando Y, Homma S, Saito T, Asano Y. Erythropoietin modulates angiotensin II- or noradrenaline-induced Ca(2+) mobilization in cultured rat vascular smooth-muscle cells. *Nephrol Dial Transplant* 2001; 16: 491-499.
 62. Kusano E, Akimoto T, Umino T, Yanagiba S, Inoue M, Ito C, Ando Y, Asano Y. Modulation of endothelin-1-induced cytosolic free calcium mobilization and mitogen-activated protein kinase activation by erythropoietin in vascular smooth muscle cells. *Kidney Blood Press Res* 2001; 24: 192-200.
 63. Hand MF, Haynes WG, Johnstone HA, Anderton JL, Webb DJ. Erythropoietin enhances vascular responsiveness to norepinephrine in renal failure. *Kidney Int* 1995; 48: 806-813.
 64. Rundqvist H, Rullman E, Sundberg CJ, Fischer H, Eisleitner K, Ståhlberg M, Sundblad P, Jansson E, Gustafsson T. Activation of the erythropoietin receptor in human skeletal muscle. *Eur J Endocrinol* 2009; 161: 427-434.
 65. Rafiee P, Shi Y, Su J, Pritchard KA Jr, Tweddell JS, Baker JE. Erythropoietin protects the infant heart against ischemia-reperfusion injury by triggering multiple signaling pathways. *Basic Res Cardiol* 2005; 100: 187-197.
 66. Shi Y, Rafiee P, Su J, Pritchard KA Jr, Tweddell JS, Baker JE. Acute cardioprotective effects of erythropoietin in infant rabbits are mediated by activation of protein kinases and potassium channels. *Basic Res Cardiol* 2004; 99: 173-182.
 67. Parsa CJ, Matsumoto A, Kim J, Riel RU, Pascal LS, Walton GB, Thompson RB, Petrofski JA, Annex BH, Stamler JS, Koch WJ. A novel protective effect of erythropoietin in the infarcted heart. *J Clin Invest* 2003; 112: 999-1007.
 68. Westenbrink BD, Ruijrok WP, Voors AA, Tilton RG, van Veldhuisen DJ, Schoemaker RG, van Gilst WH, de Boer RA. Vascular endothelial growth factor is crucial for erythropoietin-induced improvement of cardiac function in heart failure. *Cardiovasc Res* 2010; 87: 30-39.
 69. Mitsuima W, Ito M, Kodama M, Fuse K, Okamura K, Minagawa S, Kato K, Hanawa H, Toba K, Nakazawa M, Aizawa Y. Cardioprotective effects of recombinant human erythropoietin in rats with experimental autoimmune myocarditis. *Biochem Biophys Res Commun* 2006; 344: 987-994.
 70. Goldberg N, Lundin AP, Delano B, Friedman EA, Stein RA. Changes in left ventricular size, wall thickness, and function in anemic patients treated with recombinant human erythropoietin. *Am Heart J* 1992; 124: 424-427.
 71. Linde T, Wikström B, Andersson LG, Danielson BG. Renal anaemia treatment with recombinant human erythropoietin increases cardiac output in patients with ischaemic heart disease. *Scand J Urol Nephrol* 1996; 30: 115-120.
 72. Löw-Friedrich I, Grützmacher P, März W, Bergmann M, Schoeppe W. Long-term echocardiographic examinations in chronic hemodialysis patients substituted with recombinant human erythropoietin. *Blood Purif* 1990; 8: 272-278.
 73. Silverberg DS, Wexler D, Blum M, Keren G, Sheps D, Leibovitch E, Brosh D, Laniado S, Schwartz D, Yachnin T, Shapira I, Gavish D, Baruch R, Koifman B, Kaplan C, Steinbruch S, Iaina A. The use of subcutaneous erythropoietin and intravenous iron for the treatment of the anemia of severe, resistant congestive heart failure improves cardiac and renal function and functional cardiac class, and markedly reduces hospitalizations. *J Am Coll Cardiol* 2000; 35: 1737-1744.
 74. Silverberg DS, Wexler D, Blum M, Schwartz D, Wollman Y, Iaina A. Erythropoietin should be part of congestive heart failure management. *Kidney Int Suppl* 2003; 87: S40-S47.
 75. Marti HH, Gassmann M, Wenger RH, Kvietikova I, Morganti-Kossmann MC, Kossmann T, Trentz O, Bauer C. Detection of erythropoietin in human liquor: intrinsic erythropoietin production in the brain. *Kidney Int* 1997; 51: 416-418.
 76. Springborg JB, Sonne B, Frederiksen HJ, Foldager N, Poulsen L, Klausen T, Jørgensen OS, Olsen NV. Erythropoietin in the cerebrospinal fluid of patients with aneurysmal subarachnoid haemorrhage originates from the brain. *Brain Res* 2003; 984: 143-148.
 77. Juul SE, Yachnis AT, Rojiani AM, Christensen RD. Immunohistochemical localization of erythropoietin and its receptor in the developing human brain. *Pediatr Dev Pathol* 1999; 2: 148-158.
 78. Nagai A, Nakagawa E, Choi HB, Hatori K, Kobayashi S, Kim SU. Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture. *J Neuropathol Exp Neurol* 2001; 60: 386-392.
 79. Morceau F, Dicato M, Diederich M. Pro-inflammatory cytokine-mediated anemia: regarding molecular mechanisms of erythropoiesis. *Mediators Inflamm* 2009; 2009: 405016.
 80. Buck I, Morceau F, Cristofanon S, Reuter S, Dicato M, Diederich M. The inhibitory effect of the proinflammatory cytokine TNF α on erythroid differentiation involves erythroid transcription factor modulation. *Int J Oncol* 2009; 34: 853-860.
 81. Chin K, Oda N, Shen K, Noguchi CT. Regulation of transcription of the human erythropoietin receptor gene by proteins binding to GATA-1 and Sp1 motifs. *Nucleic Acids Res* 1995; 23: 3041-3049.
 82. Leviton A, Dammann O. Coagulation, inflammation, and the risk of neonatal white matter damage. *Pediatr Res* 2004; 55: 541-545.
 83. Murthy V, Kennea NL. Antenatal infection/inflammation and fetal tissue injury. *Best Pract Res Clin Obstet Gynaecol* 2007; 21: 479-489.
 84. Phares TW, Kean RB, Mikheeva T, Hooper DC. Regional differences in blood-brain barrier permeability changes and inflammation in the atherogenic clearance of virus from the central nervous system. *J Immunol* 2006; 176: 7666-7675.
 85. Tao W, Wen F, Zhang H, Liu G. The signal transduction mediated by erythropoietin and proinflammatory cytokines in the JAK/

- STAT pathway in the children with cerebral palsy. *Brain Dev* 2009; 31: 200-207.
86. Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, Sasaki R. In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc Natl Acad Sci USA* 1998; 95: 4635-4640.
 87. Sirén AL, Knerlich F, Poser W, Gleiter CH, Brück W, Ehrenreich H. Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. *Acta Neuropathol* 2001; 101: 271-276.
 88. Chin K, Yu X, Beleslin-Cokic B, Liu C, Shen K, Mohrenweiser HW, Noguchi CT. Production and processing of erythropoietin receptor transcripts in brain. *Brain Res Mol Brain Res* 2000; 81: 29-42.
 89. Koshimura K, Murakami Y, Sohmiya M, Tanaka J, Kato Y. Effects of erythropoietin on neuronal activity. *J Neurochem* 1999; 72: 2565-2572.
 90. Zhu X, Luo L. Effect of nitroprusside (nitric oxide) on endogenous dopamine release from rat striatal slices. *J Neurochem* 1992; 59: 932-935.
 91. Kawakami M, Sekiguchi M, Sato K, Kozaki S, Takahashi M. Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. *J Biol Chem* 2001; 276: 39469-39475.
 92. Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents *in vitro* glutamate-induced neuronal death. *Neuroscience* 1997; 76: 105-116.
 93. Yamasaki M, Mishima HK, Yamashita H, Kashiwagi K, Murata K, Minamoto A, Inaba T. Neuroprotective effects of erythropoietin on glutamate and nitric oxide toxicity in primary cultured retinal ganglion cells. *Brain Res* 2005; 1050: 15-26.
 94. Chong ZZ, Kang JQ, Maiese K. Erythropoietin fosters both intrinsic and extrinsic neuronal protection through modulation of microglia, Akt1, Bad, and caspase-mediated pathways. *Br J Pharmacol* 2003; 138: 1107-1118.
 95. Chong ZZ, Kang JQ, Maiese K. Apaf-1, Bcl-xL, cytochrome c, and caspase-9 form the critical elements for cerebral vascular protection by erythropoietin. *J Cereb Blood Flow Metab* 2003; 23: 320-330.
 96. Um M, Gross AW, Lodish HF. A "classical" homodimeric erythropoietin receptor is essential for the antiapoptotic effects of erythropoietin on differentiated neuroblastoma SH-SY5Y and pheochromocytoma PC-12 cells. *Cell Signal* 2007; 19: 634-645.
 97. Villa P, Bigini P, Mennini T, Agnello D, Laragione T, Cagnotto A, Viviani B, Marinovich M, Cerami A, Coleman TR, 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.
 98. Agnello D, Bigini P, Villa P, Mennini T, Cerami A, Brines ML, Ghezzi P. Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. *Brain Res* 2002; 952: 128-134.
 99. Marti HH, Bernaudin M, Petit E, Bauer C. Neuroprotection and Angiogenesis: Dual Role of Erythropoietin in Brain Ischemia. *News Physiol Sci* 2000; 15: 225-229.
 100. 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.
 101. Sirén AL, 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 metabolic stress. *Proc Natl Acad Sci USA* 2001; 98: 4044-4049.
 102. Martínez-Estrada OM, Rodríguez-Millán E, González-De Vicente E, Reina M, Vilaró S, Fabre M. Erythropoietin protects the *in vitro* blood-brain barrier against VEGF-induced permeability. *Eur J Neurosci* 2003; 18: 2538-2544.
 103. Ismailov RM. Erythropoietin and epidemiology of Alzheimer disease. *Alzheimer Dis Assoc Disord* 2013; 27: 204-206.
 104. Assaraf MI, Diaz Z, Liberman A, Miller WH Jr, Arvanitakis Z, Li Y, Bennett DA, Schipper HM. Brain erythropoietin receptor expression in Alzheimer disease and mild cognitive impairment. *J Neuropathol Exp Neurol*. 2007; 66: 389-398.
 105. Zhi-Kun S, Hong-Qi Y, Zhi-Quan W, Jing P, Zhen H, Sheng-Di C. Erythropoietin prevents PC12 cells from beta-amyloid-induced apoptosis via PI3K/Akt pathway. *Transl Neurodegener* 2012; 1: 7.
 106. Jang W, Park J, Shin KJ, Kim JS, Kim JS, Youn J, Cho JW, Oh E, Ahn JY, Oh KW, Kim HT. Safety and efficacy of recombinant human erythropoietin treatment of non-motor symptoms in Parkinson's disease. *J Neurol Sci* 2014; 337: 47-54.
 107. Elliott S, Sinclair AM. The effect of erythropoietin on normal and neoplastic cells. *Biologics* 2012; 6: 163-189.
 108. Ehrenreich H, Weissenborn K, Prange H, Schneider D, Weimar C, Wartenberg K, Schellinger PD, Bohn M, Becker H, Wegrzyn M, Jähnig P, Herrmann M, Knauth M, Bähr M, Heide W, Wagner A, Schwab S, Reichmann H, Schwendemann G, Dengler R, Kastrup A, Bartels C, and for the EPO Stroke Trial Group. Recombinant Human Erythropoietin in the Treatment of Acute Ischemic Stroke. *Stroke* 2009; 40: e647-e656.
 109. Mestas J and Hughes C. Of Mice and Not Men: Differences between Mouse and Human Immunology. *J Immunol* 2004; 172: 2731-2738.
- Peer reviewers:** Elena Masselli, MD, Hematology and BMT Unit, Parma University Hospital, Parma, Italy; Periklis Dousdampanis, MD, PhD, Consultant Nephrologist, Hemodialysis Unit, Kyanos Stavros, Patras, Greece.