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Differential Effect of Erythropoietin and Carbamylated Erythropoietin in Erythroid and Endothelial Cells

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Abstract

This revision provides recent information regarding the signaling pathways involved in cell mechanisms induced by Epo and cEpo that could predict their differential effects on erythroid and endothelial cells as well as open interesting research areas concerning the potential role of tyrosine-phosphatases in controlling growth factor functions.

Keywords: Erythropoietin; Carbamylated erythropoietin; Cell proliferation; Erythropoietin receptor, β-common receptor; Tyrosine phosphatase 1B; Erythropoiesis; Neuroprotection; Endothelial cells

Abbreviations: Epo: Erythropoietin; cEpo: carbamylated Erythropoietin; EpoR: Erythropoietin Receptor; βcR: β-common Receptor; PTP1B: Protein Tyrosine Phosphatase 1B; Jak2: Janus Kinase-2; PI3K: Phosphoinositide 3-Kinase; FOXO3a: Transcription Factor FOXO3a; AKT: Protein Kinase B; IL-3: Interleukin 3; IL-5: Interleukin 5; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; $p27^{kip1}$: Cyclin Inhibitory Protein.

Introduction

Erythropoiesis is an essential process that takes place in the bone marrow and is characterized by the proliferation and maturation of cells committed to the erythroid lineage. The glycoprotein erythropoietin (Epo), predominantly secreted by the adult kidney, is regarded as the major promoter of erythropoiesis, as it stimulates proliferation and prevents the apoptotic death of early (BFU-E, Burst-Forming Units-Erythroid) and late (CFU-E, Colony-Forming Units-Erythroid) erythroid progenitors, thus allowing their further differentiation to the erythroblast and reticulocyte stages, and finally to erythrocytes.

More recently, research aimed at understanding the basic mechanisms of its biological effect has revealed a pleiotropic cytoprotective activity of Epo beyond the regulation of erythropoiesis. These findings have placed Epo as a compound of therapeutical interest in neurodegenerative and cardiovascular diseases, apart from the treatment of anemia.

Experimental evidence of Epo activity in tissues outside the bone marrow has suggested the participation of different membrane receptors for the cytokine. In this regard, the association between EpoR and the *β-common* subunit shared by the IL-3, IL-5 and GM-CSF receptors (βcR or CD131) [1], as well as experimental evidence collected from βcR knockout mice support the hypothesis of a cytoprotective effect of Epo mediated by the EpoR- βcR heteroreceptor [2].

With the aim of broadening the therapeutic spectrum of rhuEPO, the ability of the cytokine to treat anemia of chronic disease is not the only point to be considered, but also its protective activity against inflammation andapoptotic stimuli. In these situations, the erythropoietic activity of Epo could be detrimental for non-anemic patients, as it induces an unnecessary and potentially harmful increase in erythrocyte mass. This has prompted researchers to find derivatives of Epo incapable of erythropoiesis but with a cytoprotective action. One such compound is carbamylated erythropoietin (cEpo), obtained by incubation of rHuEpo with potassium cyanide. This reaction yields a structurally modified cytokine in which one carbamyl group is irreversibly coupled to each of the lysine residues in the Epo molecule, potentially causing functional alterations. In vivo, the isocyanate generated by the dissociation of urea is responsible for the carbamylation of different proteins, which may play a role in the development of various diseases. In this regard, the carbamylation of endogenous Epo could be involved in chronic renal failure patients showing resistance to treatment with recombinant human Epo.

Moreover, carbamylation renders erythropoietin unable to bind the classical $EpoR_2$ receptor [3,4]. Competence assays performed by our group showed that an excess of cEpo inhibited the ability of GM-CSF to stimulate proliferation of TF-1 cells [5], thus suggesting that cEpo binds to the β-common subunit and could utilize the EpoR-βcR heteroreceptor.

After cEpo administration, rats displayed no alterations in their hematological parameters, supporting a loss of biological activity after carbamylation [6]. Comparative studies between the native and the carbamylated Epo have demonstrated an inability of the latter to stimulate the formation of hematopoietic colonies from CD34+ bone marrow cells [7], and to promote erythroid proliferation and survival in mice bone marrow-derived CFU-E, as well as in cultures of the UT-7 and TF-1 human erythroid cell lines [5].

The development of cEpo paved the way for new pharmacological studies in non-hematopoietic tissues, with neuroprotection being one of the most important endpoints. In mice, cEpo was found to prevent tissue damage due to spinal hemisection [8], hipoxia [9], experimental autoimmune encephalomyelitis [10] and focal brain ischemia [11]. Results obtained by our group confirmed that the modification of Epo by carbamylation does not affect its neuroprotective action on SH-SY5Y cells [5]. By carring out inhibition and competence assays, we also observed that cEpo requires both the EpoR and the βcR subunit to prevent apoptosis in neuronal cells. In recent years, the neuroprotective action of the carbamylated erythropoietin has continued to attract attention, as newly reported research shows experimental results about the cellular and molecular mechanisms involved in the process of neuroprotection of cEpo [12,13].

Apart from the nervous system, cytoprotection has also been tested in the cardiovascular system. In this regard, cEpo was shown to prevent myocardium damage in a mouse model of ischemia-reperfusion injury [14] as well as epilepsy-derived myocardiocyte apoptosis [15]. However, the effect of cEpo on endothelial cells has not yet been deeply explored.

Unlike Epo, cEpo was found unable to promote proliferation of mature endothelial cells [7,16]. Similarly, carbamylation impaired the promigratory action of the native cytokine in cultures of the human EA.hy926 endothelial cell line. An investigation of the membrane receptors involved in such effect showed that Epo does not require the βcR subunit to promote endothelial cell migration [16], thus suggesting that Epo may utilize similar transduction pathways in erythroid and endothelial cells. However, in competence assays the promigratory effect of Epo on endothelial cells was impaired by an excess of cEpo [17]. Given that the same tendency was observed in the erythroid lineage, where a high concentration of cEpo blunted the effect of the native cytokine on mice CFU-E cells and on the human UT-7 and TF-1 cell lines [5], it is possible that upon cEpo exposure, the formation of the heteroreceptor reduces the availability of the EpoR subunit, thus interfering with the activity of Epo.

The differential effects of Epo and cEpo in erythroid cells have prompted research to clarify its underlying mechanisms. Cytokine receptors are generally devoided of kinase activity. Instead, they are associated to kinases such as Jak2, which activate by transphosphorylation and become able to phosphorylate other substrates. Although in cultures of erythroid UT-7 and TF-1 cells cEpo induced Jak2 phosphorylation, a research work by our group demonstrated for the first time that cEpo was unable to maintain Akt and the transcription factor FOXO3a phosphorylated for the same period of time as Epo [5]. Dephosphorylation of FOXO3a and its subsequent translocation to the nucleus stimulates the expression of the cell cycle inhibitory protein $p27^{kip1}$. Consistently with the inability of cEpo to stimulate proliferation, $p27^{kip1}$ was found increased in cell cultures exposed to the modified cytokine. cEpo was also found to induce Jak2 and Akt phosphorylation in endothelial progenitors [18] and in mature endothelial cells [17].

Given that the differential effect of Epo and cEpo on cell proliferation could be associated to the silencing of transduction pathways by an increase in the dephosphorylation rate, we also analyzed the expression and activity of the protein tyrosine phosphatase 1B (PTP1B), involved in the inactivation of Epo signalling. cEpo induced PTP1B to a significantly higher level than Epo. Moreover, co-localization of PTP1B and βcR supports the conclusion that the phosphatase inactivates the signal triggered by cEpo much faster than that of Epo, thus interrupting pathways of survival and proliferation [19]. As observed in erythroid cells, cEpo enhanced PTP1B expression in endothelial cells, and the inhibition of this phosphatase allowed this modified erythropoietin to overcome its inability to promote endothelial cell migration [16]. These results may explain, at least in part, the differential effects of Epo and cEpo on erythroid and endothelial cells.

Conclusion

Following carbamylation, cEpo maintains its neuroprotective ability based on its antiapoptotic and antioxidant properties, while it is unable to stimulate erythropoiesis. This makes cEpo a promising agent to prevent an unnecessary increase in erythroid mass in non-anemic patients under treatment for neurodegenerative diseases. However, precise focus upon interference of high doses of cEpo with Epo is required to elucidate the benefits and risks of future therapeutic strategies.

This revision provides recent information regarding the signalling pathways involved in cell mechanisms induced by Epo and cEpo that could predict their differential effects on erythroid and endothelial cells as well as open interesting research areas concerning the potential role of tyrosine-phosphatases in controlling growth factor functions. Further research is required to determine the effectiveness of erythropoietin and

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its derivatives on different tissues with the aim of setting the foundations for the development of conclusive clinical trials.

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