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Review

Erythropoietin, erythropoiesis and beyond

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ABSTRACT

Erythropoietin (EPO) is a glycoprotein that is mainly produced in the adult kidney, and it was initially highlighted for its action on the hematopoietic system. Moreover, EPO is also expressed in several non-hematopoietic tissues, where it plays a role in the protection from apoptosis and inflammation due to hypoxia, toxicity or injury. These protective effects are mainly known and studied in cardioprotection and neuroprotection but are also reported in retina degeneration, auditory injury and pancreatic-related diseases. The tissue protective effect of EPO is mainly mediated through the interaction with the heterodimeric receptor EPOR/βcR. Human recombinant EPO (HuREPO), which has been developed to treat anemia, is not adequate for tissue protection. The low affinity of the alternative receptor for EPO involves the injection of excessive concentration of erythropoiesis-stimulating agents (ESAs), implicating side effects due to the cross-talk with hematopoietic activity. For these reasons, EPO derivatives with less affinity for the EPO homodimeric receptor are under development. In this review, we provide an overview of the erythroid and non-erythroid functions of EPO by detailing the molecular mechanisms activated by the binding of EPO to its receptors in different tissues.

Contents

1. From EPO production to EPO target genes . . . 1291
2. Erythropoietic and non-erythropoietic erythropoietin . . . 1294
3. EPO-related protection . . . 1296
   3.1. Cardioprotection . . . 1296
      3.1.1. Inflammation . . . 1296
      3.1.2. Apoptosis . . . 1297
      3.1.3. Angiogenesis . . . 1298
   3.2. Neuroprotection . . . 1298
      3.2.1. EPO in brain development . . . 1298
      3.2.2. EPO in neuroprotection . . . 1298
   3.3. Other protections . . . 1299
      3.3.1. Pancreatic disorder . . . 1299
      3.3.2. Retinal protection . . . 1299
      3.3.3. Auditory protection . . . 1299
      3.3.4. Others . . . 1299
4. Conclusion . . . 1299
   Acknowledgements . . . 1300
   References . . . 1300

1. From EPO production to EPO target genes

More than a century ago, a plasmatic humoral factor was assumed to be essential for red blood cell production. It was called hemopoietin. Hemopoietin was the initial name for erythropoietin (EPO), which was later identified and described as a 34 kDa

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glycoprotein of 165 amino acids that plays the role of a hormone, cytokine and growth factor. The peritubular interstitial cells of the kidney are believed to be the main producers of EPO [1,2]. Until recently, EPO-producing kidney cells were not clearly identified as reported in various publications [3]. Indeed, the latest study from Frede et al., suggested a potential fibroblast-like/neuronal origin for the EPO-producing cells in the kidney. This work was performed by using Renal Epo Producing Cells (REPC) isolated from a tumor-free tissue. REPC that produce EPO with the typical pattern after 36 h of continuous hypoxia, present neuronal markers and respond to neural growth factors [4]. The incontestable fact is that the EPO gene is mainly expressed in the fetal liver, whereas it is expressed in the kidney after birth, and the kidney then becomes the predominant site of EPO production. Therefore, the liver is a secondary site of EPO production in the adult [5–7]. This differential site expression between prenatal and postnatal life is regulated at the gene transcription level. The EPO gene exhibits different specific sequences in its *cis*-regulatory elements, which provide tissue-specific regulation. Indeed, repression of EPO gene expression in the postnatal liver occurs *via* a specific negative regulatory liver element (NRLE) located at 3′ on the EPO gene, while the expression in the postnatal kidney is dependent on the kidney-inducible element (KIE), located at 5′ on the gene [8]. A fundamental discovery revealed that EPO gene expression is dependent on hypoxic conditions in two hepatocarcinoma cell lines HepG2 and Hep3B, attesting that individual cells are sensitive to oxygen (O_2) tension variations [9]. The EPO gene is indeed hypoxia inducible through a 50 bp hypoxia-inducible enhancer [10–12]. The hypoxia responsive elements (HRE) are differentially located in the kidney and in the liver. Using a mutant EPO-GFP transgene containing mutations in the hepatic HRE, the authors demonstrated that the 3′ enhancer is a liver-specific and hypoxia-inducible enhancer. However, this region remains dispensable for renal EPO expression [13]. In the kidney, the HRE includes the KIE and is located upstream, 5′ on the EPO gene.

Tissue-specific transcription factors bind regulatory sequences to control gene transcription. The proximal hepatic hypoxia-inducible enhancer binds hypoxia-inducible factors (HIF). The three members of the HIF transcription factor family are known as HIF-1, -2 and -3. HIF-1α was first identified as a mediator of EPO induction in response to hypoxia *in vitro*. However, HIF-2 was later identified as the primary transcription factor that induces EPO expression [14,15] (Fig. 1).

All HIF transcription factors interact with HIF-1β, which is also known as ARNT (arylhydrocarbon-receptor nuclear translocator), to regulate the genes involved in erythropoiesis, including the EPO gene, as well as in iron metabolism, which is essential for tissue oxygen delivery. HIF transcription factors also play a role in neovascularization and hematopoiesis [16]. On the other hand, the distal site binds the hepatocyte nuclear factor HNF-4α, which cooperates with HIF [17] through interaction with the transcriptional coactivators CBP/p300. The latter factors interact with the 3′ basal transcriptional machinery in the promoter.

Beside the HRE, other regulatory elements in the 5′ promoter of the EPO gene have been identified, and these elements have a highly conserved GATA sequence as well as NF-κB binding motifs [18,19]. The GATA site preferentially binds the transcription factor GATA-2, which has been reported to inhibit EPO gene expression [20,21]. One of the NF-κB binding sites is adjacent to the minimal HRE of the EPO promoter. Through this specific site, NF-κB becomes an inhibitor of EPO expression [21] (Fig. 1). The activities of GATA-2 and NF-κB in HepG2 cells decrease in hypoxia *vs.* normoxia conditions. However, the inhibition of EPO expression in these cells was correlated with an increased activity of both of these transcription factors, which are induced by the proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)α in hypoxia conditions [21]. These data clarified the role of proinflammatory cytokines in the failure of EPO production, which occurs in the anemia of chronic inflammatory diseases and cancers. In recent investigations that examined the effect of TNFα on erythropoiesis, we confirmed that TNFα induced GATA-2 expression in hematopoietic cells. In this case, TNFα-mediated overexpression of GATA-2 was correlated with EPO receptor (EPOR) gene downregulation and the inhibition of hemoglobin production in leukemia cell lines as well as a delayed erythroid development of EPO-stimulated $CD34^+$ hematopoietic stem progenitor cells [22–24].

The induction of EPO expression is tightly dependent on the physiological conditions that control its production in the kidney. Hypoxia and anemia are the main events that are able to induce EPO gene expression. Once it is produced, erythropoietin is released in the blood flow to meet the cells expressing EPOR. EPO physically interacts with EPOR homodimers that are expressed on the erythroid cell surface [25] to stimulate erythropoiesis by generating a complex network of molecular signals involved in the control of cell proliferation, differentiation and death (Fig. 1). In the erythroid system, EPOR is highly expressed in cells from the colony forming units-erythroid (CFU-E) to the basophilic erythroblast stage [26]. The EPO/EPOR interaction triggers conformational changes in the extracellular domain of the receptor and consequently the activation of the EPOR-associated Janus Kinase (JAK)-2 by autophosphorylation [27]. JAK2 activation results in the phosphorylation of eight tyrosine residues on the cytoplasmic region of EPOR [28]. These phosphotyrosine residues recruit a variety of Src homology-2 (SH2) domain-containing proteins that initiate various signaling pathways (Fig. 1).

One of the main activated signaling pathway is the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB) AKT pathway. This pathway plays a crucial role in the regulation of erythroid cell survival by protecting them from apoptosis, although this pathway is necessary but not sufficient [29]. PI3K has a catalytic subunit p110 and a regulatory subunit p85, which binds directly to the EPOR *via* its tyrosine Y479. However, PI3K can also be recruited to EPOR indirectly by other proteins [30] (Fig. 1). Using murine models that do not express PI3Kp85α, Huddleston et al. showed that p85α was necessary for fetal erythropoiesis development [31]. The binding and activation of PI3K leads to the phosphorylation of AKT, which in turn activates the proteins involved in erythropoiesis regulation. The PI3K/AKT signaling pathway phosphorylates serine S310 of the transcription factor GATA-1 *in vivo* and *in vitro*, and this phosphorylation enhances GATA-1 activity in erythroid cells [32]. GATA-1 is indeed a key transcription factor for the regulation of erythro-specific genes as well as the antiapoptotic Bcl-X_L gene transcription [33–35] (Fig. 1). The forkhead box O3A (Foxo3A) is another AKT-mediated phosphorylation-relevant transcription factor for erythropoiesis [36–38]. In fact, the phosphorylation of Foxo3A results in the inhibition of its transcriptional activity and subsequently the downregulation of Foxo3A target genes such as the cell cycle inhibitor protein $p27^{Kip1}$/cyclin dependent kinase (CDK) inhibitor [37,39]. Other Foxo3A target genes that have antiproliferative or proapoptotic functions include the B cell translocation gene 1 (BTG1), the Bcl2-family member BIM and the TNF-related apoptosis-inducing ligand (TRAIL) genes [26,36] (Fig. 1). EPO-mediated activation of PI3K/AKT can also occur *via* adaptor proteins such as Grb2. In fact, Grb2 is recruited by the phosphorylated tyrosine Y464 of EPOR, triggering the activation of the G-protein RAS and subsequently PI3K phosphorylation. In a similar mechanism, the other EPO-mediated signaling pathway the RAS/RAF/mitogen-activated protein kinase (MAPK)/MEK/ERK1/2 pathway is activated *via* adaptor proteins [40]. RAF1 activation is correlated with cell proliferation, and importantly, RAF1 inhibits caspase-3 (CASP3) activity, which leads to the arrest of cell differentiation [41,42] (Fig. 1). Moreover, EPO mediates the modulation of MAPKs

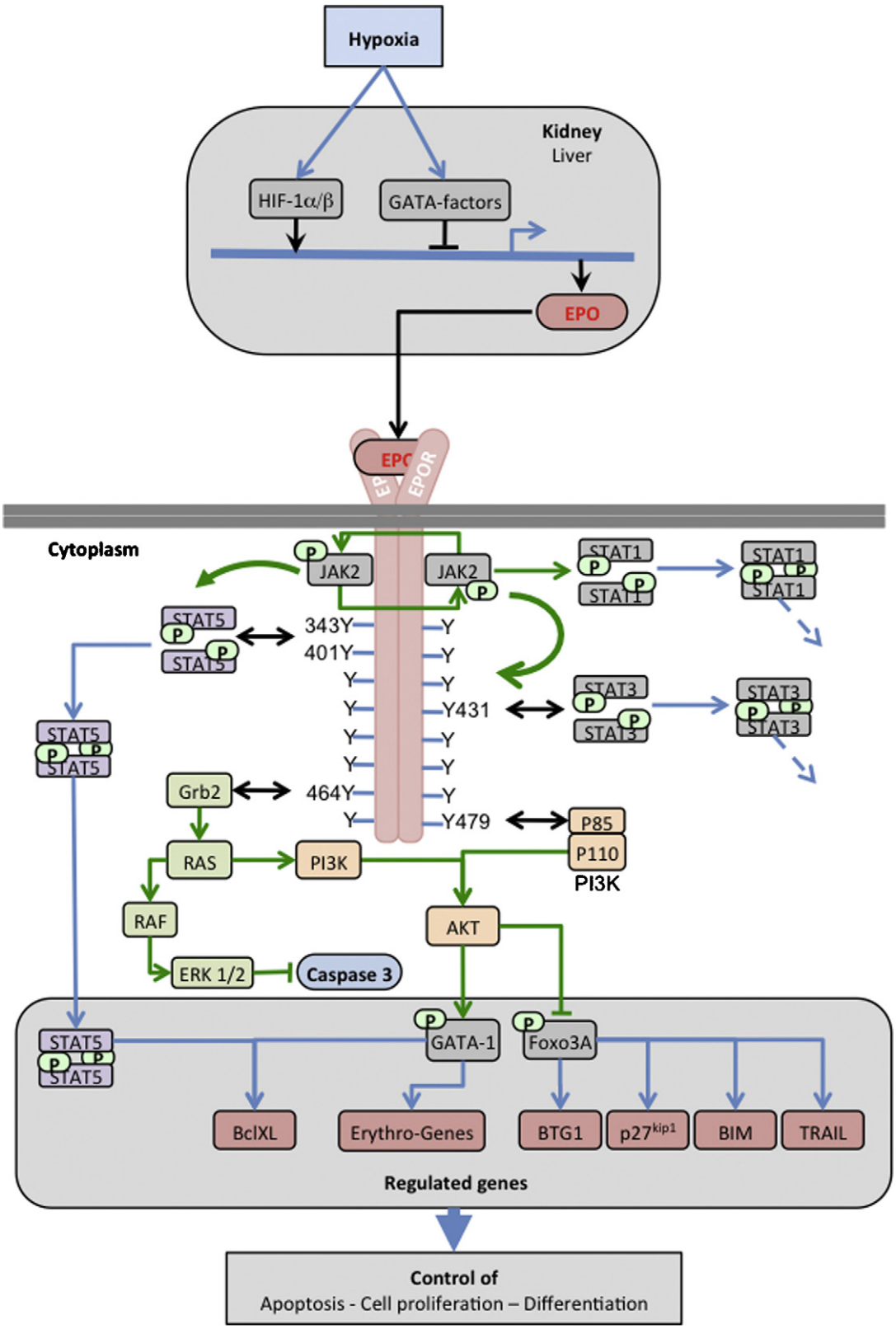

Fig. 1. Under hypoxia conditions, EPO production is induced in adult kidney through the activation of HIF and the down-regulation of GATA transcription factors, acting as inhibitors of EPO gene transcription. Following posttranslational modifications, the glycoprotein EPO is released in blood circulation to reach EPOR expressing hematopoietic cells in bone marrow. Interaction of EPO with EPOR triggers homodimerisation and activation of pre-existing associated JAK2 by trans-phosphorylation. JAK2 activation results in the phosphorylation of eight tyrosine residues (Y) on the cytoplasmic region of EPOR. Phospho-tyrosines are docking sites that recruit a variety of Src homology-2 (SH2) domain-containing proteins. Localisation of the tyrosine residues in the primary structure of EPO protein are indicated only for the described signaling pathways: JAK2/STAT5 (STAT3), PI3K/AKT, Grb2/RAS/RAF/MEK/ERK1/2. Green circles with P represent phosphates and green arrows and bars are phosphorylation activation and inhibition respectively. Double arrows represent protein interaction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

activation in erythropoiesis, while different studies revealed divergent results concerning EPO-mediated activation of p38 and SAPK/JNK. Indeed, p38α and p38γ are expressed during erythroid differentiation [43], and p38 is activated by EPO and is required for EPO-induced erythropoiesis [44,45]. Many cytokines activate SAPK/JNK and p38 in several murine cell lines [30,40,44]. However, proinflammatory cytokines, mainly TNFα, are responsible for the anemia in cancers and chronic inflammatory diseases [46,47]. As for EPO, TNFα is capable of activating p38 by phosphorylation [48,49]. Indeed, in a study examining the TNFα-inhibiting effect on erythropoiesis, we showed that the cytokine induced a rapid phosphorylation of p38 in human leukemia TF-1 cells (10 min), whereas the EPO-mediated activation of p38 occurred later (8 h). The use of p38α inhibitor SB203580 prevented the inhibitory effect of TNFα on EPO-induced erythroid differentiation, which was shown by hemoglobin production as well as γ-globin and GATA-1 upregulation. In this experimental model, phosphorylated p38α had an inhibitory effect on the EPO-mediated erythroid differentiation of the TF-1 cell line [22].

The signal transducer and activator of transcription (STAT) factors contain the SH2-domain. Among the seven members of the STAT family known in mammals, EPO activates STAT1, STAT3 and STAT5a/b [22,30,50,51] following their binding to the docking sites of the phosphorylated tyrosines of EPOR. They are then phosphorylated by JAK2, which allows their dimerization and activation as transcription factors. JAK2/STAT5 is the classical pathway activated by EPO in erythroid cells. Notably, EPO-mediated activation of JAK2/STAT5 leads to the upregulation of the antiapoptotic Bcl-X_L gene, therefore protecting proerythroblasts from apoptosis [52] (Fig. 1).

Dysfunctions in EPO/EPOR-mediated signaling pathways lead to serious perturbations of erythropoiesis by affecting cell differentiation, proliferation and apoptosis. Indeed, pathologies such as polycythemias (polycythemia vera (PV) and primary familial and congenital polycythemia (PFCP)) show an absolute increase in the red blood cell mass and the development of erythrocytosis. A constitutive activation of JAK2 caused by a V617F mutation has been incriminated in some patients [53,54]. JAK2V617F activity leads to a permanent activation of STAT5 and therefore of Bcl-X_L in erythroid progenitors, inducing EPO-independent differentiation and colony formation [55]. In PFCP patients, dominant gain-of-function EPOR mutations lead to EPO hypersensitivity of erythroid progenitors and hematopoietic cells, resulting in the prolonged EPO-induced JAK2 and STAT5 activation [56,57]. On the other hand, it was reported that activation of RAS-ERK and PI3K/AKT pathways were abnormally increased in association with EPO hypersensitivity and apoptosis resistance of erythroid precursor cells in PV [58].

As a crucial cytokine for erythroid development, the effects of EPO *via* the EPOR have been studied extensively on the physiological, cellular and molecular levels. However, an increasing number of studies demonstrate that EPOR is expressed in non-hematopoietic tissues and mainly in the brain and heart. Moreover, EPOR is expressed in many cancer cell types. These findings suggest new roles for EPO in non-hematopoietic tissues, while importantly, EPO has been shown to activate tumor cell proliferation. This observation constrains the clinical use of EPO through human recombinant EPO (HuREPO), which requires rigorous care, particularly as anti-anemia treatment for cancer patients. Nonetheless, numerous promising HuREPO molecules have been developed, and have been engineered to be more specifically efficient and have fewer side effects.

2. Erythropoietic and non-erythropoietic erythropoietin

Erythropoietin was initially discovered and purified in small quantities from the urine of patients with aplastic anemia [59]. In the mid 1980s, the human EPO gene was cloned, and recombinant DNA technology allowed for the large-scale production of erythropoiesis-stimulating agents (ESAs) [60,61]. ESAs mimic the cytokine's physiological function and act on the homodimeric EPOR (Fig. 2).

The first commercialized HuREPO, also known as epoetin, is a full-length unmodified EPO polypeptide that has the identical amino acid sequence to that of endogenous EPO. HuREPO is administered by subcutaneous or intravenous injections two to three times per week, and its half-life is approximately 6 h. HuREPO is the most successful recombinant medicine widely used to correct the low hemoglobin levels in anemia. It is a symptomatic treatment, the action of which triggers the recruitment of erythroid progenitor cells to commit to the erythroid lineage. It is used to treat anemic patients who have low EPO levels, including those patients with chronic diseases (inflammation and cancer) and those with renal failure. HuREPO treatment depends on the type and degree of anemia. The diagnosis of anemia takes into consideration different parameters. Among these are the morphological aspects of the red blood cells (shape, color and size), the amount of red blood cells (production, destruction), the red blood cell indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH)) and the circulating levels of EPO. The plasma levels of EPO generally range between 4 and 27 mU/mL. The anemic patients who are treated with HuREPO are those who present low levels of EPO, while in other cases, patients are treated with blood transfusions, nutritional administration steroid therapy and, more drastically, by splenectomy or bone marrow transplants. Of those anemic patients treated, 90% successfully respond to HuREPO, but some patients never achieve satisfactory levels of hemoglobin, or HuREPO may progressively lose its efficacy as a treatment. The hypo-responsiveness of this minority of anemic patients is mostly due to chronic inflammation, infections, iron deficiency, chronic blood loss and EPO antibody-mediated pure red cell aplasia (PRCA) [62–65].

To relieve the patients from the frequent and regular administration of HuREPO and to improve their quality of life (QOL), efforts have been made to produce molecules with longer half-life but with the same efficiency. In this way, different strategies are followed. Darbepoetin α, a novel erythropoiesis stimulating protein (NESP), contains specific modifications such as mutations creating N-linked-glycosylation sites on the glycosylated sites of the native EPO. This product has a 3-fold longer half-life in circulation and permits less frequent administration of the product (intravenous or subcutaneous injections every 2 weeks or monthly) [66,67]. Darbepoetin α is used in patients with chronic kidney disease and in cancer patients under chemotherapy [68–70].

Another strategy to prolong the plasma half-life of the HuREPO molecule is to bond it to different types of polymers. The synthetic erythropoiesis protein (SEP) is a fully synthetic polypeptide with a similar sequence as that of the parent EPO but is linked to negatively charged polymers. This molecule presents higher erythropoietic activity than HuREPO and has a 2.5-fold increased half-life [71]. Moreover, the continuous erythropoietin receptor activator (CERA) is a PEGylated EPO molecule with double mass of the native molecule. The half-life of CERA is remarkably high; it requires 135 h to be eliminated from circulation after intravenous or subcutaneous injection [72]. Furthermore, recombinant dimeric EPO linked *via* a flexible peptide bridge or chemically crosslinked *via* free sulfhydryl groups were also synthesized [73,74]. Finally, a screening peptide strategy allowed the isolation of oligopeptides with low affinity to the EPOR. After further modifications and testing for EPOR specificity, a 20-amino-acid peptide was synthesized (GGTYSCHFGPLTWVCKPQGG). This oligopeptide,

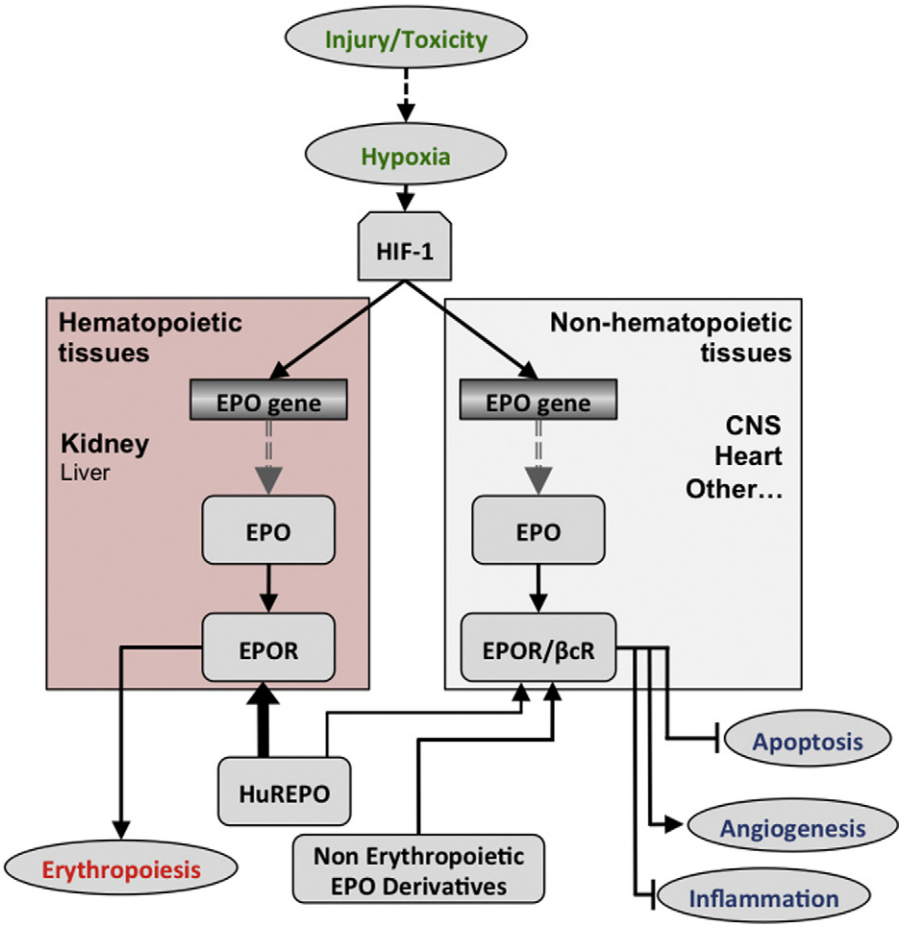

Fig. 2. Hypoxia, injury and toxicity can increase EPO and EPOR expression in hematopoietic and non-hematopoietic tissues. This increase leads to erythropoiesis in hematopoietic tissue or induction of angiogenesis and inhibition of apoptosis and inflammation in non-hematopoietic tissues.
Adapted from Xiong et al. [164].

called Hematide™, mimics EPO function and lacks sequence homology with EPO [75]. It is a very promising drug with a variety of advantages. Firstly, the manufacturing process of a synthetic peptide is cheaper compared to the production of recombinant proteins, which requires a cell line. Second, due to its pegylated form, Hematide™ is administrated less frequently than those needed for the currently marketed ESAs. Third, the fact that it is not a protein makes it more stable at room temperature without the need of cold storage conditions throughout the distribution and the administration to the patients. Moreover, immunologic cross-reactivity of Hematide™ does not occur in comparison to those occurring for rhuEPO such as PRCA. In a rat model of EPO antibody-mediated PRCA, Hematide™ could stimulate erythroid progenitors and increase hemoglobin levels without activation of an immune response [76]. These encouraging observations led to a phase II clinical trial in order to get the confirmation that Hematide™ is not neutralized by antibodies and can rescue patients from developing PRCA. Therefore, using Hematide™ could treat active EPO antibody-induced PRCA in patients. Results obtained by *in vivo* and *in vitro* studies about the biologic activity and safety profile of Hematide™ have allowed its entry into phase III trials [77–79].

In the case of anemia related pathologies, an alternative therapy could be the stabilization of HIF transcription factors by the use of the prolyl hydroxylase domain-containing protein (PHD) inhibitors. This therapeutic strategy is currently undergoing a phase II clinical trial [80].

Although EPO has beneficial effects on anemia, its administration can trigger an increase in cardiovascular and thromboembolic events. Moreover, EPO may accelerate tumor growth, but this observation remains debatable [81]. By contrast, Lundby et al., in a recent review, suggested that the administration of HuREPO in healthy humans does not alter their physiological indices and that it is safe to use for experimental purpose [82].

It was established that EPO binds EPOR to induce activation of the erythropoietic pathway, and that the affinity of EPO to its receptor is high. But an initial study with different EPO sensitive cell types and by evaluation of their EPO affinity, showed the existence of at least two types of receptors, one with a high affinity for EPO and the second with an affinity 8–16 times lower (Fig. 2) [83]. Later, a study confirmed the hypothesis that this second receptor may be a heterodimer consisting of EPOR and the β common receptor (βcR, also named CD131). This hypothesis was based on the synergistic action of cytokines IL3, IL5 and GM-CSF with EPO, to promote erythropoiesis [84]. Following that, it was proposed that this alternative receptor could be responsible for the majority of the EPO protective activity that we will mention below [85], and which constitutes a very interesting therapeutic approach. However, recent data questions the exclusivity of this receptor, showing that at least in differentiated neuroblastoma SH-SY5Y and pheochromocytoma PC-12 cells, the EPO antiapoptotic activity is due to the homodimeric EPOR [86].

The protective activity of EPO may be due, in some tissues, to the presence of the heterodimeric receptor but the affinity of EPO for the EPOR/βcR receptor is low, so the tissue-protective properties of EPO are reached with higher dosage than needed for its circulating hormonal effects and concurrently, high doses of

EPO are associated with side effects and abnormally increased erythropoiesis. To circumvent the side effects while preventing the cytoprotective activities of EPO, different non-erythropoietic erythropoietin derivatives have been developed either by chemically modifying or mutating EPO.

The carbamylation of lysines is a characteristic modification that alters protein conformation and function. Leist et al. noted that carbamylated EPO (CEPO) lacks erythropoietic activity but keeps the neuroprotective effect of EPO [87]. CEPO is also known for its capacity to protect the kidneys from ischemic injury [88] as well as for its cardioprotective and neuroprotective properties [89,90]. CEPO has the same half-life as HuREPO and overcomes the platelet reactivity induced by high doses of EPO [91].

The difficulties in the production and storage at cold conditions to preserve stability led to the development of other small alternative molecules. Based on the tertiary structure of EPO, helix B surface peptides have been developed. These peptides are less expensive to produce and more stable. Moreover, they display less immunogenicity and have tissue-protective effects [92,93]. The QEQLERALNSS peptide protects cardiomyocytes from TNFα-induced apoptosis *in vivo* and *in vitro* [94]. These oligopeptides mimic the 3D structure of EPO, and they present a promising alternative to CEPO for therapeutic use and the study of EPO-mediated cytoprotection.

Carbamylated darbepoetin alpha (C-darbe) is synthesized using the same protocol as that for CEPO. Ramirez et al. found that C-darbe protects endothelial progenitor cells from inflammation-induced apoptosis without stimulating erythropoiesis [95].

AsialoEPO is obtained by the enzymatic conversion of HuREPO using a sialidase that removes the sialic acid from HuREPO. The plasma half-life of the obtained product is between 1 and 2 h, and its binding affinity to EPOR is the same as that of HuREPO. The shorter half-life of AsialoEPO in comparison with that of HuREPO did not trigger the commitment of erythroid progenitors to mature erythrocytes in circulation, so the hemoglobin concentration did not increase. Erbayraktar et al. also demonstrated the neuroprotective capacity of AsialoEPO *in vivo* [96].

S100E is an EPO mutant generated by site-directed mutagenesis of the EPO-encoding sequence. It lacks an affinity for EPOR but retains the tissue-protective effect of EPO without any hematopoietic bioactivity [87]. S100E promotes neuroprotective, post-ischemic neurologic function improvement [90] and protects against ocular photoreceptor degeneration [97].

3. EPO-related protection

In addition to the key roles of EPO in differentiation, proliferation and inhibition of cell death, emerging evidence has suggested that EPO exerts cytoprotective effects on non-erythroid cells. Moreover, the data have often shown that EPOR is expressed in non-hematopoietic tissues, including the brain [98,99] and heart [100] as well as the small bowel [101], uterus [102], kidney [103] and pancreatic islets [104]. As described above, the initially developed recombinant EPO mimicked all of the properties of the native EPO. Then, the other derivatives of EPO have targeted some of these properties, such as the capacity for non-hematopoietic tissues protection. Cardioprotection and neuroprotection are the main areas that have been studied for the protective activities of erythropoietin.

3.1. Cardioprotection

Ischemic-reperfusion injury (I/R) refers to tissue damage caused by the return of blood supply to the tissue after a period of ischemia. I/R occurs in cell death, myocardial infarction (MI), myocardial remodeling and the functional decline of the heart.

The use of HuREPO or EPO derivatives, such as CEPO, Darbepoetin, and others, are different methods to avoid ischemia injury. Postconditioning (IPost) consists of a brief intermittent ischemia, applied during the onset of reperfusion after a long period of ischemia [105]. A recent study that compared the efficiency of this method to EPO treatment by evaluating the size of the infarct showed that EPO exerts a better protective effect than IPost against reperfusion injury [106].

The rapid effect of cardiovascular protection induced by pretreatment with EPO, with regard to I/R and MI, involves a mechanism distinct from hematopoiesis. The cardioprotective effects of EPO are achieved primarily through the inhibition of myocardial apoptosis, limiting the infarct expansion, and attenuating the post-infarct deterioration of hemodynamic function, the reduction of inflammation, and the induction of angiogenesis (Figs. 2 and 3). Interestingly, following MI, the heart seems to be able to produce erythropoietin, albeit a negligible amount, which is probably induced by HIF-1α, the main transcriptional regulator of erythropoietin [107,108] (Fig. 2).

The first mention of EPO cardioprotection was made in 2003 after *in vitro* and *in vivo* experiments in rodents [109–113]. In 2004, Wright et al. confirmed, using a rat model, the action of EPO in cardiovascular protection and the involvement of the preservation of ATP levels in the ischemic myocardium [114]. At the same time, Shi et al. showed in a rabbit model that the rapid activation of potassium channels and protein kinases by EPO represents an important new mechanism for increasing cardioprotection [115]. Primary investigations on the signaling pathways involved in EPO-mediated cardioprotection demonstrated the phosphorylation and activation of JAK1/2, STAT3, STAT5a and PI3K as well as the activation of PKCε, RAF, MEK1/2, p42/44-MAPK and p38-MAPK [116] (Fig. 3).

The cardiac inflammation and apoptosis of myocardial cells are two distinct phenomena in the deterioration of cardiac function, but both are triggered during I/R or MI. The regulation of these phenomena by EPO borrows a common part of the signaling pathway. In recent years, several independent teams working on different models have involved the same signaling pathway, with little variance in the regulation of cardiomyocyte apoptosis and inflammation by EPO. The first key molecule highlighted in this regulation is AKT, already known to be involved in the regulation of apoptosis, including cardiomyocyte apoptosis. AKT normally acts through the inhibition of proteins such as Bcl2-antagonist of cell death (BAD), Caspase 9 (CASP9) or forkhead receptor ligand (FKRL)-1 [117,118]. The implication of AKT in cardioprotection and the inhibition of apoptosis and inflammation triggered by EPO appear to involve other signaling pathways (Fig. 3).

3.1.1. Inflammation

The key molecules of inflammation are TNFα, Cyclooxygenase (COX)-2 and IL-1β. Several articles have highlighted their involvement in the inflammation of the myocardium and have shown that IL-1β and TNFα are produced in the myocardium and mainly in the cardiac fibroblast [119,120]. The first studies linking EPO and inflammation showed an inhibition of inflammation by the induction of PI3K, Activator Protein 1 (AP-1) and endothelial nitric oxide synthase (eNOS) *via* AKT activation [121], as well as the inhibition of the myocardial expression of IL-6, TNFα, and IL-1β *via* STAT3/5 signaling [122] (Fig. 3). However, a study using later time points reported the induction of IL-10 and the inhibition of IL-6, IL-1β, TNFα, NFκB and, surprisingly, AP-1 [123]. The kinetics of AP-1 activation should therefore be studied in depth.

Proinflammatory protein COX-2 was also studied in the regulation of inflammation by EPO. On the one hand, the inhibition of COX-2 myocardial expression is coupled with the activation of the extracellular signal-regulated kinase (ERK)1/2 and is indepen-

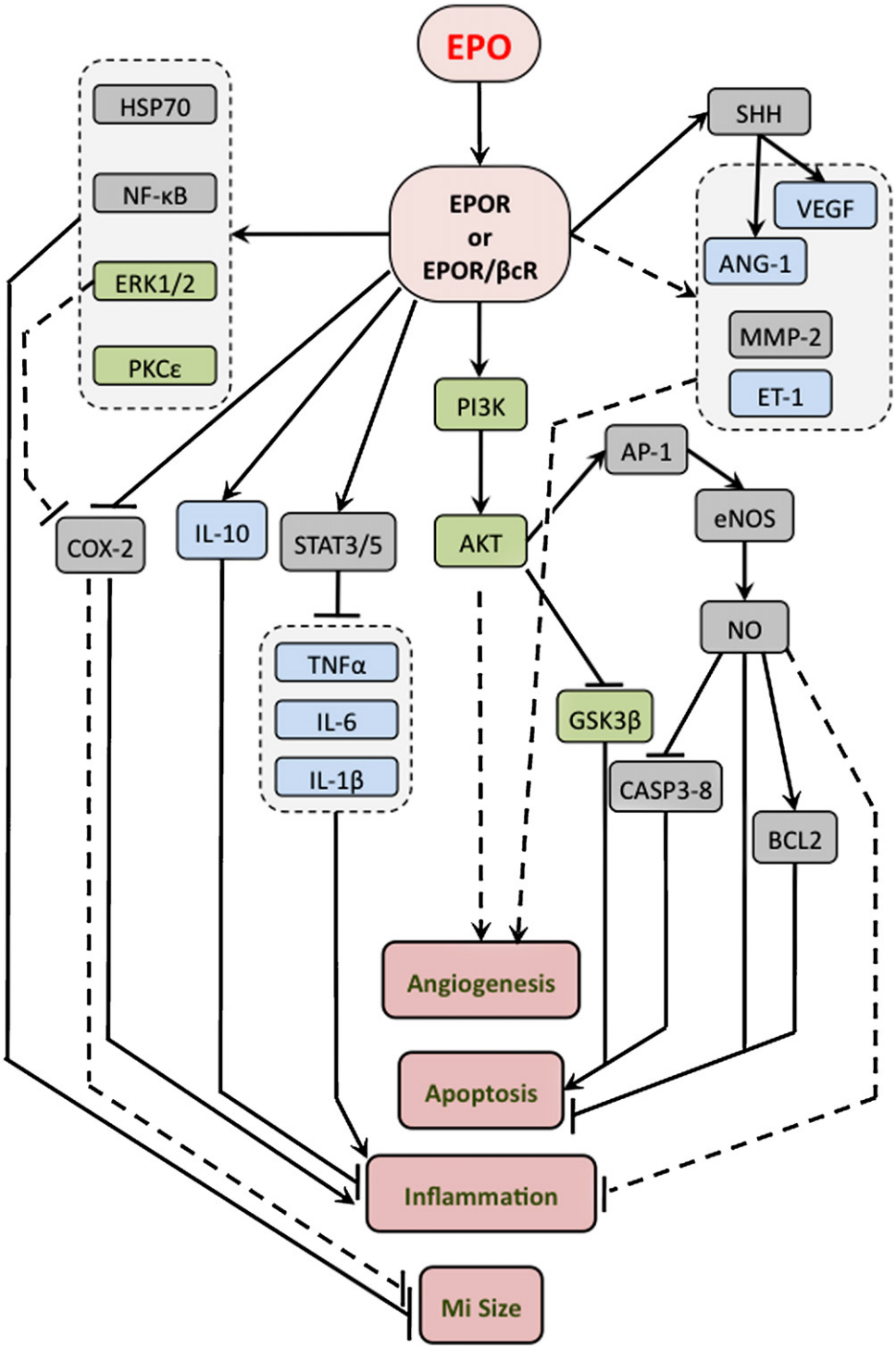

Fig. 3. Overview of molecules and pathways implicated in tissues protection and leading to inhibition of apoptosis, inhibition of inflammation, induction of angiogenesis, and decrease of Myocardial Infarction (MI) size. Solid lines: established relationship; Dashed lines: direct relationships not proved; arrow: positive regulation; Bar: inhibition. Green proteins: reperfusion injury salvage kinases (RISK); blue proteins: cytokines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

dent of AKT and STAT5 modulation [124]. On the other hand, EPO induces the expression and activity of COX-2 and subsequently the production of its products, such as prostaglandin PGE2 and PGF2α, leading to a decreased infarct size [125] (Fig. 3).

3.1.2. Apoptosis

The antiapoptotic effect of erythropoietin on cardiomyocytes was discussed for the first time by Calvillo et al. [110] and was validated in several articles. The signaling pathways were defined gradually from a first observation *in vivo* and *in vitro*, showing that AKT is the central molecule [113,126]. AKT induces eNOS, NO, and Bcl2 [127]; inhibits CASP3 and CASP8 [128]; and is activated by EPOR through PI3K [129] (Fig. 3).

Glycogen synthase kinase-3 beta (GSK-3β) is known to be involved in the apoptosis of myocardial cells by inhibiting Bcl2 and activating the proapoptotic protein BAX. EPO inhibited apoptosis through the activation of AKT and the phosphorylation of GSK-3β to its inactive form [130]. Kim et al. completed these observations,

showing that the EPO-mediated GSK-3β inactivation is PI3K-dependent in mice cardiomyocytes [131]. Some of the elements not validated in this study are in favor of the action by GSK-3β on NO and Bcl2, but this option deserves more targeted studies (Fig. 3).

3.1.3. Angiogenesis

The heart ischemia corresponds to a decrease in blood flow toward the myocardial cells. Angiogenesis is the formation of new capillary vessels from the preexisting vessels, thus optimizing the oxygen delivery to tissues. This mechanism is a solution for improving cardiac function by restoring perfusion. The endothelial cells of the blood vessels express EPOR and are able to proliferate in response to treatment with EPO [132,133]. Many studies examining the effect of EPO on endothelial cell proliferation or blood capillary formation have validated these data. However, few studies have provided a complete mechanism. The regulation of angiogenesis can be achieved through Endothelin-1 (ET-1), as the use of anti-endothelin-1 antibody blocks EPO-induced angiogenesis [134]. However, no further studies have been conducted on this mechanism. Other studies have implicated JAK2 phosphorylation or the production of matrix metalloproteinase-2 (MMP2) without validating the hypothesis by using specific inhibitors. Finally, EPO regulates the expression of VEGF, a cytokine involved in the proliferation of endothelial cells, *via* EPOR [135,136]. With regard to the regulation of VEGF expression in the mediation of EPO-induced signals, a recent *in vitro* study showed that a deletion of Sonic hedgehog (SHH) abolished the EPO-induced cardioprotection, highlighting the central role of SHH [137]. Furthermore, results also showed the involvement of angiopoietin-1 (ANG-1), and finally, unlike the aforementioned studies, there was a lack of STAT3 effect in this model of cardioprotection (Fig. 3).

In a very interesting study, Teng et al. recently focused on the *in vivo* effect of EPO on endothelial cells. The study examined ΔEPOR-mice, which expressed EPOR only in hematopoietic and endothelial cells. In addition to confirming the involvement of the AKT/NO/eNOS and the MEK/ERK pathways, the study results suggested that the endothelial response is probably sufficient for an acute EPO-mediated cardioprotection effect [138] and that therefore, this cardioprotection does not necessarily require an effect on myocardial cells.

Despite the importance of studies that validate the involvement of EPO in regulating angiogenesis, especially after myocardial ischemia, the exact principle of this regulation has not been completely elucidated; some remaining contradictions must be considered.

The term Reperfusion Injury Salvage Kinase (RISK) pathway is currently used to define the group of protein kinases involved in reperfusion injury cardioprotection, and these include PI3K, AKT, ERK1/2 and MAPK [139]. The cardioprotection mediated by EPO has some limits. Some diseases affect one or more signaling pathways activated by EPO; for example, diabetes mellitus affects the signaling pathway of RISK. In a type 1 diabetes rat model, EPO neither decreased the infarct size nor increased the phosphorylation of AKT, ERK1/2, and GSK-3β. A direct inhibition of GSK-3β was proposed to provide an alternative strategy to protect diabetic hearts against I/R injury [140] (Fig. 3).

3.2. Neuroprotection

The EPOR protein is expressed in the brain by different cell types, such as neural progenitor cells (NPC), astrocytes, neurons and oligodendrocytes. Brain cells may also produce EPO under hypoxic conditions to enable NPC proliferation and differentiation. In experimental models of hypoxia-ischemia (HI) or stroke, an injection of EPO can reduce the size of lesions and brain damage [116].

3.2.1. EPO in brain development

EPO and its receptor are expressed in specific regions of the embryonic and adult brain (shown in the rat, monkey and human), and this expression decreases dramatically during development. Liu et al. effectively demonstrated that EPOR mRNA expression decreased by 1 to 3% of the level observed in hematopoietic tissues in mice [141]. EPO appears to be mainly expressed by astrocytes, while EPOR is expressed by neurons [99], which corresponds to the neurotrophic activity of EPO in the cerebral system during development. This hypothesis was supported by mice experiments in which EPO or EPOR was knocked out, leading to embryonic death. The cause of death was due not only to erythropoiesis failure, but also to the failure of brain development, as there was slowed proliferation and increased sensitivity to hypoxia [142]. This study also showed that the conditional knockdown of EPOR leads to a specific deficit in post-stroke neurogenesis by the impaired migration of NPC to the peri-infarct cortex. Several other studies support the mechanisms of EPO in neural development. Indeed, EPO stimulates the differentiation from pluripotent progenitor cells to NPC *in vitro* [143] as well as the neural differentiation of NPC from the subventricular zone [144]. In a model of HI, EPO stimulates neurogenesis in the subventricular zone and promotes the migration of neural progenitors to the injured cortex and striatum [145].

3.2.2. EPO in neuroprotection

EPO has repeatedly been reported to possibly play a role in neuroprotection in adults, but the high molecular weight of its native form, EPO has difficulties in passing through the blood–brain barrier (BBB). For an intravenously injected dose of 5000 U/kg, less than 2% of EPO passes the BBB in the 3 h following injection [146]. Therefore, an inappropriately large injection dose of EPO to achieve effective brain concentrations may be associated with side effects (thrombotic complications, increase in blood pressure, adverse cardiovascular complications in patients with kidney disease, chronic renal failure). However, in some pathologic conditions such as the premature or HI brain, the BBB can be dysfunctional or damaged, resulting in increased permeability.

The non-hereditary injury that affects the central or peripheral nervous system is usually related to trauma, HI, or toxicity. Global or focal cerebral ischemia is followed by damage that is characterized by oxidative stress, excitotoxicity, inflammation and apoptosis.

When neural and astrocyte cells are treated under hypoxic conditions or treated with desferrioxamine (DFX) and cobalt chloride ($CoCl_2$) and/or inhibited by H_2O_2, the hypoxic stress increases the *in vitro* expression of EPO [147]. HI accelerates neuronal cell death not only by necrosis, but mainly by apoptosis [148]. Moreover, EPO protects against neuronal apoptosis through JAK2/STAT5 activation and regulates the balance between proapoptotic and antiapoptotic pathways, with the induction of Bcl-X_L and Bcl-2 expression [149]. EPO also inhibits CASP3 and CASP9 [150] and regulates BAX through the JAK2 signaling pathway [151] (Fig. 3).

EPO protects against HI injury through angiogenesis *via* NF-κB phosphorylation and activation of AKT and PI3K [152]. This mechanism affects neurovascularization through the revascularization of the ischemic zone [153] to improve oxygen delivery in the brain. Moreover, this neurovascularization can promote the production of neuronal stem cells and neuronal and neural progenitors [143] to increase neurogenesis in the ischemic zone [145]. Finally, EPO promotes the differentiation of neural stem cells into astrocytes [154] or oligodendrocytes [155]; such differentiation is correlated with ERK and NF-κB activation. The EPO capacity to improve the elongation of neurites in spinal ganglion cells has also been reported [156] (Fig. 3).

A recent study improved our knowledge regarding neural angiogenesis and the TNFα/EPO/VEGF relationship [157] by showing that TNFα interacts with its receptor TNF-receptor 1 (TNFRI) and sensitizes neural endothelial cells by inducing the expression of EPOR, thereby increasing EPO-induced angiogenesis by amplifying the activation of the VEGF/VEGFR2 and ANG-1/TIE2 pathways [158] (Fig. 3).

In neural inflammation, IL-1β corresponds to the early response and induces the synthesis of other cytokines and of the infiltration of leukocytes. An injection of EPO delays the increase in IL-1β expression and decreases leukocyte infiltration [159]. Moreover, EPO inhibits the production of proinflammatory cytokines that is induced by toxicity or infection [160,161].

The excitotoxicity/neurotoxicity of glutamate is mediated by the N-methyl-D-aspartate receptor and can be improved by treatment with EPO, which involves the cross-talk between the JAK2/STAT and PI3K/AKT signaling pathways [162] and may be through the PI3K-dependent activation of Ca^{2+}-activated K^+ channels [68]. In a similar manner, EPO reverses the increase of lipid peroxidation and the decrease of antioxidant levels induced by alcohol intake [163].

A recent study provided another insight of the neuroprotection induced by EPO. In this *in vivo* study, which uses EPOR-null mice, EPO protection occurs through the upregulation of antiapoptotic proteins p-AKT and Bcl-X_L in the ipsilateral hippocampus and cortex [164], suggesting that a separate pathway independent of EPOR may be borrowed to mediate this protection.

3.3. Other protections

3.3.1. Pancreatic disorder

In 2003, Fenjves et al. showed that pancreatic islets express the EPOR protein [104]. Subsequently, several studies have examined the effects of EPO on islet cells and especially the effect of this cytokine in the case of types 1 and 2 diabetes or other pancreatic disorders. Types 1 and 2 diabetes mellitus are chronic disorders induced by insulin insufficiency, which results in the deregulation of glucose homeostasis, inducing hyperglycemia and then vascular complications. Diabetic pathologies have distinct mechanisms, but the common element between types 1 and 2 is the insufficiency of the functional pancreatic β-cell mass that is required to maintain euglycemia.

Several papers demonstrated that erythropoietin can provide a beneficial or possibly protective effect in diabetic patients through a direct effect on β cells [165–168]. Data from He et al. suggested that EPO can protect neonatal islet cells (in the porcine model) through the upregulation of Bcl-2 and downregulation of BAX and CASP3 [169] (Fig. 3). Finally, results provided recently by Shuai et al. suggested the requirement of the PI3K/AKT pathway [167], which was already described for cardioprotection. There is also the possibility of an acute necrotizing pancreatitis protection by EPO, but more investigation is needed to clarify that role [170].

Importantly, pancreatic cancer cells also express EPOR, and a study on the effect of EPO in pancreatic cancer showed that EPO enhanced the proliferation of these cells (in a rat model), and this proliferation may correspond to an acute side effect of long-term treatment with EPO [171].

3.3.2. Retinal protection

The retina is the most metabolically active tissue in the human body and is highly sensitive to reductions in oxygen tension or traumatisms. EPO is expressed in the adult human retina [172], and EPO has a potent neuroprotective effect in the retina. EPOR expression has been detected in the mouse retina [173] and in the healthy human fetal retina [174]. More recently, EPO expression was highlighted in not only the retina of diabetic retinopathy patients, but also in non-diabetic donors [175]. Moreover, EPO and EPOR are present in the vitreous of healthy donors, again with a higher level found in diabetic donors.

Numerous studies show the protective effect of EPO in non-inherited or inherited retinal degenerations [97,176]. This phenomenon is independent of erythropoiesis and is most likely mediated by the EPOR/βcR receptor [97]. Recently, it was shown that the resistance of retinal progenitor cells (RPC) to hypoxic and superoxide stress is mediated in part by EPO [177]. Nevertheless, the action of this molecule may be partly the basis of the resistance of retinal tissues to moderate and severe stresses due to low oxygen levels. This resistance is mediated by the PI3K/AKT/mTOR pathway [177] and has been validated *in vitro* using specific inhibitors of this signaling pathway and correlated with AKT phosphorylation. In the case of hypoxia and also in the case of white light damage, EPO is induced by HIF-1α [178] (Fig. 3).

3.3.3. Auditory protection

Hearing loss is one of the most relevant chronic diseases in the elderly, but indeed, 1 in 1000 children is deaf by age 3. In the cochlea, supporting cells and hair cells composes the organ of Corti. The latter cells are responsible for hearing, but they are the most fragile part of the ear. The death of these cells usually occurs as a result of trauma or exposure to toxins and occurs through apoptosis, mediated by the activation of caspases.

EPO expression is exclusively in the supporting cells of the organ of Corti [179]. EPOR is expressed in some cells of the stria vascularis, endothelial cells, auditory hair cells and the supporting cells of the organ of Corti in newborn and adults rats [180]. First, *in vitro* studies showed a protective effect of EPO on hair cells against ischemia-induced and gentamycin-induced damage, albeit with a low efficiency compared with the control [180,181]. Until now, none of the *in vivo* models were conclusive for the effect of EPO in the protection of hair cells, and this field seems poorly represented. However, a study linking HI, hearing and learning modeled the carotid HI in postnatal rats, concluding that EPO affects behavioral and neurological protection, but proposed no explanation for these results [182].

3.3.4. Others

Finally, it is impossible to list the numerous studies showing the beneficial effects of EPO on non-hematopoietic tissues; these studies do not provide a mechanistic explanation of the effects.

EPO can affect bone reconstruction. This effect was shown a few years ago in an experiment involving the injection of EPO between 1 day before and 4 days after a fracture. The results showed a significant increase in the rate of calcification and the expression of EPOR in terminally differentiating chondrocytes within the callus until 2 weeks after the fracture [183].

EPO plays a role in the restoration of dysfunctional microvasculature, which has been demonstrated in murine striated muscle [184]. This angiogenic effect observed seems to depend on eNOS but not inducible NOS (iNOS), when antiapoptotic effect of EPO is maintained is this model independently to both NOS molecules [185].

In rats with unilateral urethral obstruction and treated with a non-erythropoietic EPO derivative (CEPO), CEPO decreases tubular apoptosis and alpha-smooth muscle actin (ASMA) expression in the absence of polycythaemia, and in these same rats, the use of HuREPO induces the same results in addition to a wedge-shaped infarction [186].

4. Conclusion

Since recent years, much evidence through *in vitro* and *in vivo* studies has undoubtedly established that erythropoietin has a

tissue-protective capacity against some trauma, toxins and other damages that are hereditary and non-hereditary. Tissue protection is mainly mediated by the inhibition of apoptosis, inflammation and induction of angiogenesis. This protective ability could be characterized by an increase in native EPO in the injured tissue (such as cardiac and neural tissue); however, the non-hematopoietic tissues produce an amount of EPO unable to induce a protective effect. The protective effect can then be obtained through the injection of recombinant EPO. Nevertheless, such treatment involves high doses of EPO administration, which can lead to serious side effects. Indeed, most EPO-mediated tissue-protection occurs through binding to the heterodimeric receptor EPOR/βcR, which has a lower affinity for EPO than the homodimeric receptor. Moreover, in the case of neuroprotection, EPO needs to pass through the BBB. In this regard, EPO derivatives are being developed to cross the BBB more easily, decrease administration frequency, increase plasma half-life, decrease side effects and avoid cross-talk with hematopoietic activity.

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