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Minireview

Experimental evidence of the potential use of erythropoietin by intranasal administration as a neuroprotective agent in cerebral hypoxia

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Abstract

Stroke is a major human health problem without efficient available therapeutics. Ischemic brain injury can induce cell death as well as upregulation of endogenous adaptive mechanisms depending on the severity and duration of hypoxia, and the activity of transcription factors, such as hypoxia inducible factor 1-α (HIF-1α). HIF-1α induces gene expression as *multidrug resistance* (*MDR-1*) gene associated with drug-refractory phenotype, as well as erythropoietin (Epo) and erythropoietin receptor (Epo-R) associated with O_2 supply. The spontaneous stimulation of the Epo/Epo-R system is not enough for brain protection. Therefore, administration of exogenous recombinant human Epo (rHu-Epo) was suggested as an alternative therapy in stroke. In several experimental models of brain hypoxia, Epo and Epo variants, including rHu-Epo, showed neuroprotective effects. Intranasal administration of these Epo-compounds can reach the central nervous system and protect the brain against ischemia, avoiding hematopoietic effects. However, it has been reported that high expression of Epo-R in neurons must be available to be activated by Epo. According to these considerations, intranasal delivery of rHu-Epo could be an interesting approach in the treatment of cerebral hypoxias avoiding both (i) adverse peripheral effects of treatment with Epo in stroke, and (ii) the pharmacoresistant phenotype depending on MDR-1 expression.

Keywords: erythropoietin; hypoxia inducible factor 1-α (HIF-1α); intranasal delivery; *multidrug resistance (MDR-1)* gene; neuroprotection; stroke.

Stroke, with an incidence of approximately 250–400 in 100,000 individuals and a mortality rate of around 30%, is the first leading cause of early disability and the third frequent cause of death in the industrialized world. Approximately 85% of all strokes are ischemic and are the result of transient or permanent reduction in cerebral blood flow caused by cerebral artery occlusion. The remaining 15% are caused by cerebral hemorrhage (1, 2). The damage in the central nervous system (CNS) produced after an ischemic injury can induce a sequence of severe pathophysiological events that will produce changes on the brain tissue (Figure 1). In ischemic injury, the abrupt reduction on cerebral blood flow produces a restriction of substrates in the affected brain area as well as oxygen supply, one of the essential components of normal cell homeostasis (3). Cells at the center of the ischemic focus, named as the ischemic core, are especially vulnerable and can die within minutes of ischemic onset. The ischemic penumbra surrounding the core is an area of reduced perfusion where cells are still viable.

Oxygen deprivation is rapidly detected by a continuously expressed system, capable of producing a wide spectrum of molecular and biochemistry effects facing the emergency. The mechanism has the potential role of saving the cell as well as causing its death according to the severity of hypoxic injury and/or the balance between the amounts of pro-apoptotic vs. antiapoptotic proteins expressed in each affected cell. The main protagonist of this dual behavior is the hypoxia inducible factor 1α (HIF-1α). HIF-1α is a transcription factor that upregulates several genes associated with cellular rescue and survival (4).

In a normal situation (normoxia), HIF-1α is continuously synthesized and recognized by von Hippel-Lindau (VHL) protein. A proline hydroxylase enzyme hydrolyzes HIF-1α allowing its binding with VHL protein so it can be ubiquitinated and later degraded via proteasome. As a consequence, HIF-1α destruction impairs its enhancer effect over several genes, such as erythropoietin (Epo) and its receptor (Epo-R) (5).

Proline hydroxylase is a hemoprotein which is highly sensitive and inhibited at low oxygen supply. This inhibition

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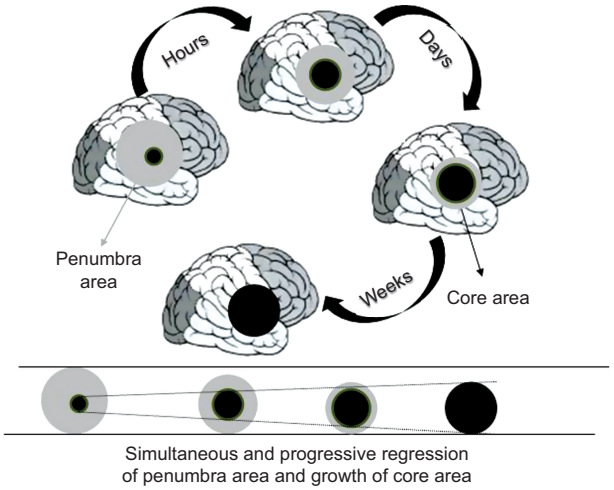

Figure 1 Schematic representation of the progressive changes observed in the brain hypoxic areas; modified from (3).

avoids the binding of HIF-1α with VHL protein, and their later degradation via proteasome. This mechanism stabilizes HIF-1α allowing its binding with ARNT (aryl hydrocarbon receptor nuclear translocator) also named HIF-1β. Nuclear translocation of this complex will upregulate several gene targets, such as Epo and Epo-R, as well as multidrug resistance (*MDR-1*) gene, and vascular endothelial growth factor, among others. However, if oxygen deprivation is in accordance with anoxic conditions, HIF-1α will bind with p-53 inducing activation of apoptotic cell death (6) (Figures 2 and 3). HIF-1α was discovered by the identification of a hypoxia response element (5′-RCGTG-3′) in the 3′ enhancer region of the erythropoietin (*Epo*) gene, a hormone that stimulates erythrocyte proliferation and undergoes its own transcription, induced by hypoxia (7).

Epo-R is expressed in the membrane cell as a preformed Epo-R homodimer with the articular conformation that in the absence of Epo, intracellular domains do not interact with each other. Binding of Epo to the homodimer induces a conformational change that brings the intracellular domains together (Figure 4) which results in phosphorylation and activation of Janus tyrosine kinase 2 (JAK2) signaling cascade to stimulate proliferation of red blood cells (8).

In addition to erythropoiesis regulation, biological functions of Epo and Epo-R are also reported to be neuroprotective after ischemic or traumatic brain injury. These effects were described as intracerebral autocrine and paracrine functions (9).

Surprisingly, it was proposed that the mechanisms of this Epo-mediated neuroprotective action could be also produced by the induction of an alternative pathway activation, which is different from the classical Epo-R homodimeric complex expressed by erythroid precursors. This mechanism is mediated by binding of Epo with a heterotrimer (Figure 5), formed by one unit of Epo-R and βCR (β common receptor) homodimer, a member of the type I cytokine-receptor superfamily (10).

It is important to note that there are different hypoxic conditions. In systemic hypoxia, a high level of Epo synthesis is induced for red blood cell (RBC) production, and this Epo has very low or null capacity to enter into the CNS. However, after focal brain hypoxia, only an intracerebral synthesis of the Epo/Epo-R system is induced, without the proliferative stimulation of systemic RBCs (11). This local production of Epo and Epo-R is not enough to protect the brain cells against hypoxic injury.

In several experimental models of cerebral hypoxia, a maximal expression of HIF-1α was observed 5 h after hypoxic stress, which declines to basal levels at 12 h, and after focal brain hypoxia, expression of HIF-1α-dependent genes, such

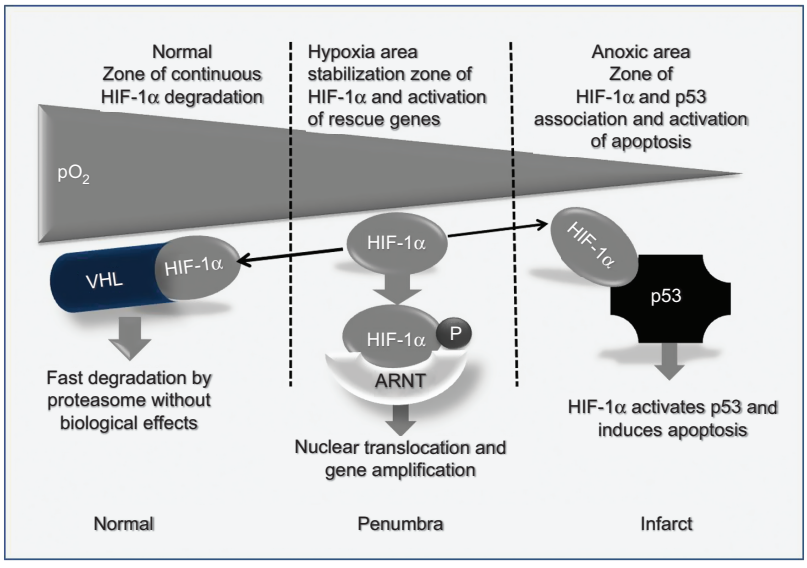

Figure 2 HIF-1α action according to hypoxia severity; modified from (6).

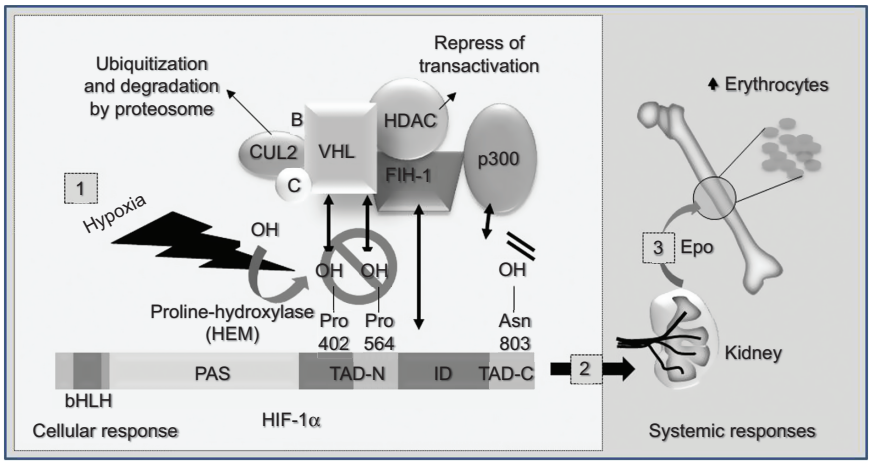

Figure 3 Regulatory mechanism of HIF-1α interaction with VHL protein (A), renal synthesis (B) and erythropoiesis induction (C); modified from (7).

as Epo-R, could be detected in neurons located outside the infarct zone longer than 12 h after artery occlusion (12–14).

Interestingly, it was recently demonstrated that a single hypoxic exposure (1 h) has no effect on the Epo-R transcript basal levels in neurons immediately after hypoxia, but repetitive hypoxic stimuli can increase the Epo-R transcription level by >85% (15). In addition, animals exposed to an experimental model of sleep apnea by intermittent hypoxia showed a persistence of elevated HIF-1α in hippocampal neurons (16).

According to this persistent hypoxic stimulation, in our experiments using intracerebral injection of cobalt chloride ($CoCl_2$) (17), long-term HIF-1α expression at the fifth day after cobalt injection was observed, suggesting that this model mimics the development of a chronic-like hypoxic insult associated with concomitant MDR-1 and Epo-R overexpression, as well as HIF-1α-related genes (18).

Because elevated expressions of Epo-R in neurons are required for optimal neuroprotection by Epo (15), it is possible that this required high expression of Epo-R could be activated only if an additionally high concentration of Epo is produced at the surrounding area. These data indicate that the Epo/Epo-R system could be a plastic endogenous mechanism that continuously modifies activity according to fluctuations or reduction in the brain blood flow and/or metabolic cell requirements. However, this active Epo/Epo-R system cannot spontaneously protect the brain against severe hypoxia as observed in the clinical follow-up of stroke. In spite of this, the controversy still remains to be resolved. This system could be useful if overexpressed Epo-R is activated with pharmacological doses of exogenously administered recombinant human Epo (rHu-Epo) (19, 20). With regard to these considerations, it was postulated that cerebral cortical neurons could be protected if sufficient Epo amount could reach and activate the overexpressed Epo-R on their membranes, confirming the paradigm that "therapeutic actions on penumbra area could be the hope of protection or recovery in stroke" (21–23).

The neuroprotective efficacy of Epo or its analogs have been evaluated in several experimental models of brain hypoxia. The middle cerebral artery occlusion (MCAO) model is the most commonly model used. A total of 14 MCAO studies showed a great disparity in different variables, such as duration of occlusion intervals as well as the doses, routes and/or the administration timing of Epo or its analogs. However, the results of these studies suggest that when administered after the onset of ischemia, Epo reduced infarct size and improved neurobehavioral deficits significantly, as demonstrated in our experiments (18). These effects were associated with both higher doses and earlier administration of the hormone after experimental stroke initiation (24).

However, the reason for these similar results in spite of different experimental designs is not clear. We suggest that regardless of time elapsed after the insult, while the Epo-R remains overexpressed, the exogenous administration of Epo could play a protective role. The hematopoietic effects of Epo (in addition to its neuroprotective activity) could be a problem in the treatment of cerebrovascular diseases, as Epo induces polyglobulia that could disturb blood flow, thus compromising the survival of the tissue. Variants of Epo that do not bind to the classical Epo-R might overcome this issue, as these compounds

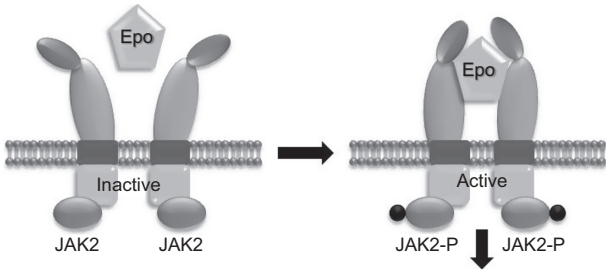

Figure 4 Erythropoietin receptor (Epo-R).
Schematic interactions between Epo and Epo-R in the hematopoietic system; modified from (8).

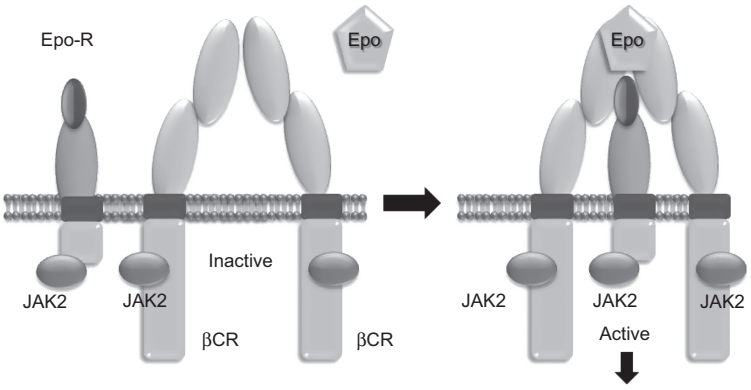

Figure 5 Schematic interactions between Epo with heterotrimeric Epo-R/(βCR)2 complex in central nervous system (CNS); modified from (10).

exert neuroprotective, but not hematopoietic, actions. Note that the modified Epo containing little sialic acid can also be applied nasally. To date, it is not known if these compounds will be finally successful in the treatment of human stroke, but the main advantages of intranasal administration of Epo or its analogs include the lack of hematopoietic activity and the lower doses required. This stratagem would open new opportunities to deliver Epo in the CNS over longer intervals, e.g., in the post-acute phase of stroke, and in this case, polyglobulia induced by Epo could be avoided with relevant clinical impact. rHu-Epo has been used for experimental stroke models, but its hematopoietic effect, as well as alterations in platelet functions and hemostasis, can result in potential complications if used in patients (25). Our experiments have demonstrated that intranasal delivery of rHu-Epo has no effect on the hematopoietic system, and a neuroprotective long-term action was observed until 5 days after brain focal injury (18).

Nasal delivery seems to be a favorable way to circumvent the obstacles for the blood-brain barrier (BBB) allowing direct drug delivery in the biophase of CNS-active compounds. Furthermore, this route also avoids the intestinal absorption limitations, enterohepatic circulation and total body biodistribution, allowing a more effective CNS treatment with lowest drug doses needed (26, 27). In different experimental models of brain hypoxia, we have described the induced overexpression of the *MDR-1* gene that involves not only vascular endothelial cells of BBB but also neurons and astroglial cells (16, 28, 29).

According to these observations, it was suggested that *MDR-1* gene overexpression could be an impairment of brain hypoxia treatment with conventional drugs (29–31).

In conclusion, our results indicate that the $CoCl_2$ brain injection mimic chronic hypoxia, as observed by the expression of HIF-1α remaining at least 5 days after injury, with a concomitant high overexpression of *Epo-R* and *MDR-1*. The overexpression of both *Epo-R* and *MDR-1* genes were documented at the same type cells, and a successful treatment with intranasal administered (IN) rHu-Epo was also observed, suggesting that IN delivery of rHu-Epo could be an interesting approach in the treatment of cerebral hypoxias avoiding both pharmacoresistant phenotype depending on MDR-1 expression and peripheral adverse effects. In summary, we think that the key for a positive neuroprotective effect of Epo is a quantitative factor, depending on the expression of Epo-R in neurons, as well as the amount of Epo in the SNC needed to activate its receptor, irrespective of restriction mediated by the *MDR-1* gene.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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