Erythropoietin in heart failure
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Erythropoietin in cardiac disease: New features of an old drug

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ABSTRACT

Erythropoietin (EPO) is a haematopoietic hormone with extensive non-haematopoietic effects. The discovery of an EPO receptor (EPOR) outside the haematopoietic system has fuelled the research into the beneficial effects of EPO for various conditions, predominantly in cardiovascular disease. Experimental evidence has revealed the cytoprotective and angiogenic properties of EPO and it seems that the EPO-EPOR system provides a powerful backbone against acute and chronic myocardial ischemia, each gaining from the different properties of EPO. Clinical trials in which EPO was titrated to achieve certain haematocrit levels have generated equivocal results. It has been suggested that a (too) high haematocrit is undesirable in cardiovascular disease. We have shown that intermittent (low-dose) EPO administration, that does not increase haematocrit substantially, suffices to activate the beneficial downstream pathways of EPO. We postulate that intermittent administration or a lower than conventional dose of EPO, not only aimed at increasing haemoglobin at high levels, will provide powerful cellular protection and will improve cardiac outcome, without the side-effects of EPO associated with increased haematocrit.
INTRODUCTION

Erythropoietin (EPO) is a haematopoietic hormone with extensive non-haematopoietic effects. The recombinant human form of EPO has been used for several decades now in the treatment of anaemia, mostly in chronic kidney disease. The use of EPO has markedly increased, as it is now widely applied e.g. in cancer patients receiving chemotherapy, HIV positive patients treated with zidovudine, and treatment of myelodysplastic syndromes. Furthermore, EPO treatment as prophylaxis to reduce blood transfusions during mayor surgery is nowadays also common practice. Recently, several large scale trials suggested an adverse effect of EPO in patients with chronic kidney disease.1,2 This has prompted a fierce discussion on the use of EPO in chronic kidney disease and other (off-label) indications. Much debate is on the optimal dosage of EPO, since both very low and very high haematocrit are associated with excess mortality.3,5 We postulate that EPO exerts its effects mainly via activation of anti-apoptotic and pro-angiogenic pathways, while the erythropoietic effects are rather secondary. To achieve this pro-angiogenic, anti-apoptotic, and haematocrit-neutral status, intermittent, not continuous, administration of EPO is warranted. By doing this, we may circumvent the problems of complicated EPO titration and (too) high haematocrit and associated side-effects.

EPO AND ITS RECEPTOR

Already a century ago, in 1906, Carnot and DeFlandre suggested the existence of a circulating erythropoietic factor.6 Fifty years later EPO was discovered and the kidneys were established as the predominant site of production.7 The glycoprotein EPO has a predominant role in red blood cell production. The EPO gene is located on chromosome seven, encoding for a polypeptide chain containing 193 amino acids. EPO is produced in the foetal liver and in the adult kidney in response to hypoxia, mainly under control of hypoxia inducible factor (HIF)-1.8 Tissue oxygen demand and oxygen transport capacity regulate EPO production and secretion.9,10 A principal effect of EPO is the prevention of physiological apoptosis, which normally adds to erythroid progenitor cell turnover.11 A functional EPO receptor (EPOR), which was previously thought only to be present in haematopoietic progenitor cells, is also expressed in non-haematopoietic systems, such as the cardiovascular system, the central nervous system, and others.12 This discovery started extensive research to the non-haematopoietic effects of EPO. EPO modulates a broad array of cellular processes that include progenitor stem cell development, cellular integrity, and angiogenesis. As we stand, EPO is emerging as a cell death blocker and a vascular growth factor with promising protective potential in the setting of acute and chronic myocardial ischemia and may potentially represent a powerful pharmacological addendum in the fight against cardiovascular diseases.
Chapter 2

EPO RECEPTOR IN THE CARDIOVASCULAR SYSTEM

In 1992 the first clues of existence of an EPOR outside the haematopoietic progenitor cells were published when Tan et al. demonstrated the existence of EPO messenger RNA in the brain. Nowadays, the expression of EPO and its receptor has been shown in numerous tissues, including the reproductive organs, liver, kidney, endothelial and vascular smooth muscle cells, brain and the heart. The EPOR is expressed in even more tissues, such as in gastric mucosal cells, pancreatic islets and even in the prostate epithelial cells. The EPOR is a member of the type 1 cytokine receptor family characterised by a single transmembrane domain. Under normal circumstances EPO and EPOR have a relatively low expression in non-haematopoietic tissue, however, expression of EPO and EPOR is rapidly increased in response to hypoxia and a number of other metabolic stressors including pro-inflammatory cytokines, hypoglycaemia and increased reactive oxygen species. These stressors directly activate the HIF-1, -2 and -3 pathways, which in turn regulate the gene transcription of EPO and EPOR. Activation of the EPOR leads to downstream activation of intracellular pathways such as phosphoinositide 3 kinase (PI3K) / protein kinase B (Akt), mitogen activated protein kinase (MAPK) and signal transducers and activators of transcription (STAT). These pathways are associated with cell survival. These pathways originate with the binding of EPO to the EPOR to activate Janus-tyrosine kinase 2 (Jak2). Next, by phosphorylation, PI3K and Akt are activated. Activation of the specific gene product STAT5 can regulate EPO mediated cell proliferation and protect against apoptosis, which are direct substrates of Jak2. EPO maintains cellular integrity and prevents apoptosis through a number of pathways, such as the modulation of apoptosis protease activating factor-1 (Apaf-1), the release of cytochrome C, and the activation of caspases 1, 3 and 9. EPO also modulates cellular inflammation by inhibiting cellular phosphatidylserine membrane exposure and subsequent targeting of cells for phagocytosis. In two recent studies, EPO increased endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) production in cardiomyocytes. Blocking eNOS activity significantly decreased the anti-apoptotic effects of EPO, suggesting that the anti-apoptotic effects of EPO in cardiomyocytes are, partially, mediated by eNOS-derived NO production and is crucial for the anti-apoptotic effects of EPO as well. As a result of this upregulation of the EPOR, EPO robustly protects the cell, mainly by inhibiting the apoptotic mechanisms of injury, including the preservation of cellular membrane asymmetry to prevent inflammation. Genetically engineered mice provided more in-depth insight into the importance of the EPOR. EPOR expression in the erythropoietic lineage cells is necessary for normal development of mammals. EPOR knock-out mice, lacking the EPOR in their erythroid lineage, die of severe anaemia between embryonic day 13 and 15. EPO and EPOR play an essential role in proliferation, survival and differentiation of erythroid progenitor cells. Wu et al. demonstrated that EPO and EPOR have a major function in embryonic heart development, since EPO⁻/⁻ and EPOR⁻/⁻ mice experience ventricular hypoplasia and defects in the intraventricular septum. In addition to these findings, Suzuki et al. developed a mouse model in which EPOR expression is restricted to the erythropoietic lineage, by targeted knock-in of the EPOR gene ligated to the
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GATA-1 promoter, a transcription factor exclusive to erythroid lineage cells (EPOR⁻/⁻-rescued mice). These mice express the EPOR exclusively in their erythropoietic cells, while other organs lack the EPOR. These mice develop normally and are fertile, so that it appears that EPO and EPOR are dispensable for normal development. However, these mice were subjected to various acute and chronic models of cardiovascular disease, and EPO and EPOR seem to play a major role in protection to cardiovascular damage. A deficiency of the endogenous EPO-EPOR system deteriorates cardiomyocyte survival after ischemia-reperfusion (I/R) injury and subsequent left ventricular remodelling. This effect is partially due to enhanced apoptosis of cardiomyocytes. In the setting of peripheral artery disease the EPO system plays an important role in angiogenesis in response to hind limb ischemia through upregulation of vascular endothelial growth factor (VEGF) and the VEGF receptor (VEGFR), both directly by enhancing neovascularisation and indirectly by mobilising endothelial progenitor cells (EPCs). Asaumi et al demonstrated that the deletion of EPOR in non-haematopoietic cells results in enhanced
susceptibility to the development of heart failure (HF) in mice with pressure overload of the left ventricle. The enhanced susceptibility to left ventricular failure was associated with impaired phosphorylation of STAT3 and p38, decreased expression of VEGF and impaired left ventricular neovascularisation.\cite{59} In pulmonary hypertension, mobilisation of EPCs from the bone marrow and their incorporation into the pulmonary endothelium are impaired in EPOR\(^{-/-}\)-rescued mice, with a resultant potentiation of pulmonary hypertension and pulmonary vascular remodelling in response to chronic hypoxia.\cite{60} EPO treatment was also associated with less cardiac and pulmonary vascular remodelling in flow-associated pulmonary hypertension. Molecular studies in this model suggested that the VEGF-VEGFR system may be involved in mediating these effects of EPO.\cite{61} Upregulation of VEGF results in angiogenesis in hypoxic tissue.\cite{62-65} The group of Folkman et al has shown that angiogenesis in the atherosclerotic plaque can lead to instability of the plaque and at a final stage rupture of the plaque. When inhibiting this angiogenesis in the atherosclerotic plaque, intimal neovascularisation and plaque growth are diminished.\cite{66,67} This process of VEGF mediated angiogenesis might be a side effect of prolonged, chronic use of EPO (figure 1). Together, current evidence supports a pivotal role of the EPOR in the complex cascades of acute and chronic hypoxic damage in the body and, more specifically, the cardiovascular system.

**EPO TREATMENT FOR ACUTE MYOCARDIAL ISCHEMIA**

Apoptosis and necrosis are the major forms of cell death contributing to the extent of damage after myocardial infarction (MI) and are a major determinant of the final infarct size.\cite{68-71} Furthermore, apoptosis contributes to the final injury size during injury caused by revascularisation.\cite{72} It is postulated that this reperfusion injury might contribute up to 50% of the final size of damage,\cite{73} in turn suggesting a large potential for therapeutical interventions.

**Ischemia-reperfusion injury**

In several experimental studies it has been established that EPO exerts cytoprotective effects.\cite{74-84} The anti-apoptotic effect of EPO is the acute protecting mechanism of EPO during ischemia (figure 2).\cite{85,86} Clearly, this effect is independent from haematocrit-increasing effects of EPO, but is rather exerted via distinct apoptotic pathways. EPO exerts its potent anti-apoptotic effects in a number of cellular systems, including cultured endothelial cells and neonatal rat cardiomyocytes.\cite{87,88} Cavillo et al. administered a high-dose of EPO (5.000 IU/kg) for seven consecutive days in an I/R injury model in rats. This reduced cardiomyocyte loss by 50%, an extent sufficient to normalise haemodynamic function within one week after reperfusion.\cite{89} Other experimental *in vivo* studies using an I/R injury model showed decreased infarct size,\cite{90-95} enhancement of left ventricular function,\cite{96,97} and less apoptosis,\cite{98,99} even when EPO was administered after the onset of reperfusion.\cite{100} We have shown a 16% reduction (measured at a random, given moment, thus underestimating the true number of apoptotic cells) in number of apoptotic cells in pre-treated animals with EPO in an I/R injury model.\cite{101,102} Furthermore, apoptosis was significantly attenuated in animals treated with EPO at the start of ischemia (29% reduction) and after the onset of reperfusion (38%).\cite{103} Importantly, these positive effects of EPO are observed both at a high-dose administration and at a low-dose administration of EPO.\cite{104,105}
Acute coronary syndromes

Experiments using permanent occlusion of the left coronary artery to induce MI show smaller infarct size, prevention of left ventricular dilatation, improved left ventricular ejection fraction (LVEF) and increased capillary density.\textsuperscript{106,107} Amongst others, Moon et al. found a reduction of the infarct size up to 25% after permanent ligation of the coronary artery using a single dose of EPO (3.000 IU/kg) compared to untreated animals examined eight weeks later.\textsuperscript{108} From our own group, Lipsic et al. has recently shown that low-dose (0.4 μg/kg/3 weeks) EPO administration had no effect on haematocrit levels (figure 3A). Low-dose EPO significantly improved cardiac function, reflected by increased left ventricular developed pressure and improved contractility (dP/dtmax) and relaxation (dP/dtmin), indices of the left ventricle at nine weeks after MI (p<0.05 compared to MI) (figure 3B).\textsuperscript{105}

In the above-mentioned studies, EPO was administered at different times before and after I/R injury and permanent occlusion of the coronary artery, and in different dosages. All show the beneficial effect of EPO administration, indicating a broad window of opportunity for the potential treatment of MI in the human setting. From these data, it becomes clear that EPO administration represents a powerful anti-apoptotic and pro-angiogenic treatment. These effects are seemingly irrespective of the dose and dosage interval chosen, so that it may be proposed to low-dose and use EPO-free interval in order to prevent undesired haematocrit increases.

\textbf{Figure 2.} Schematic view of the protective properties of erythropoietin in time after ischemic insult.
Clinical studies
We performed a single centre, safety trial exploring the effects of low-dose EPO in the setting of acute MI. Only small and non-significant changes in haematocrit levels were observed, while EPCs were increased at 72 h (2.8 vs. 1.0 cells/μl in control group, p<0.01). No adverse events were recorded during the 30-day follow-up. Together with the available experimental data this we considered a promising role for EPO as a cytoprotective agent in the setting of an acute MI and reperfusion injury afterwards. To evaluate this strategy in patients, in The Netherlands the HEBE III trial is currently conducted in which the effect of a single bolus of EPO is evaluated in patients with acute MI on LVEF (Clinical Trials number NCT00449488). In total, 466 patients will be included. The REVEAL study, which has a somewhat similar design, is currently performed in the United States of America (Clinical Trials number NCT00378352).

Figure 3. A. Effect of erythropoietin (EPO) treatment on longitudinal changes in haematocrit (p<0.01 vs. sham) in a model of experimental heart failure in rats. B. Effects of myocardial infarction (MI) and high-dose and low-dose EPO treatment on haemodynamic parameters, dP/dtmax and dP/dtmin; * p<0.05 vs. MI, ** p<0.01 vs. MI. C. Representative pictures of the effect of EPO treatment on incorporation of endothelial progenitor cells after MI into new vasculature. Staining of three different groups: sham animals, MI animals and MI animals treated with EPO (green: hPAP, red: His 52; endothelium and blue: DAPI; nucleus). More double positive cells are found in the EPO treated group, suggesting incorporation of bone marrow derived endothelial progenitor cells in ischemic tissue contributing to neovascularisation. Sham: sham operated animals; MI: myocardial infarction, placebo treated animals; MI-EPO high: myocardial infarction animals, treated with high-dose EPO; MI-EPO low: myocardial infarction animals treated with low-dose EPO. A and B reprinted from Lipsic et al, Eur J Heart Fail 2008;10:22-29. Copyright (2008), with permission from Elsevier. For colour figure, see supplement 1.
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**EPO TREATMENT FOR CHRONIC HEART FAILURE**

Sustained EPO signalling may also have salutary effects beyond erythropoiesis (figure 2): it brings about neovascularisation and recruitment of EPCs and improves cardiac function in rats with HF.\textsuperscript{110,111} Irrespective of epicardial coronary anatomy, perfusion of the myocardium in HF becomes insufficient due to disproportionate cardiomyocyte hypertrophy relative to (micro) vascular growth, resulting in ischemia and upregulation of the different HIFs.\textsuperscript{112,113} Therapies aimed at improving cardiac microvascularisation might therefore result in improvement of myocardial contractility and might favourably affect outcome in patients with chronic HF (figure 1). EPO has potent angiogenic properties, promoting proliferation and tube formation of endothelial cells \textit{in vitro} and stimulating angiogenesis in numerous experimental models \textit{in vivo}.\textsuperscript{114-116} In addition, EPO induces the proliferation, differentiation and adhesion of a subset of bone marrow derived progenitor cells with an endothelial phenotype \textit{in vitro} and results in marked mobilisation of EPCs \textit{in vivo}.\textsuperscript{117-119} EPCs have been shown to specifically home to ischemic tissues and stimulate neovascularisation by incorporating into newly formed vascular structures and by paracrine secretion of angiogenic factors.\textsuperscript{120,121} In patients with chronic HF, EPO levels are increased. This increase might be a protective but insufficient response to promote angiogenesis in the heart.\textsuperscript{122} Considering these angiogenic properties, we evaluated the therapeutic potential of darbepoetin alpha (long acting EPO analogue, 40 μg/kg/3 weeks) in rats with chronic myocardial dysfunction after MI.\textsuperscript{123} EPO treatment was initiated three weeks after the MI and although this did not result in a reduction of infarct size, left ventricular contractility and relaxation was significantly improved. As expected, the improvement of cardiac function was associated with increased capillary density and increased capillary-to-myocyte ratio, indicating neovascularisation. These beneficial effects of EPO on cardiac function and microvascularisation in post-MI left ventricular dysfunction has recently been confirmed by four independent studies.\textsuperscript{124-127} EPO-induced neovascularisation in HF is consistently associated with increased numbers of circulating EPCs.\textsuperscript{128} Therefore, we recently evaluated the contribution of EPCs to EPO induced neovascularisation by replacing bone marrow of rats with genetically labelled cells, which allowed us to quantify the contribution of EPCs and in situ proliferation of endothelial cells to neovascularisation.\textsuperscript{129} In our study, EPO significantly increased mobilisation and incorporation of EPCs into the myocardium, accounting for approximately 30% of the new vessels. The remaining 70% of the new vessels thus originated from in situ proliferation of myocardial endothelial cells, which was associated with a five-fold increase expression of VEGF. EPO-induced neovascularisation in post-MI HF therefore relies on a combination of EPC recruitment from the bone marrow and increased myocardial expression of VEGF. Moreover, the presence of ischemia is pivotal for EPO induced myocardial regeneration, since EPO treatment in healthy rats does not affect cardiac function nor does it improve myocardial microvascularisation (figure 1).\textsuperscript{130} Interestingly, EPC-mediated endothelial cell turnover was significantly improved by EPO, associated with marked improvement of endothelial function suggesting a potential therapeutic role in endothelial dysfunction.\textsuperscript{131} The effects of EPO on EPCs seem mediated
through the Akt-eNOS pathway, whereas in situ endothelial proliferation has been linked to the Jak2-STAT3 and MAPK-p38 pathways.\textsuperscript{132}

The dosing regimens used in previous studies, all resulted in a significant increase in haematocrit levels. When applied to the clinical situation, this could lead to hypertension, seizures, vascular thrombosis and death, possibly related to abruptly increased haematocrit levels.\textsuperscript{133} Therefore, we recently evaluated the effect of a low-dose EPO bolus that had no effect on haematocrit. Similar to high-dose EPO, low-dose treatment resulted in statistically improved cardiac function and improved myocardial microvascularisation, although the effect was slightly less pronounced.\textsuperscript{105} These results do not only suggest that the beneficial effects of EPO on the heart may be independent of an increased haematocrit but also suggest that low-dose EPO might provide a safe and effective strategy in patients. Another option to avoid the potentially negative effects of chronic EPO therapy in haematocrit values could be the use of recently discovered non-erythropoietic derivates of EPO, retaining the tissue protective properties, without the undesired effect on erythropoiesis.\textsuperscript{134} Two independent studies have demonstrated that these non-erythropoietic EPOs retain their cardioprotective potential in models of acute MI.\textsuperscript{135,136} It is however uncertain whether these new EPOs will improve cardiac function in chronic HF.

SAFETY ISSUES

All dose regimens used in clinical studies resulted in a significant increase in haematocrit levels. Raised haematocrit levels have been linked to hypertension, vascular thrombosis, and (rarely) death (figure 1).\textsuperscript{137} In the case of EPO treatment in acute myocardial ischemia, this danger might not apply, because a single bolus does not seem to significantly raise haemoglobin and haematocrit levels.\textsuperscript{138} Nevertheless, the increase in haematocrit associated with the use of EPO may offset its beneficial effects. A possibility for future research is to use EPO derivates which only display the non-haematopoietic properties of EPO.\textsuperscript{139,140} These derivates have shown to be as effective in cardioprotection as recombinant human EPO in a model of acute MI.\textsuperscript{141,142} Another possibility would be to use a low-dose, intermittent, dose regimen that does not elicit a substantial increase in haematocrit, yet does activate the pro-angiogenic and anti-apoptotic pathways of EPO.

To take the experimental findings for MI and chronic HF to the clinic, large multicentre, double blind, randomised, placebo controlled trials were designed (HEBE III (MI), REVEAL (MI), RED-HF (chronic HF)) and results have to be awaited.\textsuperscript{143} Recent data from two randomised clinical trials using recombinant human EPO to correct anaemia in chronic kidney disease provided neutral to negative data. The CREATE study evaluated the effect of ‘complete’ haemoglobin correction (target haemoglobin 8.1 to 9.3 mmol/l) to ‘lower’ haemoglobin correction (target haemoglobin 6.5 to 7.1 mmol/l) in chronic kidney disease patients. In this study no change in risk on cardiovascular events was seen.\textsuperscript{144} Second, the CHOIR study, which evaluated the effect on cardiovascular complications of achieving ‘high’ haemoglobin levels (target haemoglobin 8.4 mmol/l) compared to ‘lower’ haemoglobin levels (target haemoglobin 7.0 mmol/l) in chronic kidney disease patients. This study was terminated prematurely, because
of an increased adverse event rate in the high haemoglobin group. Especially the CHOIR has fuelled the debate on the safety of recombinant human EPO. As a result, the Food and Drug Administration (FDA) has recommended “the lowest possible dose to slowly raise the haemoglobin concentration to the lowest level that will avoid the need for a blood transfusion”. A third study in anaemic chronic kidney disease patients is currently running (TREAT). This study evaluates the effect of recombinant human EPO treatment (to achieve haemoglobin levels of 8.1 mmol/l) on cardiovascular events in this patient group. The current data was evaluated by the Data and Safety Monitoring Board and till date there is no evidence to terminate this study prematurely.

It may be that the beneficial effects of EPO in chronic HF patients are different from chronic kidney disease patients. Several randomised, double blind, placebo controlled studies showed improved quality of life in anaemic chronic HF patients receiving recombinant human EPO. More important, both studies do not show a difference in the incidence of adverse events. The main difference between EPO treatment in chronic HF and chronic kidney disease patients is the interval of treatment, i.e. chronic kidney disease patients are treated for a longer period of time and generally need higher dosages of EPO. Long term treatment with EPO resulting in angiogenesis in the atherosclerotic plaque could lead to instability of the plaque and finally to rupture. On the other hand, inhibiting angiogenesis reduces intimal neovascularisation and plaque growth. Chronic EPO administration, as practiced in chronic kidney disease patients may increase angiogenesis in the atherosclerotic plaque by increased VEGF production, which may be associated with plaque rupture and acute coronary syndromes, MI and death and this may explain the unexpected effects of EPO observed in trials with chronic kidney disease patients. For sure, current trials will be monitored carefully.

CONCLUSIONS

The discovery of an EPOR outside the haematopoietic system has fuelled the research into the beneficial effects of EPO for various conditions, predominantly in cardiovascular disease. Experimental evidence has revealed the cytoprotective and angiogenic properties of EPO and it seems that the EPO-EPOR system provides a powerful backbone against acute and chronic myocardial ischemia, each gaining from the different properties of EPO.

However, clinical trials in which EPO was titrated to achieve certain haematocrit levels have generated equivocal results. It has been suggested that a (too) high haematocrit is undesirable in cardiovascular disease. We have shown that intermittent (low-dose) EPO administration, that does not increase haematocrit substantially, suffices to activate the beneficial downstream pathways of EPO. We postulate that intermittent administration of EPO, as opposed to continuous administration, will provide powerful cellular protection and will improve cardiac outcome, without the side-effects of EPO associated with increased haematocrit. Clinical trials in both acute and chronic myocardial ischemia are currently underway and results are eagerly awaited.
REFERENCES

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