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Flipping the molecular switch for innate protection and repair of tissues: Long-lasting effects of a non-erythropoietic small peptide engineered from erythropoietin

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ABSTRACT

Many disease processes activate a cellular stress response that initiates a cascade of inflammation and damage. However, this process also triggers a tissue protection and repair system mediated by locally-produced hyposialated erythropoietin (hsEPO). Although recombinant EPO is used widely for treating anemia, potential use of recombinant EPO for tissue-protection is limited by rises in hematocrit, platelet activation, and selectin expression resulting in a high risk of thrombosis. Importantly, the erythropoietic and tissue-protective effects of EPO are mediated by different receptors. Whereas EPO stimulates red cell progenitors by binding to an EPO receptor (EPOR) homodimer, a heterodimer receptor complex composed of EPOR and β common receptor (βcR) subunits, termed the innate repair receptor (IRR), activates tissue protection and repair. The IRR is typically not expressed by normal tissues, but instead is rapidly induced by injury or inflammation. Based on this understanding, EPO derivatives have been developed which selectively activate the IRR without interacting with the EPOR homodimer. The latest generation of specific ligands of the IRR includes an 11 amino acid peptide modeled from the three dimensional structure of the EPO in the region of helix B called pyroglutamate helix B surface peptide (pHBSP; ARA-290). Despite a short plasma half-life (~2 min), pHBSP activates a molecular switch that triggers sustained biological effects that have been observed in a number of experimental animal models of disease and in clinical trials. This review summarizes pharmacokinetic and pharmacodynamic data and discusses the molecular mechanisms underlying the long-lasting effects of this short-lived peptide.

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Abbreviations: ACTH, adrenocorticotropic hormone; AMPK, AMP-activated protein kinase; eNOS, endothelial nitric oxide·synthase; EPO, erythropoietin; EPOR, EPO receptor; FGF, fibroblast growth factor; GLP-1, glucagon-like peptide-1; HIF, inducible factor transcription factors (HIF); hsEPO, hyposialated erythropoietin; I/R, ischemia/reperfusion; IRR, innate repair receptor; JAK2, Janus kinase 2; MI, myocardial infarction; pHBSP, pyroglutamate helix B surface protein; STAT-5, signal transducers and activators of transcription-5; β cR, beta common receptor (CD 131).

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1. Introduction

Tissue damage or stress generally activates an evolutionarily ancient inflammatory reaction that has been termed the innate immune response. Central to this process is a self-amplifying production of pro-inflammatory cytokines that cause further tissue damage through necrosis and apoptosis via a positive feedback loop. Additionally, the microenvironment becomes relatively hypoxic due to a disrupted microcirculation coupled with increased metabolic activity of both resident cells and infiltrating immune cells. This process is a prominent component in a range of inflammatory disorders, such as chronic infection, or inflammatory bowel disease (Taylor & Colgan, 2007; Werth et al., 2010). Within this system, hypoxia- and inflammation-responsive molecular pathways are closely inter-related and co-regulated (Palazon et al., 2014). One critical molecular component of this closely choreographed biological response is the family of hypoxia inducible factor transcription factors (HIF) that activate a host of metabolic pathways to restore cellular integrity (Semenza, 2012). Tissue injury has been observed to cause an increase in HIF expression (Bernaudin et al., 2002; Shein et al., 2005). Prominent among HIF-regulated gene transcription is erythropoietin (EPO).

EPO is an evolutionarily ancient protein (Nogawa-Kosaka et al., 2010) that can be synthesized by many cells. A major form of EPO is as a hormone, a 31 kDa glycoprotein predominantly produced in the adult kidney and primarily known for its role in promoting proliferation, differentiation and survival of erythroid progenitor cells (Watowich, 2011). It is clinically used for the treatment of patients with anemia secondary to chronic kidney disease or myelodysplasia following chemotherapy or radiotherapy.

When anemia develops, hypoxia is detected within the kidney which stimulates the production and secretion of EPO which travels to the bone marrow. There, EPO increases the production of erythrocytes and therefore the circulating red cell mass, and in this manner, reduces hypoxia. Within the setting of immune activation or tissue damage throughout the body, however, the production of EPO acts as a master regulator of apoptosis and pro-inflammatory cytokine production, as well as of the repair of tissue damage.

2. Erythropoietin and its receptor isoforms

EPO produced by the kidney possesses 4 oligosaccharide chains terminated by sialic acids that provide for a plasma half-life of 4–6 h. In contrast, EPO that is produced locally as the result of innate immune system activation is poorly sialated (Masuda et al., 1994), as would be expected for a molecule operating in a paracrine/autocrine mode, and therefore has a short half-life (Imai et al., 1990). Notably, completely desialated EPO has a plasma half-life of approximately 1.4 min (Erbayraktar et al., 2003). Hyposialated EPO (hsEPO) is believed to be the ligand activating the innate repair system.

The EPO-receptor that mediates erythropoiesis is comprised of two identical EPO receptor monomers (EPOR) which become localized rapidly within the microdomains of the membrane rafts of hematopoietic cells following stimulation (within several minutes) (McGraw et al., 2012). Here, the EPOR subunits spontaneously dimerize when in close proximity, forming EPOR-EPOR [$(EPOR)_2$] and assemble with Janus kinase 2 (JAK2) and other members of the molecular signaling machinery (McGraw et al., 2012). Subsequently, when EPO binds to and bridges across the receptor subunits, a conformation change occurs, leading to activation of JAK2, which in turn phosphorylates tyrosine residues within the EPOR cytosolic domain. The phosphorylation of $(EPOR)_2$ results in the activation of several signal transduction proteins, including the mitogen-activated protein kinase extracellular-regulated kinase (ERK)-1/2, phosphatidyl inositol 3 kinase (PI3K)/Akt, and signal transducers and activators of transcription (STAT)-5. STAT-5 that stimulates mitochondrial anti-apoptotic proteins, such as Bcl-XL, and consecutively inhibits cytochrome c-dependent caspases, thus, suppressing erythroid progenitor cell apoptosis (Chong et al., 2002). Additionally, $(EPOR)_2$ also mediates platelet and endothelial cell activation, including up-regulation of E-selectin (Heinisch et al., 2012). These actions serve to limit blood loss following vascular trauma by activating physiologically relevant thrombosis, but also serve to initiate and maintain inflammation. One prominent characteristic of $(EPOR)_2$ signaling pathways is that activation consists of both binary (switching on) as well as graded responses, for example, as in STAT-5 signaling (Porpiglia et al., 2012).

EPO has a high affinity (~200 pmol/L) for $(EPOR)_2$, and as only a few percent occupancy is needed for adequate signaling (Krzyzanski & Wyska, 2008), the normal human serum EPO concentration is in the 1–10 pmol/L range. The endothelial cell was the first non-hematopoietic cell found to respond to EPO for which activation of the receptor causes endothelial cell mitosis and migration. However, the effective concentrations of EPO needed for this activity (1–2 nmol/L) were significantly higher than those required for hematopoiesis (Anagnostou et al., 1990). Subsequently, the nervous system (Konishi et al., 1993), the kidney (Westenfelder et al., 1999), and the heart (Calvillo et al., 2003) were also shown to respond to EPO, but similar to endothelial cells, required higher EPO concentrations than that needed for hematopoiesis. The substantial difference between the affinity of the $(EPOR)_2$ expressed by erythrocyte precursors and the one on non-hematopoietic cells suggested that a different receptor might transduce tissue protection. Additionally, the EPO receptor isolated from neuronal-like PC-12 cells was characterized as having a different molecular weight and with distinctive accessory proteins (Masuda et al., 1993).

Using a derivative of EPO with the binding sites to $(EPOR)_2$ blocked, Leist and colleagues demonstrated that the erythropoietic and cytoprotective effects of EPO could be separated (Leist et al., 2004). Subsequent experimental data have provided good evidence that the extra-hematopoietic effects of EPO are mediated, at least in part, by an alternative receptor that is proposed to consist of a hetero-complex between the EPOR and the β-common receptor (βcR; known also as CD131) (Brines et al., 2004). βcR is a subunit also used for the receptors of other type 1 cytokines, i.e., interleukin (IL)-3, IL-5 and granulocyte-macrophage colony stimulating factor (Murphy & Young, 2006). We have named this alternative EPO receptor as the innate repair receptor (IRR) to differentiate it from hematopoietic effects and to emphasize its protective role in inflammation and tissue injury to reduce damage, while also initiating healing and repair.

A distinct temporal-spatial relationship exists between components of the innate immune response and initiation of tissue protection and repair via IRR activation (Fig. 1). In quiescent cells, EPOR and βcR are typically localized within the intracellular compartment and not present on the cell surface. Stress, e.g. by hypoxia or inflammation, induces a rapid (several minutes) movement to the cell surface (Bohr et al., 2015) with a likely coalescence into membrane rafts, as has been described for other heteroreceptors that utilize βcR (Saulle et al., 2009), and as has been described above for the EPOR dimer (McGraw et al., 2012). Assembly of the mature receptor would then allow for stimulation if hsEPO is available in the surrounding locale. In this way, the innate repair system acts as a complex lock and key system that requires a specific time and spatial resolution to be present before being activated.

As in the case of the dimeric erythropoietic receptor $(EPOR)_2$ complex, binding of EPO to the EPOR-βcR complex causes phosphorylation of JAK2. This activation of JAK2 then switches on three principle signaling cascades that depend upon the specific tissue examined: STAT-5, PI3K/Akt, and mitogen-activated protein kinases (see below). These signaling pathways induce regeneration, inhibit apoptosis and inhibit inflammation (Brines & Cerami, 2008).

The binding affinity of the classic $(EPOR)_2$ complex is significantly greater than the binding affinity of the tissue-protective EPOR-βcR complex (Brines & Cerami, 2008). Thus, to induce local tissue protection, considerably higher systemic doses of recombinant EPO (rEPO) are

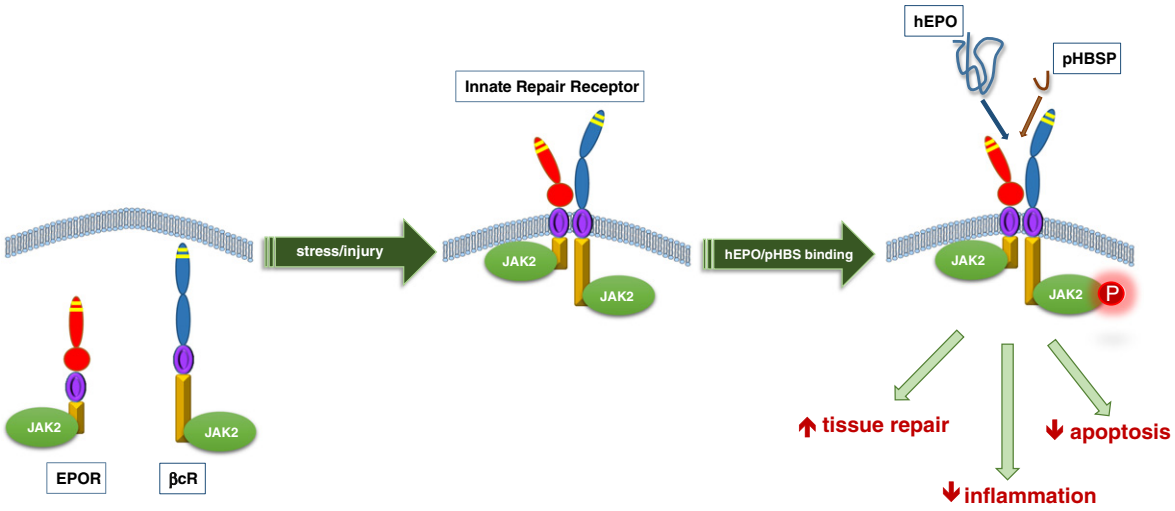

Fig. 1. The innate repair system is characterized by temporal and spatial separation of the innate repair receptor and its endogenous ligand, hyposialated EPO (hEPO). At baseline, the EPOR and βcR subunits are maintained within the cell. Following cellular stress, e.g., hypoxia and inflammatory mediators, these receptor units are transported to the cell surface rapidly (minutes) where spontaneous assembly occurs, probably within lipid raft micro-domains, forming the innate repair receptor. On the cell surface the βcR exists as an intertwined dimer. After a significant delay (hours in the case of central nervous system injury) hyposialated EPO is synthesized by cells in the vicinity of injury. If sufficient ligand is present to diffuse into the injury site, its binding to the innate repair receptor causes phosphorylation of JAK2, which then switches on signaling cascades leading to long lasting biological protective and reparative responses are turned on. When the innate repair receptor is formed, exogenous selective ligands, e.g., pHBSP, can be administered to directly activate the tissue protective response.

required than the therapeutic doses used for stimulation of erythropoiesis. As a result, the EPOR-βcR complex does not respond to EPO at the 1–7 pmol/L concentrations normally present in the circulation and is probably only activated by high levels of locally-produced EPO. Additionally, as described above, the EPOR-βcR heterodimer is not usually expressed until after an injury occurs, and only requires a brief exposure to EPO to trigger sustained biological activity. This is in contrast to the $(EPOR)_2$ homodimer complex, which is normally expressed in hematopoietic cells, and requires sustained levels of EPO to support erythropoiesis.

A host of tissues and cells under stress have been shown to express and co-localize EPOR and βcR, including the central (Brines et al., 2004) and peripheral (Loesch et al., 2010) nervous systems, retina (Colella et al., 2011), heart (Xu et al., 2009), kidney (Kitamura et al., 2008), skeletal muscle (Collino et al., 2014), the endothelium (Su et al., 2011), as well as macrophages and bone marrow derived mesenchymal cells (Bohr et al., 2015).

3. Erythropoietin: an overview of its clinical potential beyond the treatment of anemia

The potential therapeutic relevance of EPO in the clinical setting of several diseases beyond the treatment of anemia, mainly in patients with organ ischemic injury, has been recently investigated and shows variable efficacy (Patel et al., 2011a). The Gottingen EPO Stroke Study, a pilot, double-blinded study, was the first trial demonstrating that EPO improves clinical outcome in patients with stroke as determined 1 month after the incident by neurological scoring and MRI (Ehrenreich et al., 2002). However, the more recent German Multicenter EPO Stroke Trial has unexpectedly documented that a combination of rEPO and recombinant tissue plasminogen activator is not advantageous, and may even be detrimental (Ehrenreich et al., 2009), as EPO also interacts with the tissue metalloproteinases which are involved in tissue remodeling (Zechariah et al., 2010). Clinical trials with low-dose EPO in patients with myocardial infarction (MI) showed only minor improvements of cardiac function (Ozawa et al., 2010; Taniguchi et al., 2010; Voors et al., 2010), whereas a trial that used a higher dose of EPO showed a tendency toward an increased incidence of adverse events (Ott et al., 2010). Finally, a randomized, double blind, placebo-controlled trial did not find significant reduction in biomarkers of renal 'injury' in patients with acute kidney injury, which had been treated with EPO (Endre et al., 2010). However, the biomarkers used in this trial are poorly validated to detect acute kidney injury, and there were some differences in baseline between placebo and EPO-treated patients, which make this study difficult to interpret.

EPO has also been reported to reduce the degree of insulin resistance in patients who have end-stage renal disease. In a case–control study on 59 age- and sex-matched patients with end-stage renal disease treated by hemodialysis (stage 5), many of whom had diabetes, the mean plasma insulin levels of hemodialysis patients treated with recombinant human EPO were significantly lower than in patients which had not received EPO; and duration of EPO treatment negatively correlated with insulin resistance (Khedr et al., 2009). This is in agreement with other studies showing that hemodialysis patients treated with EPO are more insulin sensitive when compared with hemodialysis patients not treated with EPO (Spaia et al., 2000; Tuzcu et al., 2004).

Any potential therapeutic application of EPO in patients with chronic diseases, including metabolic disorders, is limited by rises in hematocrit and platelet count (hematopoietic effect), resulting in an increase in thrombotic risk. For instance, in a large randomized, placebo-controlled trial which enrolled 1460 critically ill patients (within 48 to 96 h of admission to medical and surgical ICUs) with EPO ($3 \times 10{,}000$ IU for a maximum of three weeks) resulted in a significant reduction in mortality in patients with trauma, but also in a significant increase in thrombovascular events (Corwin et al., 2007). The authors speculate that the observed beneficial effects of EPO are secondary to the reported tissue-protective effects, while the adverse effects are due to an activation of the 'classical' hematopoietic $(EPOR)_2$. Additionally, a large multicenter trial in traumatic brain injury recently has shown an increase of adverse thrombotic events in the EPO treatment arm without clear benefit on brain injury (Robertson et al., 2014). Thus, the development of drugs that maintain the tissue-protective effects of EPO without causing hematological complications may represent a significant advance in the treatment of several diseases, especially when the interventions have to be administrated over weeks and months to be effective.

Another concern of long-term administration of EPO is its potential to promote tumor growth (Barbera & Thomas, 2010), which has prompted regulatory agencies to issue warnings for the use of erythropoiesis-stimulating agents in oncology patients, despite reassuring recent findings reporting a lack of clinical evidence for

enhanced tumor progression in patients treated with EPO (Aapro et al., 2012). Notably, as the tumor progression by EPO is mediated by the EPOR homodimer, drugs that selectively activate the innate repair receptor are unlikely to exert this effect.

4. pHBSP: structure, pharmacodynamics and pharmacokinetics

Efforts have been made to design and validate EPO analogues with specificity for the IRR (EPOR-βcR), enabling tissue-protective, but not erythropoietic activity. These include either molecules based on the modification of the EPO protein (for example, asialo-erythropoietin, obtained by enzymatic desialylation of EPO (Erbayraktar et al., 2003)) and carbamoylated EPO (Leist et al., 2004) (synthesized by cyanate carbamoylation of EPO) and peptides that mimic the three-dimensional structure of EPO (Brines et al., 2008). In general, modified proteins are quite effective (Leist et al., 2004), but as potential pharmaceutical agents, there are also the inherent problems of poor long-term stability, antigenicity, as well as high costs of production. Here, we describe the latest generation of a non-erythropoietic derivative mimicking the spatial configuration of EPO, pyroglutamate helix B surface peptide, pHBSP, also known as ARA-290, illustrating the most recent findings covering its tissue protective effects.

EPO is a globular protein due to the self-assembly of its 4 α-helices A–D. Three of the helices (A, C and D) are hydrophobic and participate in the two binding sites within the hematopoietic $(EPOR)_2$, while the remaining helix B, that is hydrophilic and points away from the EPOR, appears to be involved in tissue-protection (Brines, 2010). It is notable that helix B of EPO has been highly conserved in vertebrate evolution, in spite of the fact it is not involved in binding to the homodimeric EPOR underlying hematopoiesis (Brines & Cerami, 2013). Modeling peptides on the 3-dimensional surface of helix B (surface simulation modeling (Kazim & Atassi, 1980)) ultimately resulted in the generation of a low molecular weight peptide, the Helix-B Surface Peptide (HBSP). HBSP is an 11-amino acid, linear peptide that mimics the external, aqueous face of helix B resembled EPO (including amino acids 58–85 of EPO that are exposed at the helix B surface as well as three residues from the loop between helices B and C). The spontaneous cyclization of the N-terminal glutamine gives rise to pyroglutamate HBSP (pHBSP). The pharmaceutical product has pyroglutamate substituted for the N-terminal glutamine, resulting in the amino acid sequence of Pyr-Glu-Gln-Leu-Glu-Arg-Ala-Leu-Asn-Ser-Ser-OH (Pyr represents pyroglutamic acid) and a molecular weight of 1258 Da. The N-terminal substitution with pyroglutamate results in a peptide that is stable for at least 2 years at 4 °C or up to 12 months at 25 °C. In vitro testing has confirmed that this small peptide derived from the binding site of EPO to the EPOR-βcR complex binds the EPOR-βcR complex, but not the erythropoietic $(EPOR)_2$ homodimer (Brines & Cerami, 2008). In vivo tests have shown that it exerts the tissue-protective activities representative of the full EPO molecule, without stimulating hematopoiesis (Brines et al., 2008).

The pharmacokinetic behavior of pHBSP was determined after a single i.v. dose to male Sprague–Dawley rats, receiving either 60 μg/kg (48 nmol/kg) or 180 μg/kg (143 nmol/kg) of pHBSP, and to male New Zealand White rabbits, receiving either 30 μg/kg (24 nmol/kg) or 90 μg/kg (72 nmol/kg) of pHBSP (Brines et al., 2008). The mean peak drug concentration (Cmax) values were 254.67 (±53.59) and 1103.67 (±194.53) ng/ml for rats and 95.10 (±44.34) and 200.67 (±52.92) ng/ml for rabbits. Both Cmax and area under the concentrations vs. time curve (area under the curve) increased with increasing dose in a slightly more than dose-proportional manner, although the variability between animals within each dose group was considerable. The mean half-life was 0.028 h and 0.047 h for rats and 0.028 h and 0.038 h for rabbits.

More recently, a pharmacokinetic study on intravenous (i.v.) and subcutaneous (s.c.) administration of pHBSP in healthy volunteers has been performed, confirming similar observations (Niesters et al.). Specifically, the results showed that 2, 4 and 6 mg s.c. of pHBSP caused a dose-dependent increase in plasma Cmax from 1.8 ± 1.4 ng/mL (2 mg) to 8.3 ± 4.0 ng/mL (6 mg) occurring at t = 12 and 15 min respectively following injection with AUC of 53, 82, and 223 ng/mL/min, respectively. The elimination half-life ($t1/2_{elim}$) estimation was ~20 min. In contrast, 2 mg i.v. caused a rapid peak in C_{max} at min 1 of 111 ± 91 ng/mL with a $t1/2_{elim}$ of just 1.1 ± 0.1 min and AUC of 313 ng/mL/min.

5. Molecular mechanisms of tissue-protective effects

The rapid elimination after the i.v. injection is remarkable, suggesting a rapid passage of the drug into the effect compartment and rapid activation of the receptor followed by the initiation of a sustained cascade of events involving a series of transduction factors. The underlying mechanisms of action of the reported beneficial effects of pHBSP are intriguing, as this peptide has a short plasma half-life, while the protective effects in animals and humans develop over hours to days. From a pharmacokinetic/pharmacodynamics perspective, pHBSP behaves similarly to a number of neuropeptides: sustained biological effects in spite of very short biological half-lives. For example, adrenocorticotropic hormone (ACTH) is a 39 amino acid peptide that is secreted from the anterior pituitary in brief pulses with a biological half-life of only a few minutes (Veldhuis et al., 1987). Tetracosactin (ACTH 1–24) is an analogue which is used clinically to assess adrenal cortical function. In a normal individual, an intravenous bolus of ACTH 1–24 disappears from the circulation with a half-life of about 5 min and is eliminated from the circulation rapidly, followed after a delay of 15–30 min later with a sustained secretion of cortisol. Small versus large doses of tetracosactin stimulate the same cortisol production rate, i.e., the same peak level of cortisol and area under the curve (Alia et al., 2006). Simultaneously, a single bolus of this peptide activates the prolonged synthesis of enzymes involved in steroidogenesis (Lehoux et al., 1998).

As another example, teriparatide (1–34 parathyroid hormone) has a half-life of only 5 min when given intravenously, yet switches on receptor signaling (Castro et al., 2005) and stimulates long-lasting effects on serum calcium concentrations, beginning about 2 h after dosing and declines to baseline by 16–24 h, long after the peptide has been cleared completely from the circulation (Rubin & Bilezikian, 2005). If given once daily subcutaneously, teriparatide enhances bone formation. In contrast, more frequent or continuous administration of teriparatide mimics hyperparathyroidism and enhances bone resorption (Rubin & Bilezikian, 2005). Similar long-lasting effects have been reported for the glucagon-like peptide-1 (GLP-1), which also has a very short biological half-life (1.5–5 min). GLP-1 is a potent insulinotropic hormone, formed as a 30-amino acid peptide secreted from the L-cells of the intestinal epithelium in response to food. When GLP-1 is continuously infused to insulin resistant animals, blood glucose is normalized (Sreenan et al., 2000), with effects similar to those we recorded administering pHBSP in our mouse model of diet-induced insulin resistance (Collino et al., 2014). Notably, in an animal model of type 2 diabetes, the GLP-1-dependent amelioration in glucose tolerance was still detectable several days after the end of the infusion and the beneficial effect rather than declining, progressively increased, reaching a peak at day 9 (Hui et al., 2002). Similarly, a single injection of the dipeptidyl peptidase IV resistant analogue of GLP-1 (GLP-1-Gly8) in diabetic mice corrected fasting hyperglycemia and glucose intolerance for several hours, although this was in contrast to the rapid disappearance of the peptide from the blood stream (Burcelin et al., 1999). Relaxin is another peptide hormone, best known for the physiological role played during pregnancy, which has shown promise in the treatment of diabetes, heart failure, neurodegenerative diseases, hypertension, dyspnea and wound healing, despite its short serum half-life (1–3 min) (Cernaro et al., 2014). We have recently demonstrated that the beneficial effects of acute relaxin administration against renal ischemia/reperfusion (I/R) injury were still detectable 3 h after its last administration and they

were mediated by intracellular mechanisms similar to those required for pHBSP, including Akt-dependent endothelial nitric oxide·synthase (eNOS) activation (Collino et al., 2013). Overall, the above studies support the view that the pharmacodynamic effects of small peptides may actually be much longer than anticipated by their pharmacokinetic half-life.

In a similar manner to agents described above, pHBSP produces a uniform response once a Cmax is obtained that is >1 nmoles/L. For example, in a kidney I/R model a 10-fold increase in pHBSP dose produces the same biological effect (Brines et al., 2008). The protective effects were time-dependent, as administration of pHBSP significantly attenuated the I/R induced rise in plasma parameters of kidney function only when administered at 6 h. In contrast, early administration of pHBSP at either 1 min or 30 min into reperfusion had no significant effect when compared to vehicle control mice. In the same model, a single dose administered with a 6 h delay produces sustained effects when evaluated 48 h later as shown by an increase in Akt phosphorylation, associated with an enhanced eNOS activation, thus resulting in protection against renal I/R injury (Patel et al., 2012).

As recently confirmed in different cell lines of mesenchymal origin, the beneficial effects of pHBSP in restoring tissue homeostasis are mainly due to selective modulation of apoptotic and inflammatory pathways secondary to the activation of the heteromeric EPOR-βcR complex that is rapidly recruited to the cell surface following cellular stress (Bohr et al., 2015). Specifically, pHBSP was able to overcome a TNFα-mediated inhibition of transcription factors activation related to cell stress responses, such as the heat shock transcription factor protein-1 and the activator protein-1, thus affecting the transcriptional activation of various nuclear promoter sites related to cell stress responses and cell cycle control. All of these transcriptional responses are sustained and persist beyond exposure of the cells to pHBSP. Below we outline the most interesting signaling pathways involved in pHBSP mechanism of action (Fig. 2), which could help to explain, at least in part, its long-lasting beneficial effects.

Much experimental evidence suggest that the anti-inflammatory and anti-apoptotic effects associated with the activation of EPOR-βcR are due, at least in part, to the phosphorylation of Akt. In cultured neonatal rat cardiomyocytes and in the cardiomyopathic hamster, pHBSP activated Akt in a dose dependent manner reaching a maximum with a concentration of 2 nmoles/L. This effect was associated with significant inhibition of the apoptosis induced by TNF-α. In the same experimental models, pHBSP caused activation of other critical signaling pathways of cell survival, including ERK1/2, and STAT-3 (Ueba et al., 2010). Using a small interfering RNA approach, the authors confirmed that among the several pathways activated by pHBSP, only Akt plays an essential role in the prevention of apoptosis afforded by pHBSP. Similarly, silencing of the βcR abolished the Akt phosphorylation caused by EPO in endothelial colony-forming cells (Bennis et al., 2012).

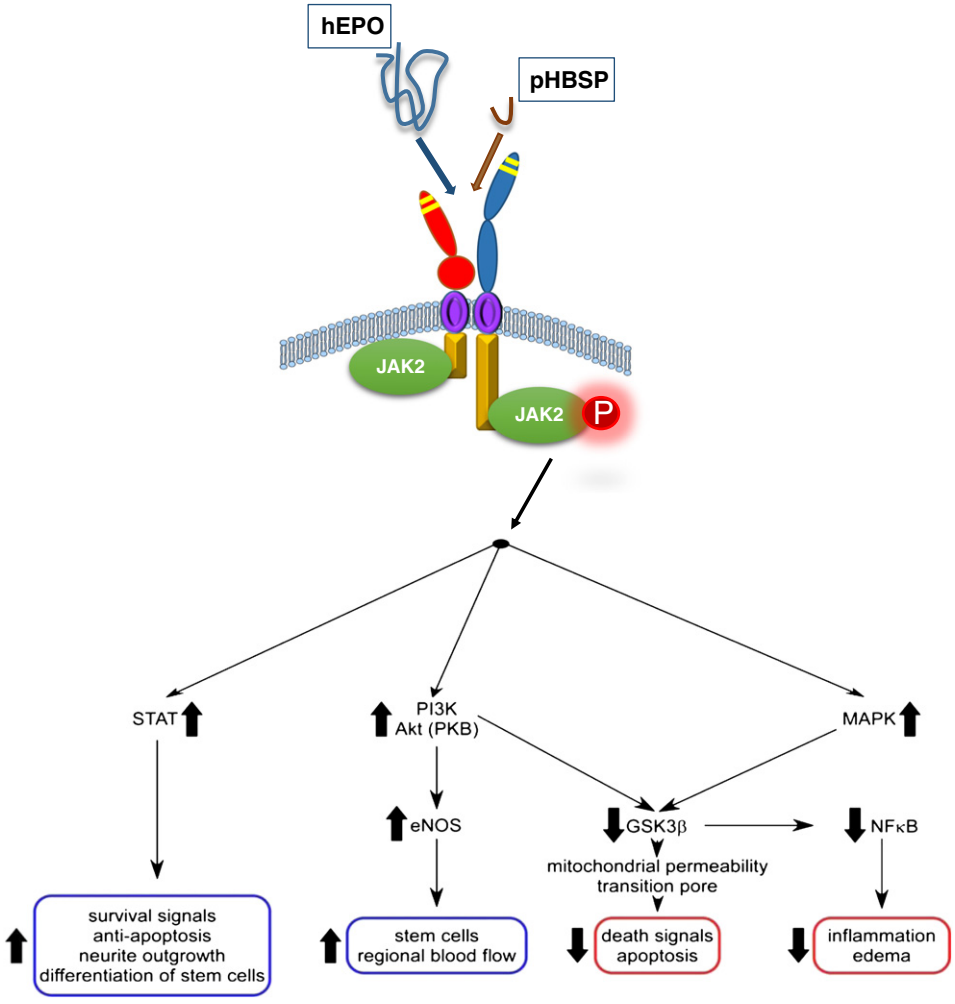

Fig. 2. Multiple intracellular signaling pathways are implicated in the biological activity of pHBSP. pHBSP or hyposialated EPO interacts with the innate repair receptor initially causing phosphorylation and activation of janus kinase-2 (JAK2). Subsequently, signal transducers and transcription factors, including STAT, Akt, AMPK, NF-kB and the mitogen-activated protein kinase systems are variably activated depending upon the tissue. Downstream sustained activation in survival, tissue reparative, and anti-inflammatory pathways leads to tissue protection and repair. Redrawn from ref (Brines & Cerami, 2012.

Additionally, inhibition of the PI3K/Akt pathway attenuates the protective effect of pHBSP in a murine model of renal I/R injury (Yang et al., 2013).

The PI3K/Akt axis is also known to regulate phosphorylation of the eNOS on Ser^{1177} in conjunction with the ERK1/2 signaling pathway. NOS activity is a physiologic regulator of cardiovascular function and physiological angiogenesis. Increased phosphorylation of eNOS causes activation of eNOS and results in an enhanced formation of NO in the microcirculation, which may contribute to the observed protective effects of EPO. The reduction of renal injury/dysfunction caused by pHBSP in a porcine model of I/R injury in the pig was associated with increased urinary nitrite + nitrate concentrations, suggesting increased eNOS activity (van Rijt et al., 2013). The phosphorylation (activation) of both Akt and eNOS afforded by EPO in the heart (Khan et al., 2013) and kidney (Coldewey et al., 2013) of septic mice is also silenced in βcR knockout mice, indicating that activation of the IRR by EPO is essential for the observed beneficial effects. Overall, these results indicate that the activation of the Akt/eNOS pathway plays a pivotal part in the observed beneficial effects of pHBSP.

In addition to causing an activation of eNOS secondary to activation of the PI3K/Akt pathway, EPO has been observed to also increase eNOS phosphorylation by enhancing the activity of AMP-activated protein kinase (AMPK) activity. This regulator of energy metabolism is integrated in EPO signaling via the βcR and inhibition of AMPK reduces eNOS phosphorylation (Su et al., 2011). In addition, an interaction between the βcR and the vascular endothelial growth factor receptor-2 has been described as being necessary for an activation of eNOS by EPO (Sautina et al., 2010). However, the signal transduction elements that link activation of the βcR by pHBSP to the vascular endothelial growth factor receptor-2 are not understood at present.

The PI3K/Akt signaling pathway is one of the most important pathways for cell survival and its activation by pHBSP via heterodimeric EPOR-βcR complex ameliorates apoptosis in models of renal I/R injury (Yang et al., 2013) and critical limb ischemia (Joshi et al., 2014). This is consistent with another study demonstrating that a peptide similar to pHBSP decreased apoptosis in response to renal I/R injury, via induction of the anti-apoptotic molecule Bcl-2 (Chattong et al., 2013).

The PI3K/Akt pathway plays also an important role in glucose metabolism and insulin signaling. Insulin receptor signaling through Akt promotes translocation of the glucose transporter type 4 by activation of AS160, resulting in increased glucose uptake. We recently demonstrated that pHBSP attenuated the impairment in insulin signaling, caused by a high fat, high sucrose (HFHS) diet in skeletal muscle (Collino et al., 2014). Specifically, pHBSP counteracted the HFHS-induced alterations in the phosphorylation of key insulin signaling molecules, Akt and its downstream substrate glycogen synthase kinase-3β. These effects were accompanied by a significant increase in the phosphorylation of AS160, followed by a marked improvement in membrane translocation of the glucose transporter type 4 and a robust increase in muscle glycogen content.

Finally, pHBSP has also been reported to alter T cell polarization and differentiation, but not proliferation (Chen et al., 2014; Cravedi et al., 2014). However, the exact mechanism of pHBSP-derived immunomodulation remains uncertain. An immune modulation in response to pHBSP was observed in a model of *Escherichia coli* invasion into urothelial cells, by reducing the activation of the protein tyrosine kinase focal adhesion kinase, which plays a prominent role in integrin-mediated cellular adhesion (Polgárová et al., 2011).

6. Long-lasting tissue-protective effects of pHBSP: from animal studies to clinical trials

6.1. Preclinical studies

In spite of a short half-life, in many systems pHBSP acts in a digital (all-in-one) manner producing prolonged biological effects. The beneficial cardiovascular effects of acute administration of pHBSP were demonstrated for the first time in two rodent models of renal I/R and middle cerebral artery occlusion injury, reporting degrees of protection similar to those recorded when EPO was administered (Brines et al., 2008). In the same study, protective capacities of prolonged pHBSP administration (s.c. daily for 10 days) in the healing of punch biopsy wounds have been provided. More recently, we demonstrated that a delayed administration (as late as 6 h into the reperfusion period) of a single dose of pHBSP was still able to improve the functional recovery of the kidney after the onset of reperfusion (after renal ischemia) in the rat (Patel et al., 2012). Similarly, van Rijt and colleagues have reported that the beneficial effects of pHBSP (when administered by an intravenous injection at 0, 2, 4 and 6 h post-reperfusion) in a porcine model of renal I/R were associated with significant reductions of biomarkers of acute inflammation (interleukin-6, and monocyte chemotactic protein-1). The protection afforded by pHBSP was still detectable at seven days post-reperfusion, and animals treated with the peptide showed both improvements in glomerular filtration rate and reductions in interstitial fibrosis (van Rijt et al., 2013). Similar protective effects were observed when HBSP or the carbamylated derivative of EPO, both devoid of any erythropoietic activity, were tested in rat and mouse models of renal I/R injury (Chattong et al., 2013; Wu et al., 2013; Yang et al., 2013).

Beneficial effects of pHBSP have also been reported in other models of injury associated with ischemia and/or reperfusion, including limb ischemia, MI and traumatic brain injury. In an in vitro model of myotube ischemia, pHBSP pretreatment decreased apoptotic nuclear fragmentation and cleaved caspase-3 expression in a multinucleated skeletal muscle cell line maintained under hypoxic conditions for 8 h. This effect was associated with a significant reduction in IL-6 release from the ischemic myotubes (Joshi et al., 2014). In a model of MI in the rat, a single systemic application of pHBSP immediately after coronary ligation reduced the infarct size to the same extent as that of EPO (Ahmet et al., 2011). This effect was associated with a significant reduction of necrosis, apoptosis and inflammation measured 24 h after MI induction in the area at risk. In addition, long-term treatment (for 10 months, two times per week) with pHBSP attenuated cardiac remodeling and reduced mortality in a rat model of post-MI-dilated cardiomyopathy (Ahmet et al., 2013). Notably, the hematocrit did not change significantly during the 10-month experiment and it did not produce any signs of arterial blood pressure elevation, thus confirming the assertion that continuous administration of pHBSP does not trigger the known undesirable erythropoietic or hemodynamic effects of EPO. In addition, pHBSP has been reported to exert protective effects against progression of coronary atherosclerotic lesions in a rabbit model that develops spontaneous MI, in part by inhibiting endothelial cell apoptosis (Ueba et al., 2013).

In a model of mild brain injury complicated by hypotension induced by withdrawal of blood, pHBSP administration every 12 h for 3 days showed protective effects, reducing contusion volume and improving recovery of cerebral blood flow in the injured brain following resuscitation (Robertson et al., 2012). Most notably, even when pHBSP was given as late as 30 to 60 min into the resuscitation period after 90 min of severe hemorrhage, single bolus injections of the peptide attenuated the renal dysfunction, liver injury, skeletal muscle injury and lung inflammation associated with severe hemorrhagic shock (Patel et al., 2011b). There are also convincing data on the neuroprotective effects of pHBSP, as shown using the experimental models of autoimmune encephalomyelitis and autoimmune neuritis, which mimic the human multiple sclerosis and the Guillain–Barré Syndrome, respectively. Therapeutic administration of pHBSP to rats reduced severity and shortened duration of the experimental autoimmune encephalomyelitis, probably by inhibiting the expression of inflammatory cytokines in the spinal cord, suppressing lymphocyte proliferation and altering T helper cell differentiation (Chen et al., 2014). Similarly, pHBSP greatly decreased the severity of the neurological symptoms and shortened the recovery time and total duration of the experimental autoimmune

neuritis, by decreasing the incidence of perivascular inflammatory cell infiltration in peripheral nerves and exerting direct cyto-protective and anti-inflammatory effects on Schwann cells, which are critical for the remyelination of the injured nerves (Liu et al., 2014). The neuroprotective activity of the EPO derivative in the peripheral nervous system has also been examined in rat models of post-status epilepticus model and neuropathic pain. Specifically, pHBSP mediated effects on hippocampal cell proliferation and neurogenesis, with significant improvement in epileptogenesis-associated cognitive deficits (Seeger et al., 2011). In addition, administration of pHBSP was effective in providing long-term relief of nerve injury-induced mechanical allodynia in both the spared nerve injury model, based on sciatic nerve surgical transection, and the neuritis model, which lacks gross nerve pathology (Swartjes et al., 2011; Pulman et al., 2013). A suppression of the neuro-inflammatory response, shown by reduction in microglia activation, was also involved in the protective effects of pHBSP against chronic neuropatic pain (Swartjes et al., 2014). Similarly, a reduction of the innate inflammatory response, mainly suppression of TNF-α mediated signaling, significantly contributed to pHBSP-induced prevention of the conversion of partial- to full-thickness burn injuries, in a mouse model of deep dermal burns (Bohr et al., 2013).

Very recently, the use of βcR knockout mice has enabled us and others to confirm that the activation of the heteromeric EPOR-βcR complex is indeed essential for the reduction of both kidney and cardiac dysfunction associated with sepsis, as well as in producing long-term relief of neuropathic pain (Swartjes et al., 2011; Coldewey et al., 2013; Khan et al., 2013).

There are some limited, but exciting, recent data that the beneficial effects of pHBSP may also be of benefit in animal models of diabetes mellitus and its complications. pHBSP is highly effective at preventing clinically relevant lesions of diabetic retinopathy, when administered for 1 month at 6 months after the induction of diabetes, dampening microglia activation and associated pro-inflammatory cytokine expression (McVicar et al., 2011). In a mouse model of diabetic autonomic neuropathy, treatment with pHBSP for 7 weeks decreased neuritic dystrophy, thus further confirming its neuroprotective effects (Schmidt et al., 2011). Very recently, we demonstrated that the chronic administration of pHBSP protects against the metabolic abnormalities, including insulin resistance, caused by a diet containing high concentrations of both fat and sugar. These effects were associated with pHBSP-induced modulation of signal transduction pathways involved in both insulin signaling and inflammation in the murine skeletal muscle. For instance, pHBSP counteracted the diet-induced alterations in the phosphorylation of IRS-1 protein as well as the activities of the downstream key insulin signaling molecules, Akt and GSK-3β, an Akt substrate, and it prevented local and systemic overproduction of myokines, such as IL-6 and FGF-21 (Collino et al., 2014). We also recorded protective effects against the mitochondrial dysfunction, which is a cardinal feature of insulin resistance.

6.2. Clinical studies

The above encouraging pre-clinical data have stimulated the following clinical trials. The first ones were two proof-of-concept clinical trials in patients with chronic neuropathic pain aimed to obtain indication of efficacy and safety of pHBSP in patients with either sarcoidosis or type 1 or 2 diabetes, with a pain score of at least 5/10. In the first study (Niesters et al., 2013), patients were treated with intravenous pHBSP injections (2 mg in 9 mL saline, given over 2 min). A total of three injections were administered at 2-day intervals. The one-week pHBSP treatment in patients with sarcoidosis was associated with a reduction in pain scores of 40% with the effect lasting for the 3 days following the end of treatment. Similarly, in diabetic patients the treatment resulted in a reduction in pain scores of 30% until at least day 8. The second trial was aimed to assess whether pHBSP produces analgesia greater than placebo in sarcoidosis-related neuropathic pain (Heij et al., 2012). This double-blind, randomized, placebo-controlled trial was performed with 22 sarcoidosis patients, of which 12 received a 4-week pHBSP treatment (2 mg IV with 3 injections/week); the others received normal saline instead of pHBSP. The preliminary data analysis revealed that pHBSP-treated patients had a significant greater decrease in neuropathic symptoms and a significant improvement in pain scores as determined from a quality of life questionnaire during the 4-week treatment period. No safety issues or adverse effects were noted by clinical or laboratory assessments (e.g., as expected no changes in hemoglobin concentration occurred). In light of the findings from the open-label studies, a double-blind, randomized, controlled trial has been recently performed in patients with sarcoidosis with moderate to severe neuropathic pain (the NERVARA trial). In contrast to the two previous trials, a subcutaneous formulation of pHBSP has been used in order to allow the patients to self-inject daily. This double-blind, randomized, placebo-controlled phase II trial confirmed the observations made in the open-label study showing that pHBSP (administered three times weekly for 4 weeks and 12 weeks of follow-up period) was associated with a significant improvement in neuropathic pain and autonomic symptoms, with no effect on hemoglobin concentrations (Dahan et al., 2013).

An extensive double-blind, randomized, controlled trial of the effects of 28 days of treatment with pHBSP in type 2 diabetes-related small-fiber neuropathy has recently been completed (Brines et al., 2014). Neuropathic symptoms, as assessed by the PainDetect questionnaire, improved to a clinically significant degree in the patients treated with pHBSP. Additionally, patients that received pHBSP also exhibited improved HbA1c and lipid profiles throughout the 56-day observation period. These observations suggest that pHBSP may improve metabolic control and reduce neuropathic symptoms in patients with type 2 diabetes. These findings are very important and warrant further clinical evaluation.

7. Conclusions

There is now good evidence that activation of the EPOR-βcR heterodimer by pHBSP (or EPO itself or other molecules that mimic the tissue-protective effects of EPO) when reaching a critical minimum concentration (>1 nmole/L) flips a molecular switch that activates long-lasting survival pathways (e.g., PI3K/Akt, eNOS), resulting in inhibition of inflammation, apoptosis, and activation of repair. These effects were observed in animal models of acute illness (e.g. MI, acute kidney injury, stroke, hemorrhagic shock) in which the peptide was administered after the injury, namely when up-regulation of the IRR has occurred. These effects were also observed in patients with chronic disease (diabetes mellitus or sarcoidosis) when the peptide was given repetitively (therapeutic administration). These preclinical and early clinical data provide a very good indication that the reported pharmacodynamic effects of pHSBP may last significantly longer than anticipated by its pharmacokinetic half-life. This enables therapeutic intervention via a pulse dosing protocol, which provides adequate receptor triggering while limiting the potential side effects and improving patient compliance. Most notably, the well-documented adverse effects of a chronic administration of EPO (e.g. thrombovascular events) have not been reported for pHSBP, making this molecule an ideal candidate to explore the reported tissue-protective and anti-inflammatory effects of the activation of the IRR in chronic diseases, including diabetes and neuropathy. Further phase 2 clinical trials evaluating the potential beneficial and adverse effects of pHSBP in chronic diseases are warranted, and their results are expected with great anticipation.

Conflict of interest statement

MC and CT declare no conflicts of interest to report. AC and MB are officers and own stock and/or stock options in Araim Pharmaceuticals, Inc, which is developing pHBSP for clinical use.

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