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Chapter 3

The Role of Erythropoietin-Derived Peptides in Tissue Protection

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Additional information is available at the end of the chapter

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Abstract

Erythropoietin (EPO), recognized as a tissue protective agent, can trigger anti-inflammatory and anti-apoptotic processes to delimit injury and promote repair by the binding tissue-protective receptor. However, only at a high dosage can EPO exert tissue protective effects, which simultaneously elicits some severe erythropoiesis-related side effects. Thus, the structural modification of EPO for the prevention of side effects is undoubtedly required. This chapter reviewed the development from EPO to its peptide derivatives with tissue protective efficacy. We also discussed are the therapeutic effects and limitations of each peptide, signaling pathways involved and the benefits for translation.

Keywords: erythropoietin, peptide, helix B peptide, cyclic helix B peptide, tissue protection

1. Introduction

Tissue injury refers to the histologic lesions and the subsequent functional insufficiencies of tissues and organs that are caused by multiple sources, such as ischemia followed by reperfusion [1], trauma [2], autoimmune inflammation [3], oxidative stress [4], drugs and toxicants [5], etc. In histology, it is characterized by the infiltration of pro-inflammatory cells and cytokines and the cellular necrosis and apoptosis induced by damage-associated molecular patterns [6, 7]. Considering the high morbidity and mortality it has caused, the protection from tissue injury is a major concern in the basic and clinical medical academies worldwide. Erythropoietin (EPO), recognized as the erythropoietic hormone secreted in response to anemia, was reported to trigger anti-inflammatory and anti-apoptotic processes to attenuate tissue injury and promote the repairing of injured tissues by binding to tissue protective erythropoietin receptor (EPOR)/β common receptor (βcR) heterodimer [8]. However, only at a high dosage can EPO exert tissue

protective effects, which simultaneously elicits some severe erythropoiesis-related side effects [9]. Thus, the structural modification of EPO for the prevention of side effects is undoubtedly required. This chapter reviewed the development from EPO to its peptide derivatives with tissue protective efficacy. Also discussed are the therapeutic effects and limitations of each peptide, signaling pathways involved and the benefits for translation.

2. Erythropoietin: more than erythropoiesis

EPO is a glycoprotein hormone, which is predominately produced and secreted by the adult kidney in response to anemia [10, 11]. After sensing the hypoxic signal in anemic conditions, the fibroblasts in the interstitial area of the renal cortex are activated to produce EPO [12, 13]. EPO stimulates erythropoiesis through binding to its EPOR expressed on the surface of the red blood cell progenitors and precursors in bone marrow [14, 15]. In normal conditions, the level of erythropoietin in the blood is quite low [10]. Under hypoxic stress, however, EPO production may be increased up to 1000-fold, reaching 10,000 mU/ml in the blood [13]. In structure, EPO consists of four long alpha-helixes (A, B, C, and D) running in an up-up-down-down direction, connected by two long cross-over loops (AB and CD) and one short loop (BC). Among the four helixes, only helix A, C, and helix D and the loop connecting helix A and B within the dimensional structure are essential for interacting with EPOR, while helix B faces away from the interior of the receptor in the tertiary structure [13, 16–18].

During the past decade, greater interest has been paid to the pleiotropic biologic actions of EPO beyond the stimulation of erythropoiesis, which include anti-apoptosis, anti-inflammation, neurogenesis, and angiogenesis, as well as their consequent tissue protection [8, 19–21]. An increasing body of studies demonstrated that EPO exerts a powerful tissue-protective effect on a variety of organs and can prevent cellular apoptosis from some sources, including reduced or absent oxygen tension, ischemia–reperfusion, toxicity and free radical exposure. Our study in a murine model of renal injury showed that EPO protected kidneys against injury through decreasing positive myeloperoxidase neutrophils and suppressing the expression of pro-inflammatory cytokines and chemokines by the inhibition of NF-kB signaling pathway [22]. In the study with isolated porcine renal allografts, it was demonstrated that EPO promoted inflammatory cell apoptosis, drove inflammatory and apoptotic cells into tubular lumens, thereby leading to inflammation clearance in the involved tissue and organs [23]. We also reported in our recent study that EPO could affect the dynamics of macrophages in the model of rhabdomyolysis-induced acute kidney injury. In this study, EPO was found to ameliorate kidney injury by reducing macrophages recruitment and promoting phenotype switch from M1 to M2 [24]. In vitro study, EPO was shown to directly suppress pro-inflammatory responses of M1 macrophages and promote M2 marker expression [24]. These results refreshed our understanding about the immunoregulatory capacity of EPO.

The mechanisms involved in the tissue protective effect of EPO was not well illustrated until the specific receptor mediating tissue protection was revealed, which was recently defined as a heterodimer composed of EPOR and CD131 [25]. CD131 also forms the receptor complexes with α receptor subunits specific for GM-CSF, IL-3, and IL-5 and thus is named as β common receptor [25]. In aqueous media, helix B and parts of the AB and CD loops face the aqueous

medium but away from the erythropoietic binding sites, which indicates that helix B mediates tissue protective effect of EPO via binding with EPOR/βcR heterodimer [26]. Following the interaction of EPO with EPOR/βcR, the Akt involved signaling pathway is reported to play a vital role in mediating intracellular signal transduction. The activation of PI3K/Akt pathway by EPO maintains the mitochondrial membrane integrity, prevents cytochrome C from release and modulates the activity of caspase cascade during cellular apoptosis [27, 28]. The blockade of Akt phosphorylation abrogates the anti-apoptotic and anti-inflammatory effect after EPO administration [28, 29]. Moreover, the activation of Akt after EPO treatment is also reported to protect against genomic DNA degradation and membrane phosphatidylserine exposure [28, 30]. Several transcriptional modulators in the downstream pathways of Akt have also been shown to mediate the tissue-protection of EPO. For example, EPO could down-regulate the activity of forkhead transcription factor (FOXO3a) through inhibitory phosphorylation, which renders FOXO3a ineffective to activate the transcription of nuclear genes involved in apoptosis [31]. Other mechanisms include the inhibition of glycogen synthase kinase-3β [32], a serine-threonine kinase that plays a significant role in the induction of apoptosis of neurons, vascular smooth muscle cells and cardiomyocytes, and the up-regulation of the anti-apoptotic Bcl-2 family member Bcl-xL [33].

3. Helix B surface peptide: a specific tissue protective peptide without erythropoietic effect

The role of EPO in anti-inflammation and anti-apoptosis inspired us to investigate if EPO could also be served as a potential therapy for tissue injury caused by the exposure to drugs, chemicals, or physical ischemia. However, the tissue protective effect of EPO only occurs at the dosage that is well above normal, which may simultaneously elicit severe side-effects associated with its erythropoietic effects such as polycythemia and hypercoagulation in the circulation [9]. The demand of high dose to exert the tissue protective activities may be caused by the relatively low affinity of EPO to the tissue-protective EPOR/βcR dimer receptor relative to the erythropoietic $(EPOR)_2$ homodimer [15, 20, 34]. Being enlightened by the finding that helix B plays a dominant role in mediating the tissue protective effect by binding with EPOR/βcR receptor, Michael Brines et al. first synthesized the nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin which comprises the amino acid sequence corresponding to helix B as well as the three residues within the proximal portion of the BC loop. The design of the novel peptide aims to mimic the three-dimensional structure that interacts with EPOR/βcR receptor to reproduce the tissue-protective activities of the full molecule [26]. This 11-mer peptide derivative was named as helix B surface peptide (HBSP) [26]. Following studies demonstrated that HBSP is sufficient to activate tissue-protective pathways representative of the full molecule and protect from injury in a wide variety of tissues and organs but without causing erythropoietic effects.

3.1. In cardiovascular system

HBSP was demonstrated to exert tissue protective effects first in the cardiovascular system after it was designed. The designers of this peptide showed that HBSP protected cardiomyocytes

from TNF-α apoptosis in an Akt-dependent pathway both in vitro and in vivo [26, 35]. In this study, the levels of serum creatinine kinase activity and of cardiac expression of atrial natriuretic peptide, a marker of chronic heart failure, were down-regulated in animals treated with HBSP [26, 35]. Then, the anti-atherosclerotic effects HBSP were investigated in vitro and in vivo [36]. In vitro, HBSP inhibited C-reactive protein induced apoptosis in human umbilical vein endothelial cells and THP-1 cells to a great extent [36]. In the hyperlipidemic spontaneous myocardial infarction model of rabbits, HBSP was shown to significantly suppress the progression of coronary stenosis and myocardial ischemia caused by atherosclerotic lesions and inhibit coronary artery endothelial cell apoptosis through the activation of Akt pathway and the corresponding decreased the production of TNF-α as well as modified macrophage M1/M2 polarization [36]. Similarly, in the myocardial ischemia-reperfusion injury model of mice, HBSP administration before reperfusion significantly reduced the myocardial infarct size, decreased cardiomyocyte apoptosis, reduced the activities of superoxide dismutase and partially preserved heart function through the upregulation of Akt/GSK-3β/ERK and STAT-3 [37]. In an in vitro study performed by using a rodent cardiomyocyte cell line subjected to hypoxia-reoxygenation injury, HBSP was reported to have protective effects by reducing cellular apoptosis, mitochondrial reactive oxygen production, $\Delta\Psi m$ collapse, and cytochrome C release from mitochondria to the cytosol. Furthermore, HBSP inhibited the activation of caspase 9 and caspase 3 as well as the alteration of Bcl-2 family proteins induced by hypoxia-reoxygenation [38]. Diabetes is also one of the major causes of myocardial lesions. Diabetic cardiomyopathy (DCM) is a ventricular dysfunction independent of coronary artery disease and hypertension, which is associated with inflammation, myocardial apoptosis and fibrosis [39–41]. In a study regarding the protection of HBSP on DCM, HBSP notably improved cardiac function, attenuated cardiac interstitial fibrosis, inhibited myocardial apoptosis, and ameliorated mitochondrial ultrastructure in mice with diabetic cardiomyopathy through an AMPK-dependent pathway [42]. HBSP promoted aortic endothelial cell repair under hypoxic conditions in a model of aortic endothelial injury, in which HBSP enhanced scratch closure by promoting cell migration and proliferation [43]. Furthermore, EPO protected bovine aortic endothelial cells from staurosporine-induced apoptosis under hypoxic conditions. Hypoxia was associated with a reduction in nitric oxide (NO) production. HBSP notably increased NO production, in a manner sensitive to NO synthase inhibition, under hypoxic conditions but not under normoxic conditions [43]. In summary, multiple studies proved the protective effect of HBSP on myocardial tissues and endothelial cells in the cardiovascular system following the injury caused by insufficient oxygen supply and implied that Akt pathway played a critical role in this process.

3.2. In nervous system

Another field of research in which the protection of HBSP was well investigated is in the nervous system. In one study by Robertson et al., the effects of HBSP on early cerebral hemodynamics and neurological outcome post-injury were investigated in a rat model of mild cortical impact injury followed hemorrhagic hypotension [44]. The results demonstrated that both EPO and HBSP treated groups improved recovery of cerebral blood flow in the injured brain following resuscitation, and showed more rapid recovery in the performance of functional neural tests. This study suggests that HBSP has neuroprotective effects similar to EPO in this

model of combined brain injury and hypotension [44]. They later reported that the treatment with HBSP resulted in significantly improved performance after the rats were suffered from mild traumatic brain injury (mTBI), which was associated with decreased infiltration of CD68-positive inflammatory cells in the damaged brain tissue [45]. The results suggest that HBSP may improve cognitive function following mTBI [45]. Among the patients with neuritis, neuropathic pain is a quite common symptom, which may be due to nerve inflammation. The neuropathic pain results from several overlapping pathways, which then merges into a magnified pain status with symptoms such as allodynia and hyperalgesia [46, 47]. The study by Pulman KG et al., examined the effects of HBSP on pain behavior in the rat model of neuritis [48]. The results showed that treatment with HBSP prevented the development of mechanical allodynia caused by neuritis but not affect heat hyperalgesia [48]. In another study, HBSP treatment could reduce allodynia coupled to the suppression of spinal microglia response in a dose-dependent manner, which may result from HBSP-induced suppression of inflammation in central nervous system [49]. In the study on the therapeutic effects of HBSP in experimental autoimmune encephalomyelitis (EAE), the administration of HBSP to EAE rats significantly reduced the severity and shortened the duration of injury, reduced the infiltration of pro-inflammatory cells and suppressed expression of pro-inflammatory cytokines such as IL-1β, IL-17, TNF-α, IFN-γ. The expression of inducible NO synthase and transcription factor T-bet at mRNA level was also reduced in spinal cords following HBSP treatment [50]. In the in vitro study, HBSP inhibited antigen-specific and non-specific lymphocyte proliferation and promoted the polarization of Th2 and regulatory T cells (Treg) while suppressed the polarization of Th1 and Th17 cells in EAE lymph nodes [50]. In summary, these studies regarding the role of HBSP in nervous system revealed that HBSP could also protect the nervous tissue from injury, which was associated with the alteration of the cytokine and cell milieu to limit inflammation in the damaged nervous tissue.

3.3. In obesity and diabetes related disorders

Several recent studies focused on the evaluation of the effects and potential mechanisms of HBSP in obesity modulation and diabetes-related disorders. One study was performed by using male C57BL/6 J mice fed with high-fat high-sucrose (HFHS) diet [51]. HFHS diet treated mice exhibited insulin resistance, hyperlipidemia, hepatic lipid accumulation and kidney dysfunction, which was related to the impaired insulin signal pathway and reduced membrane translocation of glucose transporter 4 [51]. However, treatment with HBSP ameliorated renal function, reduced hepatic lipid deposition, and normalized serum glucose and lipid profiles, which were associated with improved insulin sensitivity and glucose uptake in skeletal muscle. The mechanism included that HBSP attenuated the HFHS-induced overproduction of IL-6 and fibroblast growth factor-21, and enhanced mitochondrial biogenesis in skeletal muscle [51]. In another study regarding the effect of HBSP on obesity, HBSP was found to protect against obesity and insulin resistance by suppressing adipogenesis, adipokine expression as well as attenuating macrophage inflammatory activation in lipid tissue [52]. The retinopathy is one of the most common complications of diabetes and remains one of the leading causes of non-congenital blindness [53]. In the diabetic retina, vasodegenerative phase is accompanied by neuroglial abnormalities and eventual depletion of ganglion cells [54]. HBSP was shown to

significantly reduce microglial activation and protected against neuroglial and vascular degeneration but without exacerbating neovascularization in the retina [55]. These findings suggest that HBSP has therapeutic implications for metabolic disorders, such as obesity, diabetes, and diabetic retinopathy.

3.4. In kidney

Our research mainly discussed the tissue protection of HBSP on kidney injury. In 2013, we investigated effects of HBSP and the expression of EPOR/βcR heterodimer receptor in a murine renal ischemia-reperfusion (IR) injury model [56]. We found that HBSP could significantly ameliorate renal dysfunction and tissue damage, reduced apoptotic cells in the kidney and inhibited the activation of caspase-9 and caspase-3 [56]. The expression of EPOR/βcR in the kidney was up-regulated following ischemia-reperfusion injury but was down-regulated by the treatment of HBSP [56]. Further investigation revealed that the PI3K-Akt pathway was dramatically activated by HBSP. The treatment of the PI3K inhibitor, Wortmannin, abolished improved renal function and histologic structure by HBSP [56]. This study suggests that HBSP could protect the kidney from IR injury in a PI3K-Akt dependent pathway. Then, we also investigated the role of HBSP in IR and cyclosporine A (CsA) induced kidney injury since both of them are unavoidable after kidney transplantation and associated with allograft dysfunction [57]. We found that the level of creatinine and blood urea nitrogen was increased by CsA but decreased by HBSP. HBSP also significantly ameliorated tubulointerstitial damage and interstitial fibrosis, which were gradually increased by IR and CsA [57]. In addition, apoptotic cells, infiltrated inflammatory cells, and active caspase-3 positive cells were greatly reduced by HBSP. It was demonstrated for the first time that HBSP effectively improved renal function and tissue damage caused by IR and/or CsA, which might be through reducing caspase-3 activation and synthesis, apoptosis, and inflammation [57]. Similar findings were also reported by Nimesh SA Patel's and Willem G van Rijt's groups that HBSP has renoprotective capacities by anti-inflammation and anti-apoptosis in the injured kidney tissue [58, 59].

3.5. In liver

Very recently, the protective effect of HBSP on acute liver injury was investigated in Wu's study. In this study, the acute liver injury was induced by the administration of carbon tetrachloride (CCl4) [60]. HBSP was demonstrated to significantly decrease serum alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and pro-inflammatory cytokines in liver tissues after CCl4 injection. The infiltration of CD3, CD8, and CD68 positive cells and the expression of cleaved caspase-3 were also significantly decreased by HBSP treatment. The glutathione peroxidase activity and survival rate increased, while the total apoptotic rate was reduced in the HBSP-treated group. As to the mechanism, the authors reported that HBSP activated the PI3K/Akt/mTORC1 pathway [60]. Thus, HBSP showed convincing protective effects on CCl4-induced acute liver injury by ameliorating inflammation and apoptosis [60].

4. Thioether-cyclized helix B peptide: a cyclized peptide with increased instability and tissue protective potency

Despite the powerful tissue-protective function exhibited in various organs by inhibiting inflammation and apoptosis, the property of poor permeability to biomembranes, unstable secondary structure, and short half-time restricts the application of HBSP in translation study [26]. Therefore, the structurally optimized transformation of HBSP is urgently required. It is acknowledged that peptide cyclization could provide an efficient strategy to overcome these problems [61]. Provoked by this, we for the first time introduced the head-to-tail cyclization to the structure of HBSP to improve its stability, since the backbone of the peptide was constrained by the cyclization in which the linkages between main chains were formed by thioether [62]. This newly designed and synthesized peptide was named as thioether-cyclized helix B peptide (CHBP) [62].

4.1. Novel properties and mechanisms

In the following study, we demonstrate that CHBP is significantly stable in the human plasma and has a 2.5-fold longer half-life time than HBSP, suggesting that CHBP is highly resistant to proteolytic degradation both in vitro and in vivo [63]. We also found in our study that due to its stability, this long-acting peptide could ameliorate renal IR injury to a greater extent than HBSP, for only one dose of CHBP exerted persistent renal protective effect throughout the one week post IR injury [63]. Autophagy is demonstrated to play a renoprotective role in IR injury and is closely related to cellular apoptosis and inflammation in kidney tissue [64]. We also found that CHBP could induce autophagy in the injured kidney by increasing LC3-II/I ratio as well as upregulating beclin-1 [62]. Furthermore, our study depicted possible signaling pathways involved in CHBP-induced autophagy, which included the regulation of mammalian target of rapamycin (mTOR) pathway and the activation of AMPK pathway. The activation of AMPK by CHBP then phosphorylated and activated tuberous sclerosis 2 (TSC2), which connected with tuberous sclerosis 1(TSC1) to form a heterodimer to inhibit the activation of mammalian target of rapamycin complex 1 (mTORC1). Meanwhile, the mTORC2-Akt pathway was activated by CHBP and autophagy was induced by the altered mTORC1/mTORC2 equilibrium [62]. Also, CHBP was reported to upregulate Treg and downregulate helper T cell 17 (Th17) after renal IR injury to restore the Treg/Th17 balance [62]. These findings revealed the mechanisms that are involved in the tissue protective function in CHBP but have not been reported in HBSP yet.

4.2. CHBP in organ preservation

During the transport of donated organs, any strategies that can effectively protect against IR injury during the cold storage (CS) and reperfusion stages would be very beneficial for preventing the delayed graft function after kidney transplant surgery. Thus, we administrated CHBP in the preservation solution and autologous blood perfusate to examine its effect on the preservation of isolated donor kidney in the following study [65]. The results showed that

the administration of CHBP during cold preservation of kidneys as well as autologous blood could ameliorate IR injury after hemoperfusion, which was associated with increased renal blood supply and improved renal tubular structure and function [65].

4.3. CHBP and anti-allograft rejection

As the professional antigen-presenting cells, dendritic cells (DCs) play a triggering role in acute rejection (AR) after transplant surgery. Thus, we investigated the effects of CHBP on DCs in the kidney transplantation model from Lewis to Wistar rats [66]. The results showed that five successive doses of CHBP administration after kidney transplantation could significantly ameliorate AR with the association of lower histological injury, apoptosis, and CD4+ and CD8+ T-cell infiltration in renal allografts. CHBP also reduced the expression of IFN-γ and IL-1β but increased the expression of IL-4 and IL-10 in the serum of receipt. The number of mature DCs was significantly decreased in renal allografts treated with CHBP [66]. Also, the incubation of DCs with CHBP in vitro led to a reduction in TNF-α, IFN-γ, IL-1β and IL-12 levels and an increase of IL-10 level at the protein level in the supernatant [66]. In the mechanism study, CHBP inhibited TLR activation-induced DC maturation by increasing SOCS1 expression through Jak-2/STAT3 signaling [66]. Our study suggested that CHBP suppressed renal allograft AR by inhibiting the maturation of DCs via Jak-2/STAT3/SOCS1 signaling.

4.4. CHBP and protection of mesenchymal stem cells

Mesenchymal stem cell (MSC) is a pluripotent stem cell originating from the mesoderm and has the potential to differentiate into multiple types of cells and tissues [67, 68]. Thus, MSC has long been considered as an ideal cell-based therapy in the repairing of tissue injuries. After adoptive transferred in vivo, however, MSCs may confront a variety of undesirable factors that could decrease their viability and activity [69, 70]. Among them, nutrient starvation is the major obstacle for MSCs within injured tissues. In our study regarding the effect of CHBP on MSCs in vitro, we found that CHBP could significantly improve the cell viability and suppress apoptosis of MSCs in a dose-dependent manner [71]. Starvation resulted in the mitochondrial dysfunction, and the treatment of CHBP could alleviate mitochondrial dysfunction by diminishing the oxidative stress from ROS, restore mitochondrial membrane potential and maintain mitochondrial membrane integrity through the activation of Nrf2/Sirt3/FoxO3a pathway [71]. Moreover, MSCs pretreated with CHBP were more resistant to nutrient starvation [71]. This study suggests that CHBP has the e prospects for sustaining stem cell survival under nutrient-deprived conditions and improving the therapeutic effect of MSC-based treatment.

5. Perspective and limitation for translation into clinic

The research about CHBP in our center also includes its effects on other kinds of injuries, for example, aristolochic acid-induced acute kidney injury [72]. The study on the role of CHBP in acute and chronic allograft rejection is in progress as well. Although our understanding about CHBP has significantly increased, there is still plenty of work to do to translate this protective

peptide into clinical practice. For example, the pharmacokinetics and pharmacodynamics of CHBP are not examined so far. The dosage form design of this new drug should be improved for oral administration or intravenous injection. The clinical trials are indispensable before it is finally applied for clinical use. In a further study, we plan to investigate the effects of CHBP in primate models of acute organ injury which could better represent the analogous disorders in the human being. We believe that this smaller but stronger peptide derivative of EPO could facilitate the treatment of acute tissue injury shortly.

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Conflict of interest

The authors declare no conflict of interest.

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