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Chapter 14

The HIF System Response to ESA Therapy in CKD-Anemia

Sandra Ribeiro, Luís Belo, Flávio Reis and Alice Santos-Silva

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Abstract

Anemia is a common complication of chronic kidney disease (CKD) associated with disease progression and increased mortality. This anemia is mainly due to inadequate production of erythropoietin (EPO) by the failing kidneys, resulting from the reduction in renal EPO-producing cells (REPC) or from dysregulation of the hypoxia-inducible factor (HIF) system that regulates several genes related to hypoxia, angiogenesis, fibrosis and glucose metabolism, among others. In this chapter, we present a review on the HIF system in CKD-anemia, the HIF response to erythropoiesis-stimulating agents (ESA) therapy and its potential involvement in the development of ESA resistance by enhancing kidney fibrosis and inflammation. Due to concerns related to ESA use, new drugs to correct anemia are under study, being the prolyl hydroxylase inhibitors the most promising candidates.

Keywords: chronic kidney disease, erythropoietin resistance, fibrosis, HIF system, Hypoxia, inflammation

1. Introduction

Anemia is a common complication of chronic kidney disease (CKD) that often develops early in the course of the disease, and its frequency and severity increase with the decline of renal function [1]. This condition is associated with a decreased quality of life [2, 3], increased hospitalizations and comorbidities [4, 5], progression of renal dysfunction [6–8], enhanced cardiovascular complications [9, 10] and mortality [11–13]. The main cause for anemia in CKD patients is erythropoietin (EPO) deficit, due to decreased hormone production by the failing kidneys; other factors can also contribute to the development or worsening of CKD-anemia, such as iron deficiency, inflammation and uremic toxins, among others [14].

EPO is a glycoprotein that presents several functions acting as a hormone, cytokine or growth factor on target cells that express the EPO receptors (EPOR), through different pathways. In the bone marrow, EPO controls cell proliferation, differentiation and death of erythroid cells.

During fetal life, the majority of EPO is produced by the liver; after birth there is a switch to renal production, and in the adulthood, 90% of this hormone is produced by the kidneys, whereas the liver is a secondary site of production [15]. EPO is also expressed in the brain, spleen, lung and testis, but its contribution to serum EPO levels is not clarified [16]. The kidney cells responsible for EPO production are still under debate, but several studies showed that renal EPO-producing cells (REPC) include the peritubular fibroblast-like interstitial cells in the inner cortex and in the outer medulla [17, 18], the proximal and distal convoluted tubules and cortical collecting ducts [19]. REPC are sensitive to changes in oxygen (O_2) tension, and in conditions of hypoxia, the kidney responds increasing the number of REPC capable of producing EPO [20].

In CKD, the severity of the disease defines the kidney capacity to produce EPO [21, 22]. Indeed, patients with GFR >30 mL/min/1.73 m^2 are still able to induce a physiologic response to anemia, showed by the normal or even elevated serum EPO levels [23, 24]. Nevertheless, serum EPO levels may not be sufficient for the degree of anemia; actually, anemic patients with normal renal function may present a 10-fold to 100-fold increase in serum EPO levels [25, 26], to achieve correction of anemia.

The kidney is the major site of EPO production in the adults; however, it is possible that extrarenal sites contribute for the marked rise in plasma EPO in end-stage renal disease (ESRD) patients [27], as already showed in animal models of kidney injury [28, 29]. It was also reported that patients with anemia can switch EPO production from the kidney to the liver [30, 31], as can be shown by glycoform analysis of EPO. Indeed, the posttranslational EPO glycosylation is specific of the synthesizing cells, giving rise to different EPO glycoforms that can be used to localize EPO synthesis [30, 32].

Hypoxia regulates the EPO gene through the hypoxia-inducible factor (HIF) system [20]. This HIF system includes O_2-dependent HIF-1α, HIF-2α (also known as endothelial PAS domain-containing protein 1) and HIF-3α subunits, and the constitutively expressed HIF-1β and HIF-2β subunits (also known as aryl hydrocarbon receptor nuclear translocator). The HIF-α subunits are hydroxylated in specific proline residues, by the prolyl-4-hydroxylase (PHD) proteins that require O_2 as a co-substrate (**Figure 1**). The hydroxylated HIF-α subunit targets the von Hippel-Lindau tumor suppressor protein (VHL) to be recognized by an ubiquitin ligase complement that will induce a rapid ubiquitination and proteasomal degradation of HIF-α subunits. Under normoxia, HIF-α subunits are almost undetectable, but in hypoxic conditions, the hydroxylation by PHD proteins is inhibited; thus, the HIF-α accumulates in the cytoplasm, is translocated to the nucleus and binds to the HIF-β subunit, forming a complex that recruits the coactivators P300/CBP and activates the transcription of several genes [20].

Several genes are regulated by the HIF-1α and HIF-2α subunits (**Figure 1**), but recent studies showed that HIF-2α is the main regulator of EPO synthesis in the kidney and liver [33–35] and is also important for the regulation of several factors involved in iron homeostasis, as iron is an

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important element for hemoglobin (Hb) synthesis [36]. The HIF-1α subunit activates the transcription of glucose metabolism, angiogenesis and fibrosis related genes to promote wound healing [37]. The role of HIF-3α is still ambiguous and under current investigation. It is known that HIF-3α presents several isoforms with different roles [38]; the up-regulation of some HIF-3α isoforms appears to act as a negative feedback mechanism to regulate HIF-1α and/or HIF-2α subunits; however, recent studies showed that HIF-3α might share with HIF-1α the regulation of some genes [39].

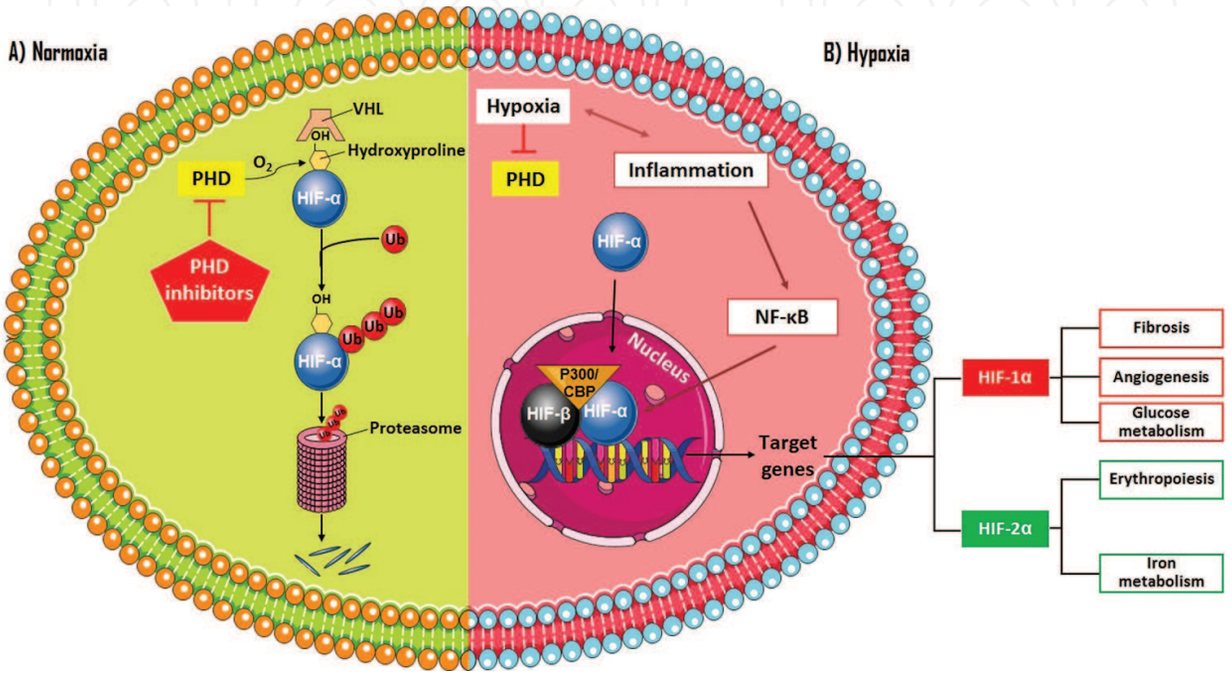

Figure 1. Regulation of hypoxia-inducible system. (A) In conditions of normoxia, the HIF-α subunits are hydroxylated, in specific proline residues by prolyl-4-hydroxylase (PHD) proteins, which recruit the von Hippel-Lindau tumor suppressor protein (VHL) a signal for rapid ubiquitination and proteasomal degradation of HIF-α subunits. PHD inhibitors are under development, as they might impair the degradation of the HIF-α subunits, improving anemia. (B) Under hypoxic conditions, the PHD proteins are inhibited, and consequently, the HIF-α subunits are not targeted by VHL protein for degradation, translocating to the nucleus and binding to the HIF-β subunit, forming a complex that recruit the coactivators P300/CBP, leading to the transcription of several genes that will depend on the type of HIF-α subunit (HIF-1α or HIF-2a) that binds to the target gene sequences. There is a crosstalk between hypoxia and inflammation, leading to the activation of the nuclear factor kappa beta (NF-κB) pathway that can also induce HIF-1α accumulation.

This chapter reviews the HIF response to erythropoiesis-stimulating agents (ESA) therapy focusing on its potential involvement in the development of ESA resistance, by enhancing kidney fibrosis and inflammation.

2. Hypoxia and progression of renal disease

Renal hypoxia is well known as an important contributor for the progression of renal disease. A study conducted in a rat model of diabetic nephropathy reported that intrarenal hypoxia develops early in the course of the disease and precedes the alterations in circulating biomarkers of kidney damage [40]. Irrespective of the initial cause of CKD, the histopathological

analysis of renal biopsies showed that fibrosis is the common final pathway [41]. The underlying mechanisms are still debatable.

Glomerular injury leads to a reduction in glomerular blood flow and consequently limits blood flow into peritubular capillaries, causing hypoxia and tubulointerstitial injury [42]. After an initial injury, the tubular cells will attempt to correct and repair the injury by recruiting and activating several cells, such as macrophages, fibroblasts and epithelial tubular cells that will release pro-inflammatory cytokines and fibrosis factors, and contribute to excessive interstitial extracellular matrix (ECM) accumulation and expansion. Transforming growth factor beta (TGF-β), a recognized pro-fibrotic factor, appears to be central for fibroblast activation, proliferation and transdifferentiation, contributing to ECM deposition [43]. TGF-β also presents immunomodulatory effects on macrophages and monocyte recruitment, leading to the production of inflammatory cytokines [44]. In early renal injuries, M2-type macrophages are recruited to promote tissue remodeling; however, if the injury is continuous, more inflammatory monocytes will be recruited differentiating their phenotype into M1-type macrophages, responsible for the release of pro-inflammatory cytokines (such as tumor necrosis factor [TNF-α], interferon [IFN]-γ, interleukin (IL)-1β and IL-6) and cell apoptosis [45]. The release of these pro-inflammatory cytokines leads to the activation of the nuclear factor kappa B (NF-κB) pathway, thus amplifying the inflammatory process [44]. The continuous activation of this system will culminate with the formation of scar tissue or fibrosis. The presence of fibrotic tissue reduces the diffusion of O_2, which will further aggravate the hypoxic environment.

Anemia caused by inadequate EPO production by the kidneys also contributes to renal hypoxia. However, the mechanisms underlying the reduced capacity for EPO production by the REPC are not well understood. It has been proposed that after renal injury, REPC can suffer a transdifferentiation, called epithelial to mesenchymal transition (EMT), into myofibroblasts, losing their capacity to synthesize EPO and increasing the synthesis of collagen, contributing to the expansion of ECM [46]. Nevertheless, this EMT phenomenon was never proved in humans. The residual capacity to increase serum EPO levels when subjected to hypoxic environment or high altitudes by renal patients, even those on dialysis [47], indicates that a dysregulation of the HIF system, more than a complete loss of REPC cells, could be responsible for the reduced EPO production. Moreover, the pharmacological inhibition of the PHD in CKD patients stimulates endogenous EPO production further supporting a deranged oxygen sensing [27]. A recent study in mice by Souma et al. [48] also strengthened this hypothesis, by showing that inflammatory cytokines and/or fibrosis factors suppress HIF activation through the over-activation of PHD even under pathologic hypoxic conditions, and that the inhibition of PHD restores EPO production.

3. Erythropoiesis-stimulating agents in CKD-anemia

The standard treatment for CKD-anemia is based on pharmacological intervention, using ESA and/or iron supplementation, in order to correct and maintain Hb concentration in the range of 10–11.5 g/dL [49]. ESA are medicines produced by recombinant DNA technology with similar

structure and biological activity of EPO. They differ from EPO by the different patterns of glycosylation that increases their half-life.

The use of ESA has beneficial effects by correcting anemia and their associated symptoms and improving patients' quality of life [50, 51]. However, the effects of ESA on the progression of renal function are controversial. Some studies showed that after starting ESA therapy and correction of anemia, renal function declines at a slower rate, delaying the need for dialysis in pre-dialysis patients [52–54]; in opposition, other studies reported that ESA do not significantly affect renal function [55, 56].

ESA were designed to correct anemia, but some evidences showed that these drugs (and EPO) may act beyond hematopoiesis. Pleiotropic effects have been attributed to EPO and ESA, such as cytoprotection, anti-apoptosis, anti-inflammatory and angiogenesis [57]. These non-hematopoietic actions appear to result from the activation of another EPOR, a heterodimeric receptor constituted by the EPOR homodimer complexed with CD131, the common beta receptor (βCR) that is involved in granulocyte macrophage colony-stimulating factor, IL-3 and IL-5 signaling [58]. The two EPOR present different affinities for EPO; in erythroid cells picomolar concentrations of EPO are sufficient to trigger activation of the EPOR homodimer, whereas on other cells and tissues high local EPO concentrations are needed to activate EPOR heterodimer [59]. This receptor was detected in several cells and tissues, such as brain (neurons, astrocytes and microglia), kidney, female reproductive system organs, vascular endothelial cells, cardiomyocytes, lymphocytes and monocytes, among others [57].

The slower progression of renal dysfunction observed in some CKD patients may result from renoprotection of ESA therapy. Several studies on acute kidney injury (AKI) reported that a single dose of recombinant human EPO (rHuEPO) reduces kidney dysfunction through anti-apoptotic mechanisms and increases NO production, only in intact vessels [60]. ESA therapy also exerts renoprotective effects by reducing the production of pro-inflammatory cytokines (e.g., IL-1β and TNF-α), acute phase proteins [e.g., C-reactive protein (CRP)], pro-fibrotic factors (e.g., TGF-β) and oxidative stress [61]. However, these effects appear to be only achieved with low doses of ESA, as high doses increase hematocrit and may activate platelets, increasing their adhesion to the injured endothelium, contributing to hemorheologic changes [60]. Indeed, other side effects are associated with ESA therapy, namely hypertension [62] and thrombotic events [63].

Despite the benefits of ESA therapy, some concerns have emerged from studies reporting a high incidence of cardiovascular events and mortality in CKD patients treated with ESA [63, 64], independently of the type of ESA used [65, 66]. Since the introduction of ESA therapy, several clinical trials aimed to define the better Hb target/ESA dose associated with lower cardiovascular risk. Indeed, recent studies reported increased cardiovascular risk and death in patients treated with high ESA doses to achieve higher Hb levels [9, 67–69].

The need for new drugs with lower associated cardiovascular risk opened a growing area of research. The most promising are the PHD inhibitors (**Table 1**) with several compounds already under evaluation in clinical trials. Some of these compounds showed to be well tolerated, corrected anemia in non-dialysis CKD and incident dialysis patients without

increasing blood pressure, and also reduced serum hepcidin levels [70–73]. However, regarding their effects in reducing cardiovascular events and slowing the progression of the renal disease, no data are still available from human studies. Yu et al. [22] showed that the administration of PHD inhibitors in a more advanced stage of CKD in the rat reduced renal fibrosis and protected renal function, whereas the administration in an early stage of CKD promoted renal fibrosis and exacerbated renal dysfunction. In another strategy to induce EPO production, the hydrodynamic gene transfer of a plasmid encoding for EPO in a rat model overexpressing TGF-β showed that this therapy increased Hb levels but had no effect on kidney fibrosis or function [74].

PHD inhibitor	Route administration	ClinicalTrials.gov Identifier
Molidustat(BAY85-3934)	Oral	• NCT02064426
Roxadustat(FG-4592)	Oral	• NCT01630889
		• NCT01887600
Vadadustat(AKB-6548)	Oral	• NCT01906489
		• NCT02648347
		• NCT02680574
GSK1278863	Oral	• NCT02689206

Table 1. Prolyl-4-hydroxylase (PHD) inhibitors in clinical trials.

4. Hyporesponsiveness to erythropoiesis-stimulating agents in CKD

The majority of CKD patients respond adequately to the currently available ESA therapy, but 5–10% of them do not respond properly, developing hyporesponsiveness to these drugs [75]. According to the KDIGO guidelines [49], CKD patients can present initial or acquired ESA hyporesponsiveness; in primary hyporesponsiveness patients, after one month of treatment with adequate weight-based ESA dose, the target Hb concentration is not achieved; in acquired ESA hyporesponsiveness, after effective treatment with stable ESA dose, achieving the target Hb concentration, the patient requires two consecutive increases (up to 50% beyond the stable dose) in ESA dose. Hyporesponsiveness (also widely referred as resistance) to ESA therapy is associated with a poor outcome, progression of renal disease, sudden death, infectious complications, sudden death and all-cause mortality, mainly due to cardiovascular events in dialysis patients [76–79]. Several causes are associated with poor response to ESA therapy, including iron deficiency, inflammation, malnutrition and hyperparathyroidism, among others [80–82].

4.1. Inflammation

A pro-inflammatory state is a hallmark of CKD, which is due to increased uremic toxins that induce the production of inflammatory cytokines. Additionally, active infections, the vascular

access for hemodialysis (HD) procedure and surgery-related inflammation (vascular surgery included) can also contribute to inflammation.

The activation of inflammatory cells is also associated with increased oxidative stress, favoring alterations in red blood cells (RBC) membrane, namely increased phosphatidylserine exposure, increased membrane bound Hb and increased membrane protein band 3 aggregation, all markers for RBC phagocytosis by macrophages and, thus, for a premature RBC removal [83, 84]. Uremic toxins and pro-inflammatory cytokines also inhibit erythropoiesis, through the inhibitory effect of IL-1β, TNF-α and IFN-γ on early erythroid cell stages in the bone marrow [85]. The macrophages of the bone marrow can also be stimulated to increase local pro-inflammatory cytokines, amplifying the effects of systemic inflammation [86]. In CKD patients, hepcidin synthesis is enhanced, due to the increase in Il-6, contributing for the limited iron availability for erythropoiesis [87]. Indeed, CKD patients often present with replete or even higher iron stores, alongside with inflammation and anemia. A disturbance in the crosstalk between inflammation, iron metabolism and erythropoiesis may, therefore, favor ESA hyporesponsiveness. The best predictors for ESA response appear to be IL-6 and CRP [88, 89]. Studies conducted by our group showed that HD patients with poorer response to ESA present higher levels of pro-inflammatory cytokines [90, 91]; moreover, in studies using a rat model of chronic renal failure, we found that the severity of the inflammatory state was related to the reduction in the rHuEPO response [92].

4.2. HIF system in the hyporesponsiveness to erythropoiesis-stimulating agents

Hyporesponsive patients to ESA therapy will develop anemia, and as already referred, it will promote the progression of renal disease. Tissue hypoxia is amplified according to the severity of anemia that will reduce O_2 availability to body tissues and organs. Within the kidney, the hypoxic environment leads to the activation of the HIF system, promoting the transcription of several target genes. In the hypoxic kidney, HIF-1α is essentially expressed in tubular and glomerular epithelial cells, whereas HIF-2α expression is limited to endothelial and interstitial cells [93]. The localization of these HIF-α subunits is related to their target genes.

Renal biopsies from CKD patients showed that increased expression of HIF-1α in tubular epithelial cells is correlated with the stage of renal disease [94]. It was reported that HIF-α activation in CKD rats presents dynamic changes, as it is activated in early CKD stages and suppressed in the moderate and end-stage of CKD [95]. Thus, the administration of PHD inhibitors may improve renal function in more advanced stages of CKD, while in earlier stages, the PHD inhibitors may increase renal fibrosis due to upregulation of the HIF-1α subunit [22].

HIF-1α subunit is involved in the activation of pro-fibrotic genes (**Figure 1**), including the connective tissue growth factor (CTGF) gene [96]; indeed, the plasma levels of CTGF appear as a good marker for staging diabetic nephropathy progression [97]. CTGF is a potent pro-fibrotic factor and a marker of renal fibrosis, increasing ECM production, promoting EMT, stimulating fibroblasts and potentiating TGF-β signaling [94, 98]. CTGF and TGF-β present similar effects, but TGF-β also presents immunomodulatory actions [44], recruiting macrophages to reduce the injury; however, a continuous macrophage activation leads to

excessive ECM accumulation and increased release of pro-inflammatory cytokines promoting fibrosis. A study by Basu et al. [99] suggested that TGF-β can in turn induce HIF-1α activation, which would amplify cell collagen expression contributing to the progression of fibrosis.

There is also a crosstalk between HIF-1α and inflammation (**Figure 1**). Inflammation favor tissue hypoxia by several mechanisms including: impaired EPO response, iron mobilization and bone marrow erythropoiesis, reduced RBC lifespan and also increased demand for O_2 by the inflammatory cells in order to increase pro-inflammatory cytokines. However, it was also reported that NF-κB can induce HIF-1α activation due to the presence of responsive elements in the promoter of *HIF-1α* gene [100]. Another mechanism is the interaction of PHD with some effectors of the NF-κB pathway, though the exact proteins involved remain unknown [101].

The majority of the studies report a beneficial effect of ESA on renal fibrosis through several mechanisms [29, 102]. However, recently Gobe et al. [103] reported that in rat model of AKI the use of higher rHuEPO doses was associated with increased TGF-β expression, oxidative stress and stimulation of fibroblasts and EMT, contributing to the progression of the disease and gradual development of CKD in the long term. In this study, the expression of HIF-α subunits was not reported, as well as the linking between HIF activation and the alterations observed. Further studies regarding this issue are warranted.

Despite the underlying mechanism, a continuous inflammatory response favoring fibrosis and a disturbance in the HIF system creates a vicious cycle, contributing to the progression of renal disease and aggravation of renal anemia [92], and reducing the response to ESA therapy creating a scenario of hyporesponsiveness to EPO.

5. Conclusions

Anemia is a common complication in CKD patients that can be corrected by the treatment with ESA. However, the development of a hyporesponse to this therapy was associated with (i) the progression of the renal disease, due to the amplification of fibrosis and inflammation through a mechanism involving activation of HIF-1α pathway; (ii) increased risks in the development of cardiovascular disorder events and all-cause mortality in patients treated with higher doses, opened a new research field, focused on the design of more effective agents to control anemia in CKD patients, with less side effects. The use of PHD inhibitors is promising, but further is needed to confirm their effects in the reduction of cardiovascular events and progression of renal disease.

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