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Review Article

Carbamylated Erythropoietin and Its Role of Tissue Protection

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ABSTRACT

Aim of review: We reviewed the study of carbamylated erythropoietin (CEPO) and its probable mechanism involved in the regulation of tissue-protection.

Methods: Recent published literatures in this area were inclusive and reviewed.

Recent findings: CEPO has equivalent tissue-protective properties as recombinant human erythropoietin (rhEPO) but with non-erythropoietic effects. It shows protective properties on nervous system, heart, kidney and some other organs, which are associated with inhibition of apoptosis, restoration of vascular autoregulation and attenuation of inflammatory responses. However, the underlying mechanism of CEPO responsible for its beneficial effects is still not well-known.

Summary: CEPO gradually turned into a promising drug candidate at least for many diseases caused by ischemia reperfusion injury. The safety and efficacy of CEPO in clinical are warranted in the future.

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Erythropoietin (EPO) is a 34 kDa pleiotropic cytokine synthesized in the renal cortex, which is originally induced by activation of hypoxia-inducible factor-1 and exerts its hematopoietic properties. It is widely used for the treatment of anemia, which is correlated with cancer, surgery, autologous blood transfusion, bone marrow transplantation, and chronic renal failure (1). Furthermore, EPO also exhibits broad tissue-protective activity which responses to tissue damage by activating its corresponding receptors distributed in different organs including neurons, astrocytes, the central nervous system (CNS) and some peripheral organs (2, 3). Recently, the tissue-protection of EPO has been extensively studied in the nervous system, the heart, the kidney and a more limited extent of other organs, including lung injury, bowel injury, skin wounds, etc (4). However, unlike the requirement to induce erythropoietic effect, high dose of EPO is required to exert such protective activities, triggering the unwanted overstimulation of the bone marrow and causing several side effects, including vascular perfusion defects, vasoconstriction, hypertension and thrombosis (5-7). Therefore, many efforts have been made to explore some compounds with selective tissue-protective actions and discard its erythropoietic effect. Fortunately, such a modified version of EPO, carbamylated erythropoietin (CEPO), was elucidated to have equivalent tissue-protective properties as recombinant human erythropoietin (rhEPO) but with non-erythropoietic effects (8). Thus, researchers opened a door to find an interesting candidate for protecting tissues with less unwanted side effects. We reviewed the study of CEPO and its probable mechanism involved in the regulation of tissue-protection and discussed as follows.

CEPO and Its Structure

The original understanding of CEPO derived from the phenomenon of carbamylation in EPO. In patients with end-stage renal disease (ESRD), urea spontaneously changed into cyanate in aqueous solutions at body pH and temperature. Then free-amino acids (F-AA) turned into carbamoyl-amino acids (C-AA) by reaction with cyanate resulting in a decrease of the essential amino acid pool. In 2000, Mun et al. (9) found the carbamylation of EPO in vitro when cyanate was added into EPO for a period of incubation and the increased level of carbamylated EPO occurred with the increasing time of exposure to cyanate and increased concentration of cyanate. The carbamoylation of EPO resulted in a derivatized α-amino group with less biologically hematopoietic than was normal EPO. However, the underlying mechanism of EPO turn to CEPO was not elucidated. It was not until 2004, Leist et al. (8) first described the method to produce CEPO and reported that although the carbamylation of EPO removed its biological activity about hematopoiesis, it exerted tissue-protective effects in multiple neurotoxicity animal models, including cerebral ischemia, spinal cord depression, diabetic neuropathy and autoimmune encephalomyelitis. Afterwards, Warren Pharmaceuticals clarified that the eight lysine residues of EPO, together with the N-terminal alanine in the polypeptide chain provided nine amino groups for carbamylation. As we know, all lysine in EPO were transformed to homocitrulline by carbamylation (Figure 1).

CEPO-Mediated Neuroprotection

Since Leist et al. preliminarily clarified that CEPO could exert neuroprotective effects in multiple neurotoxicity animal models, a series of researches were reported to show similar protective effects. It conducted central neuroprotective effects in disparate models, such as focal cerebral ischemia (10), embolic stroke (11), traumatic brain injury (TBI) (12), and traumatic brain injury-induced diffuse edema (13). Subsequently, CEPO was shown to exert peripheral nerve protective effect, through which it prevented chemotherapy-induced sensory nerve conduction velocity reduction (14), and reduced motoneuron degeneration in vitro and vivo (15). In addition, Wang et al. (16) employed a neurosphere assay to find that CEPO also enhanced neurogenesis and selectively promotes neural progenitor cell differentiation into neurons, indicating that CEPO could mediate cell differentiation. Afterwards, CEPO caused the extensive concern in the treatment of neuropsychiatric diseases including memory and/or emotional associated disorders (17), depression and anxiety (18), which were ameliorated by increasing the NeuN/BrdU double-labeled cells in the dentate gyrus field of the hippocampus. Recently, accumulated researches have documented that CEPO was neuroprotective in many CNS diseases, including Alzheimer's disease (19), Parkinson's disease (20), periventricular leukomalacia (PVL) (21) and other hypoxic-ischemic/inflammatory white matter diseases.

CEPO-Mediated Renoprotection

The kidney is an organ which is highly susceptible to hypoxia. Renal transplantation is a good choice for most patients with end-stage renal failure. For these people, renal ischemia-reperfusion (I/R) injury is an inevitable problem, which caused renal graft dysfunction and early post-transplant complications. In 1999, Westenfelder et al. (22) first demonstrated the functional receptors for EPO (EPO-R) expressed on cortex, medulla, and papilla of both human and rat kidney, suggesting that EPO receptors may play a role in regulating certain physiological functions of the kidney. Afterwards, sharples et al. (23) found a tissue-protective role of EPO, which could attenuate renal I/R injury in rats. Another study found that early treatment with erythropoiesis-stimulating agents (ESA), such as EPO, could slow the progression of chronic kidney diseases (CKD) (24). Similar to EPO, Imamura et al. (25) found CEPO exhibited protective effects to kidneys in a rat ischemia-reperfusion injury model. In the study, CEPO decreased tubular apoptosis with limited expression of α-SMA and promoted tubular epithelial cell proliferation. In a similar rat model of tubulointerstitial injury caused by unilateral ureteral obstruction

(26), a high dose of CEPO (1000 IU/kg) conspicuously suppressed obstruction-induced tubular epithelial apoptosis and decrease α-smooth muscle actin (α-SMA) expression in the absence of polycythaemia. With respect to protection of renal function, CEPO exerted both short-term and long-term therapeutic effects in multiple models. In a model of I/R injury after kidney transplantation (27), CEPO significantly reduced the serum creatinine both at post-transplant of 16 hours and 24 hours, which showed similar effect to darbepoetin alfa. In the long-term, CEPO improved serum creatinine levels than those saline-treated remnant kidney model rats both at 4 and 8 weeks (28). Although the studies about CEPO on kidney were limited, the further studies are promising and needed.

CEPO-Mediated Cardioprotection

Myocardial I/R injury is a major cause of death worldwide, increasing the economic burden to society. Despite major progress and a range of therapies have been made to reduce the impact of reperfusion injury (29), the effective therapies have not been well clarified. EPO could markedly prevent cultured adult rat myocardiocytes from apoptosis in vitro and reduced cardiomyocyte loss in a rat model of coronary ischemia-reperfusion in vivo in the absence of an increase in hemoglobin concentration (HB) (30), which indicates that EPO could directly protect cardiomyocytes independent on its increases in HB concentration. The finding was further confirmed since the discovery of EPO receptors expressed in cardiomyocytes of adult rat (31). Similar to those observed for EPO, CEPO could attenuate staurosporine-induced apoptosis of adult rat or mouse cardiomyocytes in vitro and reduce cardiomyocyte loss after myocardial infarction (MI) of rats in vivo (32). In another similar experiment of a rat model of permanent coronary artery ligation, even a single bolus injection of CEPO could reduce the apoptosis in the myocardial area at risk (33). These findings extended the protective effects of CEPO to other tissues except for the nervous system and kidney. Other than reducing myocardial infarct size after I/R, suppression of post-infarct ventricular remodeling was supposed to play an important role in exhibiting

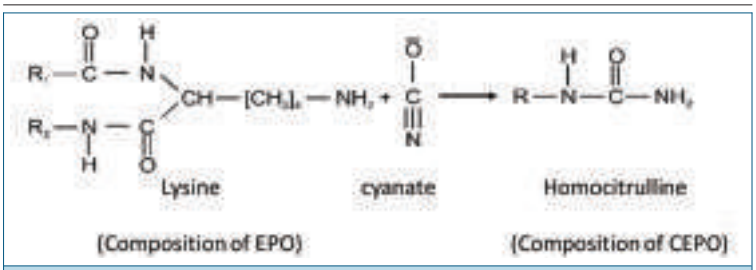

Figure 1. The Carbamylation of EPO.

myocardial protection. In a study by Xu et al. (34), CEPO improved left ventricle (LV) geometry and reduced the decline of systolic, which was consistent with early studies (32, 33). However, Sato et al. (35) recently reported that EPO exerted more potent protection against infarction than CEPO in an ischemia model of isolated rat hearts, and even increasing CEPO dose or shortening ischemia time could not improve infarct size-limiting effect. In the study, they found that EPO improve the levels of phosphorylated forms of Akt, PKC-ε and GSK-3β in mitochondria upon reperfusion than that of CEPO-treated group. EPO binds to the homomeric EPO receptors, while CEPO activating the heteromeric receptors consist of an EPO receptor monomer and βcR. Accordingly, these differences might be attributable to their different affinities to the two EPO receptor subtypes. It is concluded that different results existing in these studies might be correlated with different models, the time of CEPO administration, mode of administration and the CEPO dosage.

CEPO-Mediated Protection in Other Organs and Tissues

Despite the fact that lung is also an organ highly susceptible to hypoxia, we rarely found the report about CEPO showed protective effects to lung. The only report that can learn from the literature demonstrated CEPO could not attenuate monocrotaline-induced pulmonary hypertension in rats. This may ascribed to the receptors expressed on different organs and the lungs expressed EPOR homoreceptors, but not heteroreceptors (36). With the development of studying, recently, CEPO has been extended to other areas about the protective effects in addition to nervous system, kidney and heart as discussed

above. In these studies, CEPO was supposed to be highly effective of wound healing (37), improving breathing during hypoxia (38), and attenuating cognitive effects of electroconvulsive therapy (39).

The Mechanism of CEPO in Tissue Protection

EPO was identified that it mediated erythropoiesis through bonding to the classical erythropoietin receptor (EPOR), which is a homodimer of two identical EPOR subunits. The tissue-protective role of EPO was supposed to involve the β common receptor (βcR), which is also known as CD131 and shared by receptors for granulocyte macrophage colony- stimulating factor (GM-CSF), interleukin (IL)-3 and IL-5. One βcR subunit is associated with each EPOR monomer via a cystine linkage developing to a heterodimer (4). Overall, the differential effects of EPO and CEPO have been ascribed to their binding to different receptors depending on the relative density of each subunit. Brines et al. (40) reported that EPO and CEPO were inactive in cardiomyocyte or spinal cord injury models in the βcR knockout mouse, suggesting tissue-protective is composed of EpoR and βcR heteroreceptor. In another study by Chamorro et al. (41), EPO was supposed to through bonding to both the homodimeric (EpoR/EpoR) and the heterodimeric (EpoR/βcR) receptors in neuronal SH- SY5Y cells. This finding was confirmed by assays of receptor inhibition and competition, in which anti-EPOR addition completely blocked the anti-apoptotic effect of EPO but incubation of anti-βcR only bluntly blocked (41). However, a conflicting discovery was suggested by Um et al. (42). In the study, they found the cytoprotective effects of EPO are mediated by the classical homodimeric EpoR complex, and they failed to detect βcR expression on differentiated SH- SY5Y and PC- 12 cells. With respect to CEPO about the receptors involved in the regulation, it could not up- regulate EPOR gene expression but increase the level of CD131 gene expression (43). Xu et al. (44) demonstrated that CEPO stimulated the formation of EPOR- βcR and mediated the CEPO- induced cardioprotection against acute myocardial I/R injury. These researches indicated that CEPO is bonding to the heterodimeric (EpoR/βcR) receptors but not homodimeric (EpoR/EpoR).

CEPO has been confirmed to have broad tissue protective activities including an inhibition of apoptosis, restoration of vascular autoregulation and attenuation of inflammatory responses. In studies of EPO, the anti- apoptotic signaling pathways have been reported that EPO first activates JAK- 2 and then engages multiple downstream signaling pathways, including STAT5, MAPK, and/or AKT (45). In a model of I/R injury after kidney transplantation, CEPO preserved tubular cells by preventing p-Akt loss (27).Similar results were found in the model of tubulointerstitial, in which CEPO was showed to protect the kidney by activating Akt (28).Therefore, The AKT signaling network was considered a key determinant of the anti- apoptotic signaling pathways. Wang et al. indicated that CEPO activated sonic hedgehog (Shh) signaling pathway could up-regulate of Mash1 and promoted neural progenitor cell differentiation into neurons (16). As a downstream target of the Shh signaling pathway, PI3K-Akt pathway involved in the regulation of renoprotection, through which it attenuated apoptotic cells among the tubular epithelial cells in CEPO-treated kidneys (25).Afterwards, Xu et al. found that inhibition of PI3K could abolished the CEPO-induced cardioprotection. These studies suggested that PI3K/Akt- dependent pathway was one of the mechanism mediating CEPO-induced tissue-protection (44).

However, CEPO was also demonstrated not activate JAK-2. Therefore, other signaling pathways must be linked to AKT without activating JAK-2. In some mouse models, CEPO exhibited neurotrophic and neurogenesis function via neurotrophic factor, such as NGF and BDNF. Ding et al. (46) observed a significant increase of GDNF mRNA expression in primary neurons treated with CEPO and the GDNF neutralization could decrease CEPO-induced cell viability and proliferation. Subsequently, they found that the blockage of CD131 declined GDNF production, however it was capable to facilitate the phosphorylation of AKT when added GDNF to cultured neurons (46, 47). Therefore, the CD131- GDNF- AKT pathway may be involved in neuroprotection and neurogenesis.

Ramirez et al. (48) found that the addition of

CEPO to UT-7 cells, an EPO dependent human erythroid cell line, could enable cells to survive, however, failed to stimulate proliferation. They draw a conclusion that CEPO failed to activate proliferating cell signals (Erk1/2, NF-κB, and Stat-5) whereas remained its anti-apoptotic effect through Akt activation. In another experiment, CEPO could promote neural differentiation of human neural stem cells (hNSCs) and outgrowth of neurites, which was associated with the activation of Stat3, Stat5 and Akt (49). These findings suggested that STAT, Erk, NF-κB may be associated with neural differentiation and neurite outgrowth.

The production of proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α), was identified to be a defensive response to injury. EPO, as a member of the type I cytokine superfamily, was antagonistic to TNF-α and the EPO receptor could be induced by TNF-α after injury (4). In a chronic experimental autoimmune encephalomyelitis (EAE) model, CEPO could decrease the production of TNF-α, IL-1h and IL-1Ra in the spinal cord equivalent to those treated with EPO (50). In the model of unilateral ureteral obstruction (UUO), CEPO could dramatically attenuated the increased infiltration of macrophage, which could secrete several cytokines including TGF-β1, thus prevent the downstream inflammatory cascade and decrease the apoptosis of renal tubular epithelial cells (51).These studies indicated that CEPO exerts protective activities probable through targeting inflammation.

In addition, CEPO exhibited its therapeutic actions by a series of potential mechanism of phosphorylating histone deacetylase 5 (HDAC5) (52), modulating mitochondrial dysfunction (53), activating the PKC pathway (54) and directly inducing angiogenesis (55). Despite well-characterized tissue-protective effects of CEPO, the underling mechanism responsible for the protection by CEPO has not been identified. The probable signaling pathways involved in the regulation of CEPO are supposed in Figure 2.

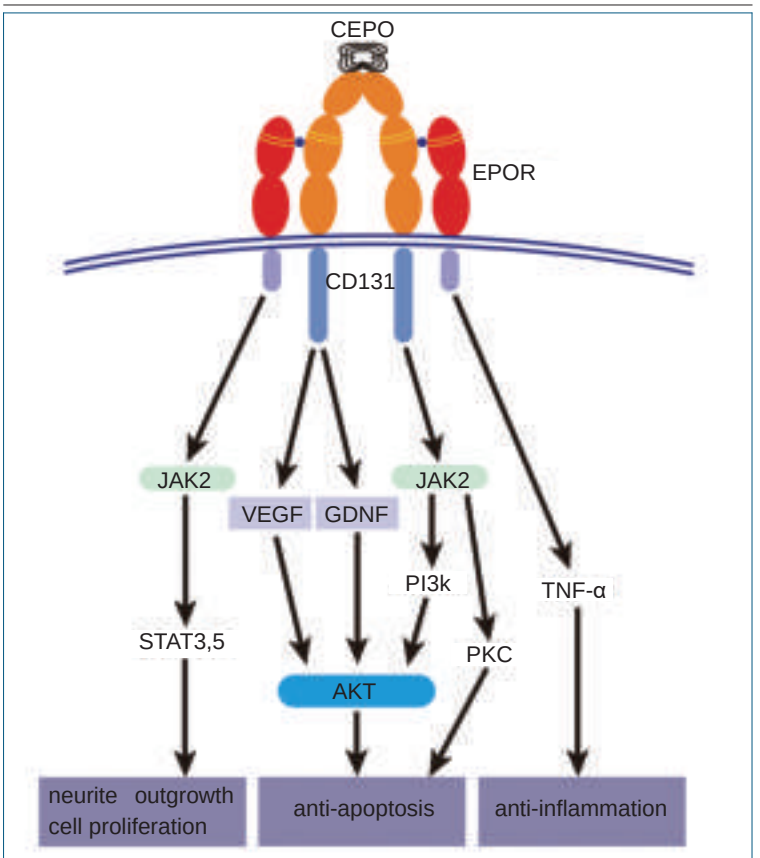

Figure 2. The Probable Signaling Pathways Involved in the Regulation of CEPO.

Conclusions and Prospects

CEPO, as a derivative of EPO, is capable of exerting tissue-protective effects without showing erythropoietic effects even after using high doses of CEPO for long periods. These protective effects have been extensively studied on nervous system, kidney, hear and other tissues in different animal models. Undoubtedly, CEPO is better than EPO due to its protective activities with less unwanted side effects, and CEPO gradually turns into a promising drug candidate at least for many diseases caused by ischemia reperfusion injury.

However, evidence for CEPO's application in clinical is inconclusive. Further studies are required to expand the researches to other fields and to determine the optimal dosing, timing regimens as well as the underlying mechanism of CEPO responsible for its beneficial effects. In addition, the safety and efficacy of CEPO is warranted before its use in clinical.

All authors have no conflicts of interest to declare for this work.

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