Erythropoietin in Heart Failure
Effects beyond Erythropoiesis

Willem-Peter T. Ruifrok

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Contents

CHAPTER 1

Introduction and aim of the thesis

INTRODUCTION

Heart failure (HF) is a common and complex clinical syndrome.[1] HF is associated with very poor outcome (1-year mortality of 20%), which becomes even worse when co-morbidities are present.[2-4] HF may arise from various aetiology and has variable clinical manifestations, notably including dysfunction of other organs than the heart, such as skeletal muscle, kidney and the bone marrow. In addition to increased mortality, high rates of hospital admission, extensive diagnostic testing and the chronic use of multiple pharmacological agents account for an immense healthcare expenditure.[5] As a result, about 1% of the entire health care budget is spent on HF. Over the past decades, treatment options for HF have improved importantly with the addition of beta-blockers, angiotensin converting enzyme (ACE)-inhibitors, aldosterone antagonists, angiotensin receptor blockers (ARBs), and device therapy such as biventricular pacing with cardiac resynchronization therapy and internal defibrillators.[6-16] However, despite all these treatment options, morbidity and mortality rates are still increasing globally. New therapeutic possibilities either focusing on the prevention of HF or on the improvement of signs and symptoms of already existing HF are therefore pivotal.

Before overt HF ensues, the heart, specifically the left ventricle (LV) undergoes changes in response to damaging stimuli, which is referred to as LV remodelling. Common aetiologic factors for HF are hypertension, ischemic heart disease with or without myocardial infarction (MI), valvular disease and myocarditis. All of these factors initiate the remodelling process. The initial response of the heart to stress is cardiomyocyte hypertrophy, while in later stages fibroblast and macrophage activation leads to apposition of extracellular matrix, and often remodelling becomes progressive and finally results in LV dilatation and congestive HF.

So although deemed initially useful, remodelling over time often becomes maladaptive. Maladaptive remodelling is characterised by excessive myocyte hypertrophy and apoptosis, interstitial and perivascular fibrosis (including replacement fibrosis) and a decrease in number of capillaries. The growth (in number and size) of capillaries, which theoretically would be needed to supply the hypertrophic LV with oxygen and nutrients, is insufficient in LV hypertrophy, thus creating an imbalance between the number of capillaries and cardiomyocytes. This imbalance is thought to further contribute to the remodelling process as it causes chronic hypoxia and less flow reserve, with further tissue damage as a result.[17,18] Without treatment, this downward spiral continues, resulting in HF, and finally in death. Current treatment targets in HF target the cardiomyocyte hypertrophy or are anti-fibrotic. However, the microvasculature is difficult to target and no specific treatments are available.

One of the common co-morbidities in HF is anaemia, which is associated with a poor outcome.[2,4] Its prevalence varies with the severity of HF and with the definition of anaemia that is used.[19] The causes of anaemia in HF are only partially understood, although several mechanisms have been implicated,[20] including treatment with ACE-inhibitors,[21] a blunted erythropoietin (EPO) production due to renal dysfunction,[22] congestion,[23] and iron deficiency.[24] However, these causes only partially explain the severity of anaemia and proved to be difficult targets for treatment.

EXPERIMENTAL FINDINGS AFTER EPO ADMINISTRATION

EPO is a haematopoietic hormone and increases red blood cell maturation and growth. Remarkably, EPO has extensive non-haematopoietic properties, which suggest that EPO has functions other than haematopoiesis alone. 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 (cardiomyocytes, endothelial cells) and the central nervous system.[25] These discoveries fuelled intense research on the non-haematopoietic effects of EPO. EPO modulates a broad array of cellular processes that include progenitor stem cell release, cellular integrity and apoptosis, and angiogenesis. EPO has emerged as a potential anti-apoptotic agent and a vascular growth factor with promising protective potential in the setting of acute and chronic myocardial ischemia. In numerous experimental studies it has been established that EPO exerts cytoprotective and angiogenic effects.[26-35] The anti-apoptotic effect of EPO is the predominant acute protecting mechanism of EPO during ischemia, while induction of neovascularisation is the predominant long-term effect of EPO.

EPO administration in ischemia-reperfusion (I/R) injury models reduces cardiomyocyte loss by 50%, decreases infarct size, enhances LV function and reduces apoptosis, even when EPO is administered after the onset of reperfusion or in low non-haematocrit increasing dosages.[27,33,34,36-42] In models of permanent MI, EPO induces neovascularisation, but only in the presence of ischemia.[42-44] Because of these non-haematopoietic effects, EPO may potentially represent a powerful pharmacologic addendum in the fight against cardiovascular diseases.

RESULTS FROM CLINICAL TRIALS WITH EPO

The very first feasibility and safety study in patients with acute MI was performed by Lipsic et al.[45] No adverse events were recorded during 30-days follow-up. Left ventricular ejection fraction (LVEF) measured with planar radionuclide ventriculography after four months, was similar in both groups. Although haematocrit levels were not significantly different in both groups, EPO treatment significantly increased the amount of endothelial progenitor cells (EPCs) (CD34+/CD45-). Further small studies showed variable results that ranged from no effect on enzymatic infarct size and LV function to increase in angiogenesis signalling proteins in peripheral blood mononuclear cells (table 1).[46-49] One of these studies, the REVIVAL-3, raised safety concerns.[46] The REVIVAL-3 evaluated the effect of a short-term infusion of high dose EPO in patients with ST-segment elevation MI treated with primary percutaneous coronary intervention (PCI). The primary endpoint was LVEF assessed by MRI at 6 months, which was similar in both groups. The secondary endpoint of this study, consisting of combined clinical events (death, recurrent MI, infarct-related artery revascularization, or stroke) occurred in 13% of EPO-treated patients as compared with 6% in the placebo group. Although this difference did not reach statistical significance, the tendency towards an increase in serious adverse events raised concerns about the safety profile of EPO in patients with MI.

Several small trials addressed the use of EPO in chronic HF. Again, these small scale studies

Table 1. Clinical studies: The effects of EPO in acute myocardial infarction

Study	Study Design	Number Patients	Patient Characteristics	Results
Lipsic *et al* [45]	Investigator-initiated, feasibility and safety study	22	First STEMI treated with primary PCI	Safe and well tolerated Stimulates EPC mobilisation
Liem *et al* [47]	Randomised, placebo-controlled study	51	NSTEMI	No effect on enzymatic infarct size
Ott *et al* [46]	Randomised, double-blind, placebo-controlled study	138	First STEMI treated with primary PCI	No difference in left ventricular function or infarct size
Binbrek *et al* [48]	Randomised, open label, blinded endpoint study	236	STEMI treated with tenecteplase	Safe, but no enhanced preservation of jeopardised ischemic myocardium
Tang *et al* [49]	Randomised, double-blind, placebo-controlled study	44	Acute MI treated with aspirin and clopidogrel after successful PCI	No alteration of markers of platelet and endothelial cell activation associated with thrombosis Increase in expression of angiogenesis signalling proteins in PBMCs (EPOR, VEGFR-1, p-PI3K)
Voors *et al* [60]	Randomised, placebo-controlled, open label, blinded endpoint study	529	First STEMI treated with primary PCI	No difference in LVEF after 6 weeks Less MACE in EPO group
REVIVAL-3 [46]	Randomised, double-blinded, placebo-controlled study	138	First STEMI treated with primary PCI	Results are awaited
REVEAL [59]	Randomised, double-blinded, placebo-controlled study	210	First STEMI treated with primary PCI	Results are awaited

STEMI = ST-elevation myocardial infarction, NSTEMI = non-ST-elevation myocardial infarction, EPC = endothelial progenitor cell, EPO = erythropoietin, EPOR = erythropoietin receptor, MI = myocardial infarction, PBMC = peripheral blood mononuclear cell, PCI = percutaneous coronary intervention, p-PI3K = phosphorylated phosphatidylinositol 3-kinase, VEGFR-1 = vascular endothelial growth factor receptor 1, LVEF = left ventricular ejection fraction, MACE = major adverse cardiac events, LV = left ventricle.

showed variable results. Some studies showed improvement in cardiac function, less hospitalisation and enhancement of exercise capacity, where other studies showed no effects on six minutes walk test, New York Heart Association (NYHA)-class and LVEF (table 2).[50-55]
Large prospective, randomised, clinical trials are necessary to provide definitive answers if EPO could be beneficial as an pharmacologic addendum against cardiovascular disease.[56] To evaluate the use of EPO in myocardial ischemia, our group therefore designed a multicenter, prospective, randomised, openlabel trial with blinded evaluation of the primary endpoint (HEBE III).[57] The primary objective was to study the effect on LVEF of a single bolus of EPO, administered directly after a primary PCI for a first MI. Eligible patients were randomly assigned to either receive standard medical care or a single bolus with 60,000 IU of EPO on top of

Table 2. Clinical studies: The effects of EPO in chronic heart failure

Study	Study Design	Number Patients	Patient Characteristics	Results
Silverberg *et al* [52]	Randomised controlled study	32	CHF with anaemia	Improvement in cardiac function Less hospitalisation and less renal impairment
Mancini *et al* [51]	Randomized, single-blind, placebo-controlled study	26		Enhancement of exercise capacity
van Veldhuisen *et al* [50]	Randomised, double-blind, placebo-controlled study	152	CHF with anaemia	Increase in Hb Improvement in Kansas City Cardiomyopathy Questionnaire score No effect on 6 min. walk distance, Patient's Global Assessment score, NYHA class, LVEF, and HF Questionnaire score
Ponikowski *et al* [53]	Randomised, double-blind, placebo-controlled study	51	CHF with anaemia	Increased in Hb Improvement in Patient's Global Assessment score No effect in Kansas City Cardiomyopathy and Minnesota Living with Heart Failure Questionnaire scores
Palazzuoli *et al* [54]	Randomised, double-blind, placebo-controlled study	40	CHF with anaemia	Improvement in NYHA-class, exercise endurance, oxygen use during exercise, renal function and BNP Reduced hospitalisation
Palazzuoli *et al* [55]	Randomised, double-blind, placebo-controlled study	51	CHF with anaemia	Improvement in LV systolic function, LV remodelling, BNP levels, and PAP
RED-HF [58]	Randomised, double-blind, placebo-controlled study	2600	CHF and anaemia	Results are awaited

(C)HF = (chronic) heart failure, NYHA = New York Heart Association, BNP = B-natriuretic peptide, LVEF = left ventricular ejection fraction, LV = left ventricle, PAP = pulmonary artery pressure, Hb = haemoglobin.

standard medical care within three hours of the PCI procedure. Primary endpoint of the study was LVEF assessed by planar radionuclide ventriculography after six weeks.

The results of the RED-HF trial (a large, randomised, clinical trial evaluating morbidity and mortality with the use of EPO in chronic HF) are expected at the end of 2011 and should give the definitive answer to the use of EPO in chronic HF.[58]

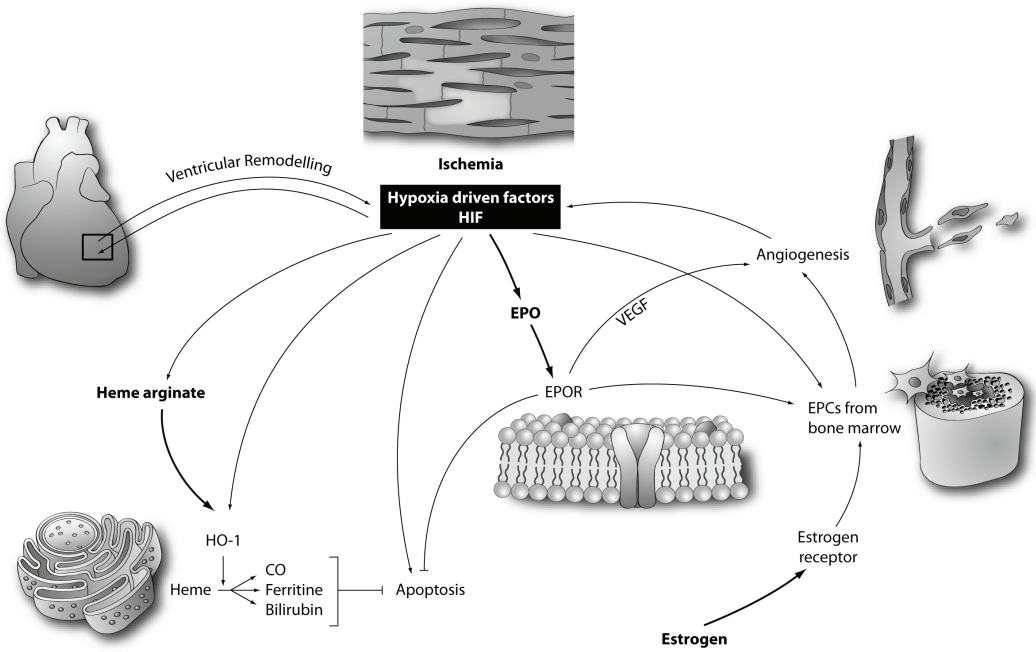

Figure 1. Figure shows the mechanisms of the various factors described in this thesis and its interplay with ischemia and left ventricular hypertrophy. HIF = hypoxia inducible factor, EPO = erythropoietin, EPOR = EPO receptor, HO-1 heme oxygenase-1, CO = carbon monoxide, VEGF = vascular endothelial growth factor, EPC = endothelial progenitor cell. For colour figure, see supplement 1.

RECENT TRIALS

Both the REVEAL and the HEBE III study were designed to evaluate the effect of EPO in first ST-segment elevation MI.[57,59] As the results of the REVEAL are still awaited, very recently the results of the HEBE III were published.[60] A single dose of EPO did not improve global LVEF after six weeks. However, more major adverse cardiovascular events occurred in the control group than in the EPO group, suggesting a potentially relevant cardioprotective effect and a favourable clinical safety profile of EPO.

Recent large trials with EPO in other diseases, i.e. in patients with chronic kidney disease (CHOIR, TREATE), haemodialysis (CREATE), and stroke (German Multicenter EPO Stroke Trial) indicated that high dose of EPO is associated with excess stroke and mortality, possibly due to the hyperviscosity and hypercoaguability associated with high haematocrit.[61-64] We therefore have postulated that EPO should be administered in low dosages,[42] so that the undesired side effects due to increasing haematocrit are avoided, yet the desirable anti-ischemic and cytoprotective effects are preserved. Alternative EPO-like compounds, which have no effects on erythropoiesis, have been advocated in this respect.[65-68]

AIM OF THIS THESIS

The aim of this thesis was to evaluate in HF the non-haematopoietic effects of EPO and associated factors with similar protective mechanisms, such as estradiol and heme arginate. In the first part of this thesis, we evaluated the extra-erythropoietic mechanisms of EPO and its role in the EPO-EPOR system. In **chapter 2**, we review the function of EPO and its receptor in the cardiovascular system and the use of EPO in both acute MI as in chronic HF. In **chapter 3** we evaluate the mechanisms of EPO-induced vascular endothelial growth factor (VEGF) production in the heart and establish that VEGF is crucial for EPO-induced improvement of cardiac performance. In **chapter 4**, the role of the EPOR in physiologic (cardiac) hypertrophy is investigated. We show that EPO-EPOR signalling is crucial for physiologic hypertrophy. **Chapter 5** describes the existence of I/R injury in ventricular tissue during coronary artery bypass graft surgery with the use of cardiopulmonary bypass. It shows that ventricular tissue maybe more sensitive to detect changes than atrial tissue. Furthermore, this chapter also includes the design and rationale to further study these mechanisms.

The second part of this thesis evaluates interventions (with estradiol and heme arginate) in downstream pathways of EPO and focuses on feasible targets. These factors were chosen as they share working mechanisms with EPO, namely anti-apoptotic and angiogenic properties. In **chapter 6**, we investigate whether estradiol administration could induce EPC-mediated neovascularisation in a model of hind-limb ischemia. In **chapter 7** we focus on the use of heme arginate as a reactive oxygen species scavenger in cardiac I/R injury. **Chapter 8** evaluates the existence of HF-associated anaemia in an experimental HF model and if EPO administration could be an efficacious treatment option.

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PART 1

Mechanistic insights in the extra-erythropoietic mechanisms of erythropoietin

Erythropoietin in cardiac disease: New features of an old drug

CHAPTER 2

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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 is 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.

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.[13] 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.[10,14-22] The EPOR is expressed in even more tissues, such as in gastric mucosal cells, pancreatic islets and even in the prostate epithelial cells.[23-26] The EPOR is a member of the type 1 cytokine receptor family characterised by a single transmembrane domain.[27] Under normal circumstances EPO and EPOR have a relatively low expression in non-haematopoietic tissue,[28] 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.[10,29,30] These stressors directly activate the HIF-1, -2 and -3 pathways, which in turn regulate the gene transcription of EPO and EPOR.[31,32] 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.[33-38] These pathways originate with the binding of EPO to the EPOR to activate Janus-tyrosine kinase 2 (Jak2).[39,40] Next, by phosphorylation, PI3K and Akt are activated.[41] 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.[42,43] EPO also modulates cellular inflammation by inhibiting cellular phosphatidylserine membrane exposure and subsequent targeting of cells for phagocytosis.[44-46] 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.[47,48]

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.[49]

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.[50-52] EPO and EPOR play an essential role in proliferation, survival and differentiation of erythroid progenitor cells.[53,54] 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.[55] 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

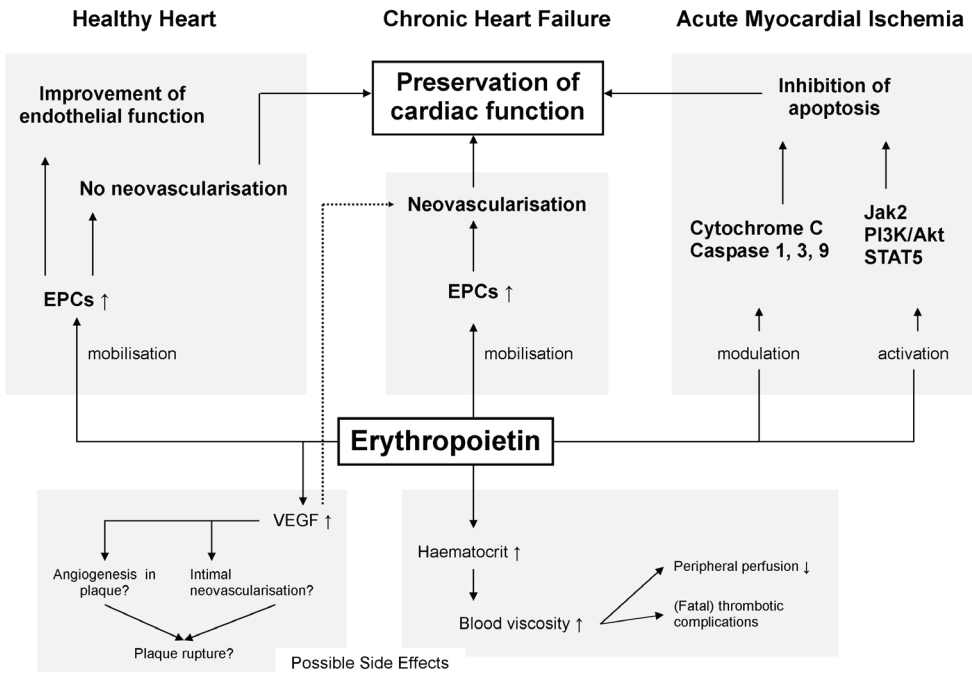

Figure 1. Overview of the different pathways of the effects of erythropoietin (EPO) administration in cardiovascular disease. In healthy hearts, number of endothelial progenitor cells (EPCs) are upregulated, but do not improve cardiac function further. However, endothelial function is improved. In acute myocardial ischemia, EPO has cytoprotective effects, preserving heart function. In chronic heart failure, EPO upregulates the number of EPCs, providing neovascularisation and preserves cardiac function. Possible side effects are raised haematocrit, heightened blood viscosity, resulting in hypertension, lower cerebral and renal blood flow and (fatal) thrombotic complications, which in turn can result in myocardial infarction or other arterial and venous thrombotic events. VEGF: vascular endothelial growth factor; Jak2: Janus-tyrosine kinase 2; PI3K: phosphoinositide 3 kinase; Akt: protein kinase B; STAT: signal transducers and activators of transcription.

GATA-1 promotor, a transcription factor exclusive to erythroid lineage cells (EPOR$^{-/-}$-rescued mice).[56] 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.[57] This effect is partially due to enhanced apoptosis of cardiomyocytes. In the setting of peripheral artery disease the EPOR 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).[58] 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.[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.[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.[61] Upregulation of VEGF results in angiogenesis in hypoxic tissue.[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.[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.[68-71] Furthermore, apoptosis contributes to the final injury size during injury caused by revascularisation.[72] It is postulated that this reperfusion injury might contribute up to 50% of the final size of damage,[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.[74-84] The anti-apoptotic effect of EPO is the acute protecting mechanism of EPO during ischemia (figure 2).[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.[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.[89] Other experimental *in vivo* studies using an I/R injury model showed decreased infarct size,[90-95] enhancement of left ventricular function,[96,97] and less apoptosis,[98,99] even when EPO was administered after the onset of reperfusion.[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.[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%).[103] Importantly, these positive effects of EPO are observed both at a high-dose administration and at a low-dose administration of EPO.[104,105]

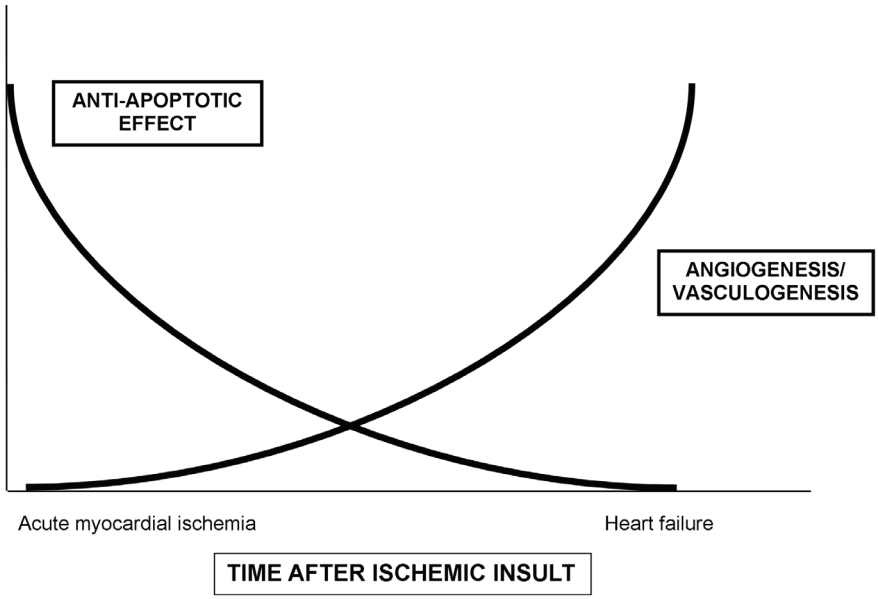

Figure 2. Schematic view of the protective properties of erythropoietin in time after ischemic insult.

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.[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.[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).[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.

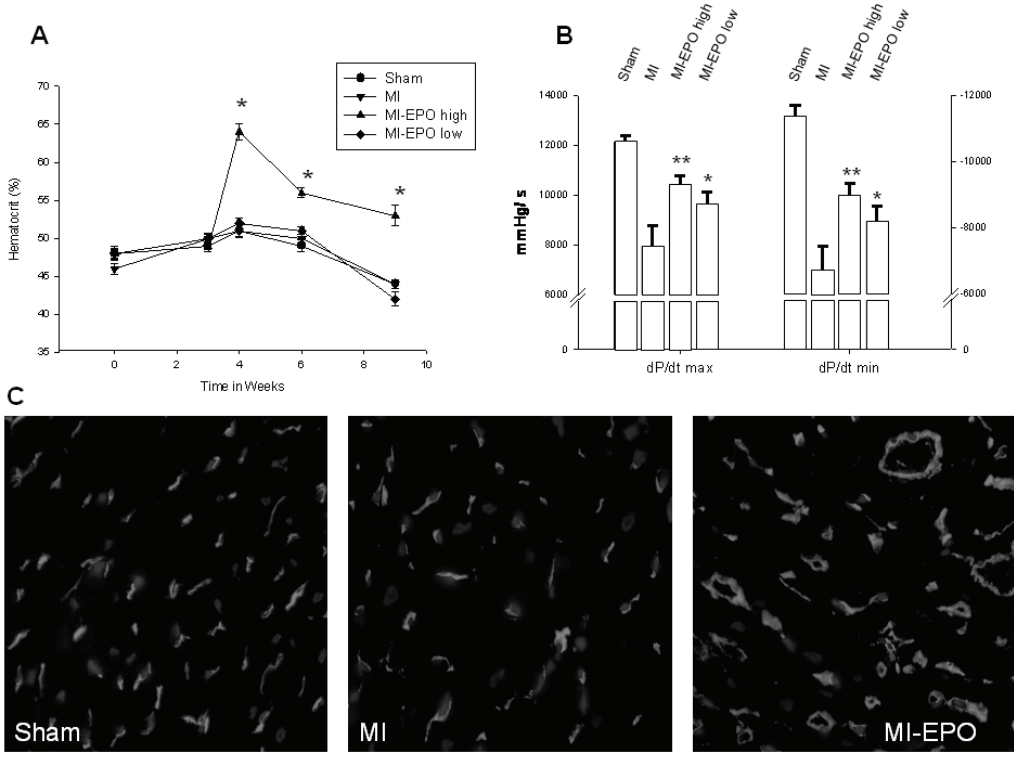

Figure 3. A. Effect of erythropoietin (EPO) treatment on longitudinal changes in haematocrit ($p<0.01$ vs. sham) in a model of experimental heart failure in rats. B. Effects of myocardial infarction (MI) and high-dose and low-dose EPO treatment on haemodynamic parameters, dP/dtmax and dP/dtmin; * $p<0.05$ 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.

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.[109] 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).

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.[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.[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 *in vitro* and stimulating angiogenesis in numerous experimental models *in vivo*.[114-116] In addition, EPO induces the proliferation, differentiation and adhesion of a subset of bone marrow derived progenitor cells with an endothelial phenotype *in vitro* and results in marked mobilisation of EPCs *in vivo*.[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.[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.[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.[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.[124-127]

EPO-induced neovascularisation in HF is consistently associated with increased numbers of circulating EPCs.[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.[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).[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.[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.[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.[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.[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.[134] Two independent studies have demonstrated that these non-erythropoietic EPOs retain their cardioprotective potential in models of acute MI.[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).[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.[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.[139,140] These derivates have shown to be as effective in cardioprotection as recombinant human EPO in a model of acute MI.[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.[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.[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.[145] 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).[146] 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.[147]

It may be that the beneficial effects of EPO in chronic HF patients are different from chronic kidney disease patients.[148,149] 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.[150,151] 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.[152,153] 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.

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3 CHAPTER

Vascular endothelial growth factor is crucial for erythropoietin-induced improvement of cardiac function in heart failure

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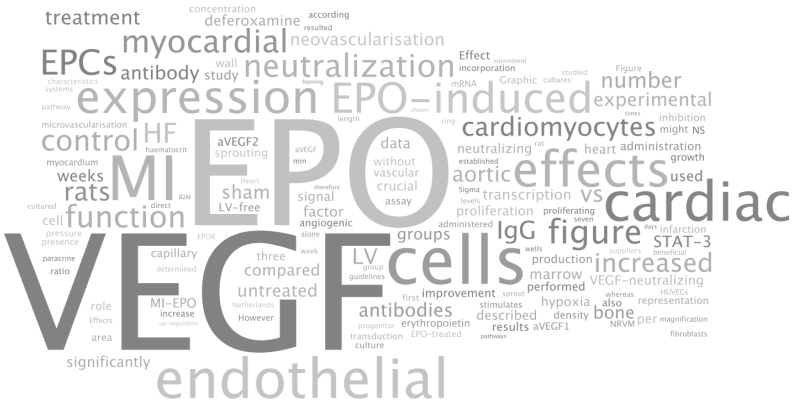

ABSTRACT

Aims

We intended to delineate the mechanisms of erythropoietin (EPO)-induced cardiac vascular endothelial growth factor (VEGF) production and to establish if VEGF is crucial for EPO-induced improvement of cardiac performance.

Methods and results

The effects of EPO on VEGF expression were studied in cultured cardiac cells and EPO-treated hearts. The role of VEGF in EPO-induced neovascularisation was studied with two distinct VEGF-neutralising antibodies or irrelevant control IgG in an aortic sprouting assay and in rats with heart failure (HF) after myocardial infarction (MI) treated with EPO. EPO-alpha (10 IU/ml) was used *in vitro* and darbepoetin alpha (40 µg/kg/3 weeks, starting 3 weeks after MI) *in vivo*. EPO stimulated VEGF mRNA expression through the signal transducers and activators of transcription-3 (STAT3) pathway in neonatal rat cardiomyocytes, but not in endothelial cells or fibroblasts. Similarly, the direct effects of EPO on endothelial sprouting were modest and VEGF independent. In rats with HF, EPO increased VEGF protein expression predominantly in cardiomyocytes, associated with a 37% increase in capillary density and improved cardiac performance. Administration of VEGF-neutralising antibodies abrogated the salutary effects of EPO on cardiac microvascularisation and function. VEGF neutralisation attenuated EPO-induced proliferation of myocardial endothelial cells and reduced myocardial incorporation of endothelial progenitor cells (EPCs) in rats with alkaline phosphatase-labelled bone marrow cells.

Conclusion

VEGF is crucial for EPO-induced improvement of cardiac function in HF. EPO fosters VEGF expression predominantly in cardiomyocytes, which in turn stimulates myocardial endothelial proliferation and incorporation of EPCs.

INTRODUCTION

Heart failure (HF) remains a prevalent medical condition with a poor prognosis. The development of new therapeutic strategies is therefore of utter importance.[1]
Insufficient microvascular adaptation in relation to the degree of cardiomyocyte hypertrophy is a key pathophysiological feature that contributes to progressive cardiac dysfunction in HF.[2,3] We and others have convincingly shown that treatment with erythropoietin (EPO) restores microvascular insufficiency, and improves cardiac performance in experimental and clinical HF.[4-7] The mechanisms of EPO-induced neovascularisation in HF are however incompletely understood.
There are several reasons to believe that the activation of vascular endothelial growth factor (VEGF) is involved in the cardiac effects of EPO. First, EPO increases VEGF expression in various ischaemic tissues and cardiac VEGF levels are strongly correlated with new vessel formation.[8-11] Secondly, EPO only stimulates neovascularisation in the heart at sites where VEGF expression is increased.[12] Thirdly, mice that lack an EPO-receptor (EPO R) in the heart, display defective VEGF expression, and dramatically accelerated development of left ventricular (LV) dysfunction during pressure overload.[8] Although these findings suggest an important role for VEGF in EPO-induced neovascularisation, the data are observational and it remains to be established whether augmented VEGF expression is crucial for EPO-induced improvement of cardiac function.
We hypothesized that VEGF upregulation in the myocardium is crucial for EPO-derived restoration of cardiac microvascularisation and performance. We first identified that cell types and signal transduction pathways are operative for EPO-induced VEGF expression in the heart. Then, we established that abolishment of VEGF expression neutralises the effects of EPO administration, specifically improvement of cardiac microvascularisation and function, using a well-established *in vivo* model of HF.

METHODS

Recombinant human EPO and experimental antibodies

We used EPO-alpha (EPREX, Janssen-Cilag) in a concentration of 10 IU/ml in all *in vitro* experiments. The long-acting EPO analogue darbepoetin alpha (Aranesp, Amgen) was administered once every three weeks in a calculated dose of 40 µg/kg bodyweight. Because we intended to explore the effect of EPO on established HF, the first Darbepoetin dose was given three weeks after the infarct when infarct healing has subsided. The VEGF-neutralising antibodies goat anti rat-VEGF affinity purified antibody (aVEGF1; R&D systems, catalogue# AF564) in a concentration of 5 µg/rat per three times per week and mouse anti-humanVEGF165 (aVEGF2, production described by Tilton et al.[13]) in a concentration of 1 mg/rat three times per week or the irrelevant control antibody goat anti-mouse-IgG (R&D systems, catalogue# AF007) at a concentration of 5 µg/rat per three times per week were used for the experimental protocol. Both VEGF-neutralising antibodies recognize and neutralise all splice variants of rat VEGF.

Effect of EPO on VEGF transcription in cardiac cells

Neonatal rat ventricular myocytes (NRVM, from Sprague Dawley rats, Harlan, The Netherlands) and human umbilical vein endothelial cells (HUVECs) were isolated and maintained as described previously[14,15] Human cardiac fibroblasts (HCF, ScienCell Research Laboratories) were cultured in RPMI 1640 medium with L-glutamin and 10% foetal bovine serum. Before the experiment, cultures with NRVM (10^5 cells/well) and HCF (100% confluence) were incubated without serum and HUVECs cultures were cultured with 2% foetal bovine serum for 22 hr. Because we have previously described that ischaemia is required for EPO-induced upregulation of VEGF in the heart, cells were pre-incubated with or without the hypoxia mimetic deferoxamine (100 μM, Sigma Aldrich) for 2 hr. The role of well-described EPOR signal transduction pathways was studied by additional pre-incubation with the phosphoinositide 3-kinase blocker wortmannin (1 μM, Sigma Aldrich), the Map-kinase kinase (MEKK) blocker PD98059 (25 μM, Sigma Aldrich), or the cell permeable signal transducers and activators of transcription-3 (STAT3) inhibiting peptide (pPYLKTK-mts, 1 μM, Calbiochem) for 30 min. After preincubation, EPO (Eprex, Janssen-Cilag, final concentration of 10 IU/ml), was added to the wells and cells were lysated after 30, 60, or 120 min with TRIZOL-reagent (Invitrogen) and RNA was isolated according to the suppliers guidelines. For measurements of STAT3 phosphorylation cells were lysated after 30 min. with RIPA-buffer containing protease and phosphatase inhibitors (Invitrogen) as previously described.[9] To confirm that the effects of deferoxamine reflected true hypoxia, we additionally performed an experiment where NRVM were placed in a humidified hypoxic chamber at 37°C with 1% O_2, 5% CO_2. We used an identical experimental design, with the exception that the cells were placed in the hypoxic chamber instead of adding deferoxamine to the wells. After the hypoxic pre-incubation wells were very briefly removed from the hypoxic chamber to administer EPO under laminar flow.

After reverse transcription with random primers (Promega), quantitative reverse transcriptase-polymerase chain reaction was performed with PCR-master mix containing Cyber Green (AbGene) and specific primers or with TAM-labelled primer/probe sets according to the suppliers guidelines (Applied Biosystems). Beta-2-microglobulin (B2M) was used as a housekeeping gene in all analyses (expression of B2M was constant under our experimental conditions). Moreover, in random subsets of the data, we additionally corrected for the housekeeping genes beta-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which revealed identical results as B2M (data not shown). Results are expressed as fold-difference compared with control and represent the average of 8-12 wells per group of at least three separate experiments, with cells from different donors.

Aortic ring assay

The aortic ring assay, which is a co-culture of endothelial cells, fibroblasts, and vascular smooth muscle cells, was performed using the method of Nicossia and Ottinetti with slight modifications.[16] Briefly, 0.6 mm long aortic rings of the thoracic aorta were embedded in growth factor reduced Matrigel (Becton Dickinson). The aortic rings were then cultured in endothelial cell culture medium [which contains endothelial cell growth factor (ECGF) and heparin[15]] with or without 10 IU/ml EPO or 1 μg/ml goat anti-rat VEGF (AF 564, R&D systems) for seven days.

After five and seven days of culture, each quadrant of the aortic ring was photographed with an inverted microscope. Maximal sprout length, defined as the length of the perpendicular line between the external circumference of the aortic ring and the tip of the longest sprout per quadrant was measured with Image-Pro (Version 4.5.0.29) and is expressed in arbitrary units.

Animals and bone marrow labelling

Male Sprague Dawley rats (270-320 gr) were purchased (Harlan). For bone marrow transplantation experiments, we used male Fischer F344 rats (200-230 gr) purchased from Charles Rivers (France) as recipients and R26-hPAP donor rats (F344 background, ubiquitously expressing human placental alkaline phosphatase). Details on transplantation have been described in detail previously.[9] Briefly, whole R26-hPAP bone marrow cells were transfused to Fischer F344 recipients after total body irradiation and left to reconstitute for six weeks before commencing with the experimental protocol. Animals were fed and housed according to the institutional rules and regulations.

Experimental protocol in rats

HF was induced by permanent ligation of the left coronary artery to produce a myocardial infarction (MI) and control rats received a sham procedure.[4] Three weeks after MI, rats were randomly assigned to treatment with darbepoetin alpha or vehicle, given once every three weeks i.p. as described above. Because we intended to explore the effect of EPO on established HF, the first darbepoetin dose was given three weeks after MI when infarct healing has subsided. The effects of VEGF in EPO-induced neovascularisation were studied by administering one of the VEGF-neutralising antibodies (aVEGF1 or aVEGF2) to rats with MI concomitantly treated with EPO or vehicle. Because VEGF-signalling is crucial for the normal control of cardiac microvascularisation in HF,[17] we only administered neutralising antibodies during the first week after EPO treatment to allow for a neutralisation-free interval. Reference and control rats in the sham, untreated MI and EPO-treated MI groups received equivalent doses of the irrelevant control IgG to control for the non-specific effects of antibody administration. This resulted in seven treatment groups: sham, untreated MI and EPO-treated MI group where control IgG was administered (sham + IgG; MI + IgG; MI-EPO + IgG); the untreated and EPO-treated MI group where aVEGF-1 was administered (MI-EPO + aVEGF1; MI + aVEGF1) and the untreated and EPO-treated MI group where aVEGF2 was administered (MI-EPO + aVEGF2; MI + aVEGF2). To evaluate the temporal characteristics of VEGF neutralisation, 400 µl of blood was drawn from the tail vein at weeks three, four, six and seven. After nine weeks, at sacrifice, haemodynamic measurements were performed with a microtip pressure transducer (Millar Instr., Inc., Houston, TX, USA) as described previously.[4] Heart rate, LV systolic pressure, and LV end diastolic pressure (LVEDP) were measured. The maximal rates of increase and decrease in LV pressure (dPdtmax and dPdtmin) and the developed LV pressure (dLVP) were determined. Next, blood was drawn and hearts were rapidly excised and weighed. Myocardial tissue was dissected transversally and processed for immunohistochemistry or snap frozen for molecular analysis.

VEGF-neutralising capacity of plasma

Ninety-six-well plates were coated with donkey anti-goat-IgG in PBS overnight. Next, plates were washed five times and incubated with plasma samples (n=4) from rats treated with the irrelevant control antibody and four rats that received aVEGF1. Recombinant human VEGF165 (R&D systems) was added to the wells to reach a final concentration of 1000 pg/ml and incubated for 60 min. Hereafter, VEGF concentration was determined by ELISA according to the suppliers' guidelines (R&D systems). Percentage neutralisation was determined as VEGF measured/VEGF added (1000 pg/ml) x 100%. The neutralising characteristics of aVEGF2 have been described elsewhere.[13]

Western blot

The expression of VEGF and GAPDH was determined in tissue homogenates of the viable LV-free wall (non-infarcted area) by standard western blotting techniques as described previously.[9] For determination of STAT3 phosphorylation, we used the PhosphoPlus STAT3 antibody kit (Cell Signalling Technology, catalogue number 9130), according to the suppliers' guidelines.

Circulating endothelial progenitor cell culture

Circulating endothelial progenitor cells (EPCs) were enumerated by culture of mononuclear cells in lineage selection medium (EndoCult medium, StemCell Technologies) according to the suppliers guidelines, as previously described.[9]

LV histology

Infarct size, cardiomyocyte cross-sectional area, the number of cardiomyocytes/mm^2, the number of capillaries per tissue area (mm^2), and the capillary/cardiomyocyte ratio were determined as described previously.[4] Other immunohistochemical stainings were performed on transverse myocardial sections at the mid-papillary level. As primary antibodies, we used mouse anti-Troponin T (JLT-12, Sigma Aldrich), rabbit anti-VEGF (A20, Santa Cruz biotechnology), mouse anti-proliferating cell nuclear antigen (PCNA, PC10, Cell Signaling Technology), rabbit anti-hPAP (Serotec), and mouse anti-rat His-52 (kind gift from Dr. J.L. Hillebrands).[9] The envision kit (Dako-Cytomation) with Mayers haematoxylin (Sigma) for nuclear staining was used for chromogenic detection. For fluorescent detection, diamidino-2-phelylindole, the biotynylrhodamine-TSA kit (Perkin Elmer) or isotype-specific anti-mouse-IgG, or anti-rabbit-IgG antibodies labelled with FITC, TRITC, or Alexa555 were used. Analysis of VEGF expression was performed in the MI border zone. Cells positive for both lectin and PCNA were considered proliferating endothelial cells, and cells positive for both hPAP and His-52 were considered bone marrow-derived endothelial cells. Cells were quantified in four to five random high power fields of the non-infarcted LV-free wall remote from the infarction by blinded observers (BDW, LY).

Statistical methods

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis among groups was performed by ANOVA with the Bonferroni post hoc test, if distributed normally or

with the Kruskall-Wallis test followed by Mann-Whitney U test when skewed distributed. All p-values are two-tailed, and a p-value of less than 0.05 was considered statistically significant. All analyses were performed using SPSS version 15.0 software (SPSS, Chicago, IL, USA).

Ethics

The investigation conforms to the principles outlined in the Declaration of Helsinki. In addition, the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). For the use of HUVECs, approval was granted by the medical ethical review board of the University Medical Centre Groningen, University of Groningen, Groningen, The Netherlands. The experimental protocol was approved by the Animal Ethics Committee of the University of Groningen.

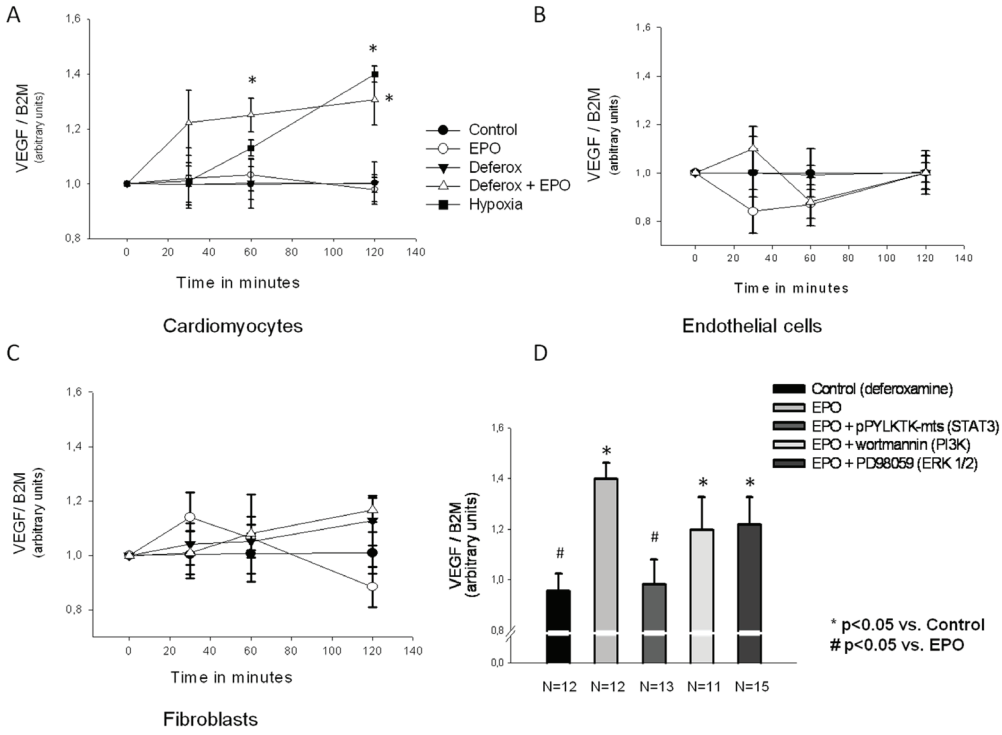

Figure 1. Differential effects of EPO on VEGF gene transcription in cardiac cells

(A) Temporal changes in VEGF mRNA expression after EPO treatment in NRVC in the presence or absence of hypoxia or the hypoxia inducing agent deferoxamine. (B) Temporal changes in VEGF mRNA expression after EPO treatment in HUVECs in the presence or absence of the hypoxia inducing agent deferoxamine. (C) Temporal changes in VEGF mRNA expression after EPO treatment in HCF in the presence or absence of the hypoxia inducing agent deferoxamine. (D) Effect of specific blockers for EPOR signal transduction pathways on VEGF mRNA expression in EPO-treated NRVM, measured at 60 min. # p<0.05 vs. EPO, * p<0.05 vs. control. Deferox = deferoxamine, EPO = erythropoietin, B2M = beta-2-microglobulin.

RESULTS

Effects of EPO on VEGF transcription in cardiac cells

VEGF mRNA expression was significantly increased by EPO in NRVM. In accordance with our recent study in rats,[9] EPO induced VEGF expression only in the presence of hypoxia or the hypoxia-mimicking agent deferoxamine ($p<0.05$, figure 1A). In endothelial cells and fibroblasts, VEGF expression was unaffected by EPO [p = non-significant (NS), figure 1B and C]. Extended incubation with deferoxamine increased VEGF expression in NRVM, HUVECs, and HCF compared with cultures without deferoxamine, but did not alter the effects of EPO on VEGF expression (data not shown). The effects of EPO on VEGF expression were blocked by STAT3 inhibiting peptide, but not by wortmannin or PD98059 (figure 1D), suggesting that the JAK2/STAT3 pathway is the dominant signal transduction pathway. To confirm that STAT-3 is activated, we additionally measured STAT3 phosphorylation, which confirmed that EPO induces STAT3 signalling in our model (see supplementary data, figure S2).

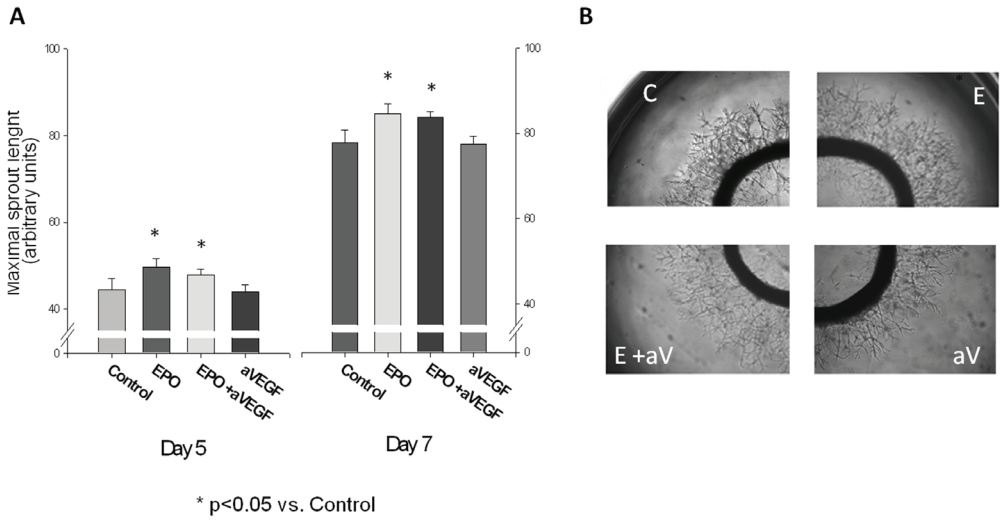

Figure 2. Effects of VEGF inhibition on EPO-induced angiogenesis *in vitro*

(A) Maximal aortic sprout length after five and seven days in a Matrigel aortic implantation assay. Aortic rings were cultured in the presence or absence of EPO (10 IU/ml) or VEGF-neutralising antibody (1 µg/ml goat anti-rat VEGF). EPO significantly increased maximal sprout length compared with control cultures. The increase was however <10%. Neutralisation of VEGF did not inhibit the effects of EPO on aortic sprouting. (B) Typical examples of aortic sprouting in the different experimental groups after seven days in culture. C = control culture, E = EPO, aV = VEGF-neutralising antibody.

Role of VEGF in EPO-induced aortic sprouting

To corroborate the previous findings, we studied the effects of EPO in the aortic sprouting assay, which is a co-culture of vascular cells without parenchyma cells such as cardiomyocytes. EPO significantly increased maximal sprout length compared with control cultures, but the increase was <10% ($p<0.05$, figure 2). Neutralisation of VEGF did not inhibit the effects of EPO on aortic sprouting.

Effect of EPO on VEGF production in failing hearts

Immunofluorescent double staining of EPO treated failing rat hearts showed that VEGF expression was especially apparent in cardiomyocytes (figure 3A). In rats with post-MI HF, EPO treatment resulted in a three-fold increased protein expression of VEGF in the viable LV-free wall (figure 3D). In addition to increased protein levels, EPO treatment also increased the VEGF-immunoreactive area, suggesting that EPO additionally increased the number of cells that produced VEGF (figure 3B and C). Importantly, administration of the neutralising antibodies did not quantitatively affect VEGF-protein levels at sacrifice (figure 3). Therefore, the results of the study were not biased by neutralisation-induced changes of VEGF expression and thus represent the effects of temporary blocking VEGF signalling.

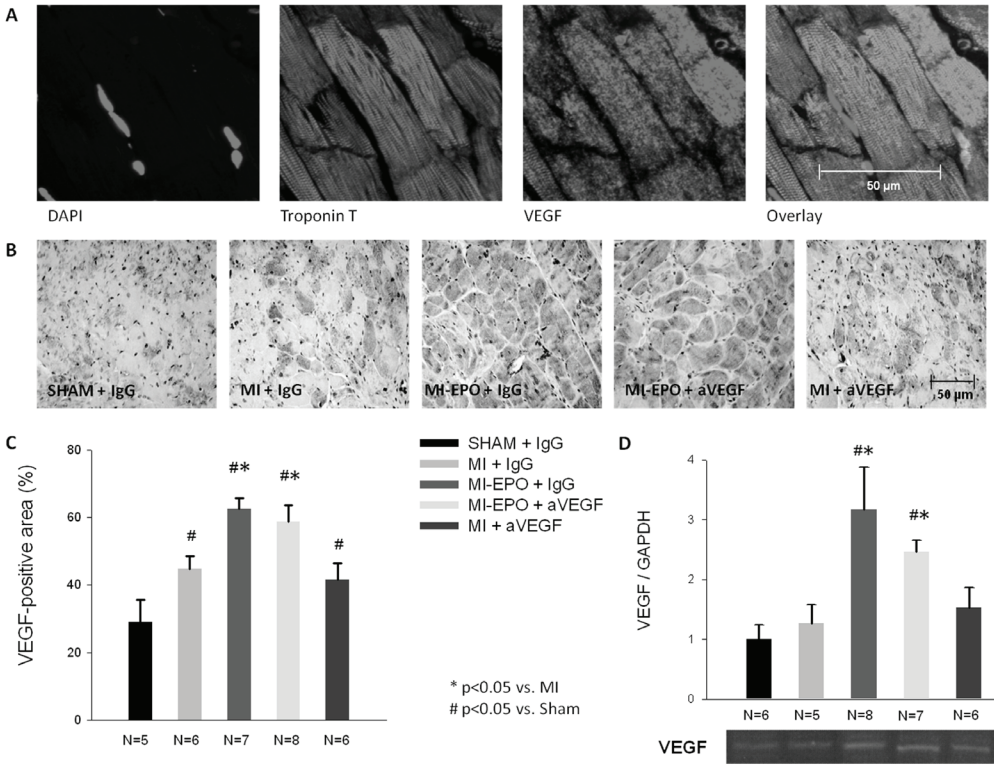

Figure 3. Effect of EPO on VEGF immunoreactivity in the myocardium

(A) Immunofluorescent staining of rat myocardium at x100 magnification. Co-localization of VEGF (red) and troponin-T (green) indicates that VEGF is expressed in cardiomyocytes. (B) Typical examples of VEGF-immunohistochemistry of myocardial sections at x40 magnification. (C) Graphic representation of VEGF immunoreactive area. (D) Graphic representation of the VEGF protein expression in the LV-free wall. MI = myocardial infarction, EPO = erythropoietin, IgG = control IgG, aVEGF = VEGF-neutralising antibody. For colour figure, see supplement 1.

Effect of VEGF neutralisation on EPO-induced improvement of cardiac function

General characteristics of the study groups are presented in table 1. Temporal characteristics of the VEGF-neutralising capacity of the plasma are presented in supplementary data. In brief, during the anti-VEGF antibody administration 78% of VEGF was neutralised. In the subsequent weeks without antibody administration, the neutralisation capacity was normalised to control values. These data indicate that intermittent administration of neutralising antibodies results in temporary neutralisation of VEGF signalling. Induction of MI resulted in a significant reduction in cardiac function, increased heart weight and increased cardiomyocyte cross-sectional area in all MI groups (table 1 and figure 4). EPO treatment significantly increased LV contractility (dPdtmax, dLVP) and LV-relaxation (dPdtmin) and decreased LV filling pressures (LVEDP) compared with the untreated MI (all p<0.01, figure 3). Neutralisation of VEGF with both antibodies blocked the salutary effects of EPO on cardiac function parameters (p=NS vs. untreated MI, p<0.05 vs. MI-EPO, figure 4), whereas intermittent neutralisation itself did not further decrease cardiac function compared with the untreated MI (p=NS, figure 4). Correction of LV contractility and relaxation indices for instantaneous pressures did not alter the results (data not shown).

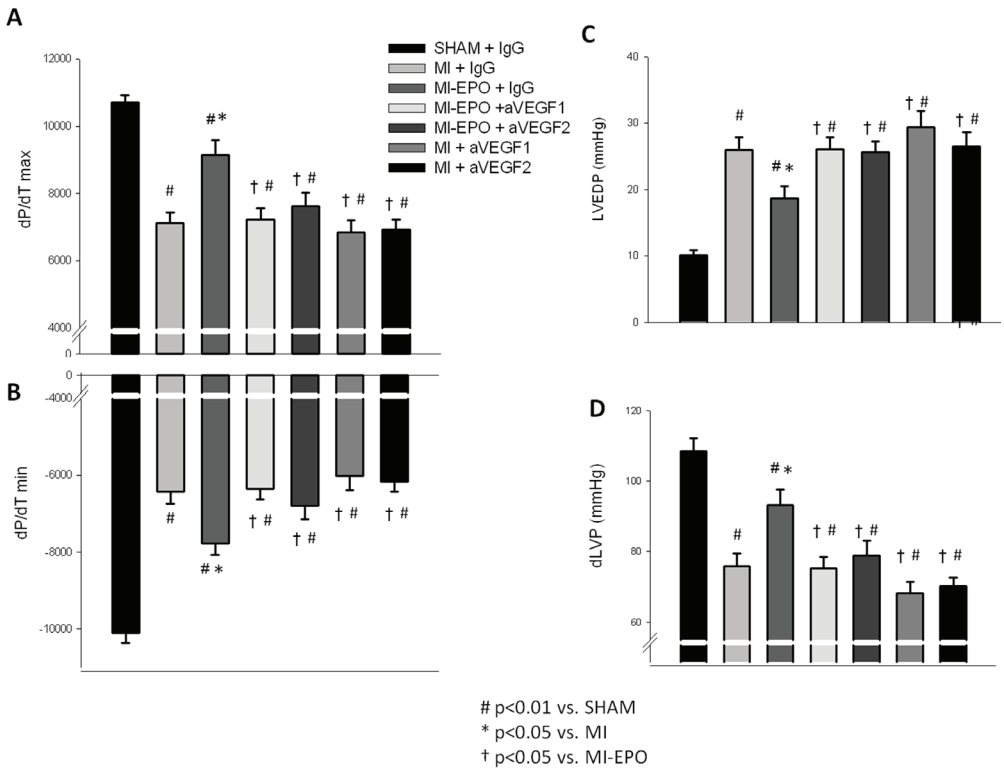

Figure 4. Effects of VEGF neutralisation on EPO-induced improvement of LV function

(A–D) Graphic representation of LV contractility (dPdtmax), relaxation (dPdtmin), LVEDP and dLVP. MI = myocardial infarction, EPO = erythropoietin, IgG = control IgG, aVEGF = VEGF-neutralising antibody.

Effect of VEGF neutralisation on EPO-induced LV neovascularisation

Cardiac capillary density was reduced in all MI groups compared with sham (p<0.001, figure 5). However, EPO treatment significantly increased cardiac capillary density by 37% (p<0.01 vs. MI, figure 5). VEGF neutralisation completely blocked the effects of EPO on capillary density (p=NS vs. untreated MI, p<0.01 vs. MI-EPO, figure 5), whereas neutralisation alone did not result in further reduction of capillary density (p=NS vs. untreated MI, figure 5). Capillary-to-cardiomyocyte ratio was slightly but non-significantly increased in all MI groups compared with sham. EPO treatment resulted in a 42% increase in capillary-to-cardiomyocyte ratio compared with sham and MI, which was also blocked by VEGF inhibition (figure 5).

Effect of VEGF neutralisation on EPO-induced proliferation of endothelial cells

The number of proliferating endothelial cells in the viable LV-free wall was comparable between sham and MI groups (figure 6A and B). EPO treatment significantly increased the number of proliferating endothelial cells (p<0.001 vs. sham and MI). VEGF inhibition resulted in a 47% reduction in of EPO-induced endothelial cell proliferation (p=0.01 vs. MI-EPO), although the number of proliferating cells was still significantly higher than in the sham and MI-group (p<0.05 vs. sham and untreated MI).

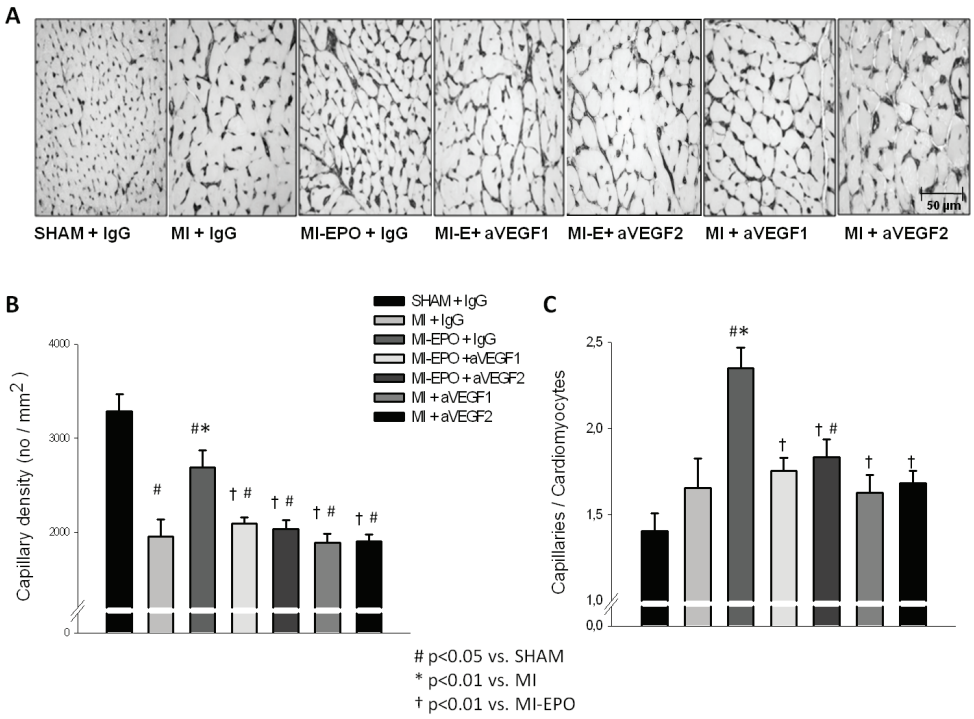

Figure 5. Effects of VEGF neutralisation on EPO-induced neovascularisation

(A) Typical examples of capillary density in the experimental groups at x40 magnification. (B) Graphic representation of capillary density expresses as the number of capillaries/mm^2. (C) Graphic representation of the capillary-to-cardiomyocyte ratio. MI = myocardial infarction, EPO = erythropoietin, IgG = control IgG, aVEGF = VEGF-neutralising antibody. For colour figure, see supplement 1.

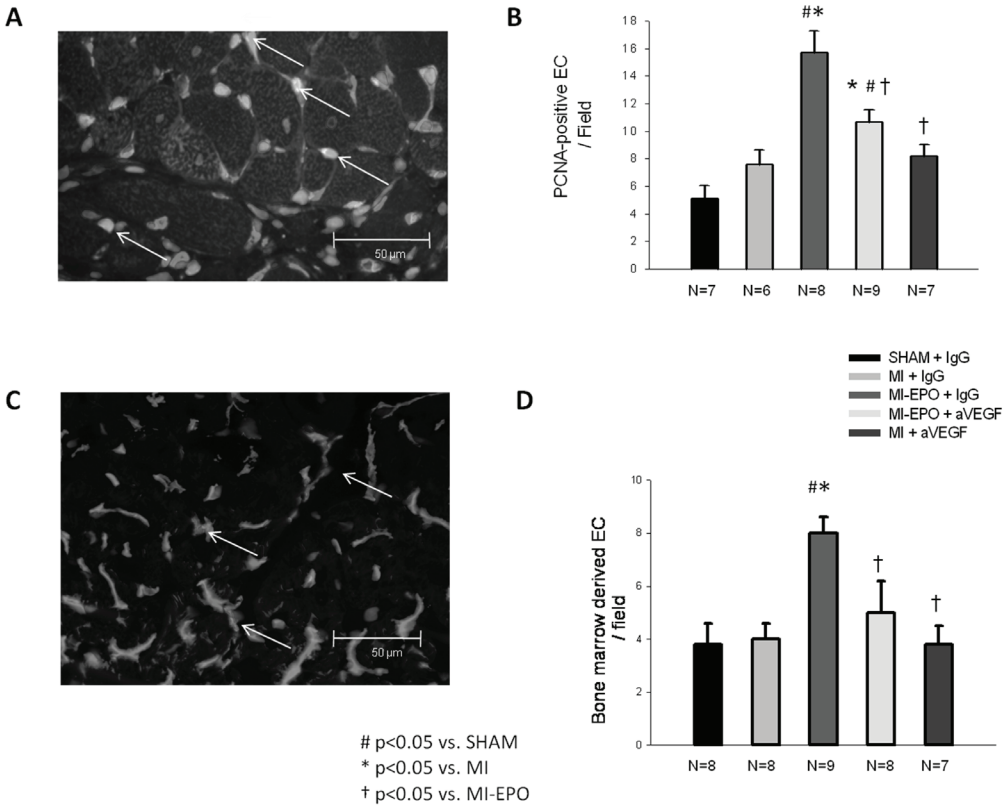

Figure 6. Effects of VEGF inhibition on EPO-induced myocardial endothelial cell proliferation and vascular incorporation of EPCs

(A) Typical example of immunofluorecent staining at x40 magnification showing PCNA-positive nuclei (purple, i.e. proliferating) in capillary endothelial cells (green) highlighted with an arrow. (B) Graphic representation of the number of proliferating endothelial cells in the LV-free wall of the experimental groups. (C) Representative fluorescent overlay of myocardial section at x40 magnification stained with hPAP (green), His-52 (endothelium, red), and DAPI (nucleus, blue) showing hPAP-positive (i.e. bone marrow derived) endothelial cells, which appears yellow and are highlighted with an arrow. (D) Graphic representation of the number of bone marrow-derived endothelial cells in the LV-free wall. MI = myocardial infarction, EPO = erythropoietin, IgG = control IgG, aVEGF = VEGF-neutralising antibody, EC = endothelial cells. For colour figure, see supplement 1.

Effect of VEGF neutralisation on myocardial homing of EPCs

EPO significantly increased circulating EPCs after EPO treatment and VEGF neutralisation did not affect the number of circulating EPCs (table 1). To evaluate whether EPO-induced myocardial VEGF upregulation is required for homing of EPCs to the myocardium, we replaced the bone marrow of rats with hPAP-labelled cells to allow tracking of EPCs. EPO significantly increased the number of bone marrow derived endothelial cells (BMDEC) in the LV-free wall compared with sham and MI ($p<0.01$, figure 6C and D). VEGF inhibition attenuated the number of BMDEC by 42% in the MI-EPO + aVEGF group (p=NS vs. untreated MI, $p<0.01$ vs. MI-EPO, figure 6C and D), whereas VEGF neutralisation alone did not further reduced the number of BMDEC (p=NS vs. untreated MI, figure 6C and D).

DISCUSSION

The present study establishes for the first time a crucial role for VEGF in EPO-induced neovascularisation and restoration of LV function. Under ischaemic conditions, EPO promoted VEGF transcription through the JAK2/STAT3 signal transduction pathway. In contrast, EPO did not stimulate VEGF transcription in fibroblasts or endothelial cells and the direct angiogenic effects of EPO in the aortic ring assay were modest and VEGF independent. In rats with post-MI HF, EPO also stimulated VEGF-production predominantly in cardiomyocytes. Most importantly, neutralisation of VEGF completely abrogated the salutary effects of EPO on cardiac function and microvascularisation. Likewise, neutralisation of VEGF blunted EPO-induced proliferation of myocardial endothelial cells and homing of EPCs to the myocardium.

Our study suggests a pivotal role of VEGF in EPO-induced neovascularisation in HF, and underscores the crucial role for the paracrine control of myocardial angiogenesis by cardiomyocytes. The results provide further evidence that the beneficial effects of EPO on cardiac function are at least in part haematocrit independent.

Table 1. General characteristics at sacrifice

Variable	SHAM + IgG	MI + IgG	MI-EPO + IgG	MI + EPO + aVEGF1	MI + EPO + aVEGF2	MI + aVEGF1	MI + aVEGF2
n	9	9	11	13	10	11	12
Infarct size (% of LV circumference)	-	41±2.3	42±1.8	43±1.5	43±2.0	43±2.1	44±1.8
Body weight (g)	396±7	409±16	414±7	406±6	415±9	416±10	402±13
Haematocrit (%)	47±1.2	48±1.2	58±1.5†‡	56±0.9†‡	55±2.6*#	49±1.4	47±0.8
Circ. EPCs (n/field)	147±15	91±25*	280±62*‡	224±34*‡	-	135±21	-
Heart/body (g/g)	3.4±0.1	5.2±0.4†	4.9±0.3†	4.7±0.3†	4.4±0.1†	5.0±0.3†	5.4±0.8†
Cardiom-cross. (mm^2)	405±28	771±46†	806±41†	756±24†	806±37†	781±47†	804±28†
Lung/body (g/g)	4.4±0.1	8.1±1.7†	8.0±1.3†	8.1±1.1†	8.4±1.1†	10.6±1.2†	7.9±1.0†
Haemodynamics							
Heart rate (bpm)	298±6.5	303±19	317±6	292±8	300±13	293±8	291±5
LV-syst (mmHg)	119±4	102±2†	112±4†‡	101±2†	105±4†	100±3†	98±2†
Aorta-syst (mmHg)	123±3	101±3†	112±3#	100±3†	103±3†	96±6†	98±2†
Aorta-diast (mmHg)	80±3	71±3	82±2	74±4	73±3	65±6	71±1

Data are presented as mean±SEM, n = number of animals, circ EPCs = circulating endothelial progenitor cells, heart/body = heart weight/body weight ratio, cardiom-cross = cardiomyocyte cross-sectional area, lung/body = lung weight/body weight ratio, bpm = beats per minute, LV = left ventricle, syst = systolic, diast = diastolic. * p<0.05, † p<0.01 vs. sham, # p<0.05, ‡ p<0.01 vs. MI.

EPO restores cardiac function in HF by targeting the myocardium

In patients with HF, correction of anaemia with EPO has been associated with restoration of cardiac function for more than three decades.[18] Moreover, in experimental HF without anaemia, EPO consistently results in sustained improvement of cardiac function and microvascularisation

even in a dose that does not increase haematocrit levels.[19,20] These observations suggested that EPO mediates these beneficial effects by targeting cardiac cells instead of bone marrow progenitor cells. Our study importantly substantiates this hypothesis. First, EPO induces VEGF production by cardiomyocytes and inhibition of VEGF abolishes the beneficial effects of EPO. From this, we postulate that the EPO-induced VEGF production by cardiomyocytes conveys the beneficial effects of EPO on the heart. Secondly, EPO failed to improve cardiac function when VEGF was antagonized, despite markedly increased haematocrit levels, thus confirming that these cardiac effects of EPO are haematocrit independent. Thirdly, although EPO-induced mobilisation of EPCs was not affected by VEGF neutralisation, myocardial neovascularisation was significantly attenuated, which indicates that the stimulation of EPCs alone is not sufficient to improve cardiac function.

EPO stimulates the paracrine angiogenic response of cardiomyocytes

Disproportional (micro) vascular growth during cardiac hypertrophy causes an impaired vascular supply of cardiomyocytes.[2,3] Exhaustion of VEGF release by cardiomyocytes during prolonged ischaemia has been proposed as an underlying mechanism.[2] We show that EPO increases VEGF expression in cardiomyocytes strictly under ischaemic conditions. Therefore, EPO seems to restore the adequate paracrine angiogenic response of cardiomyocytes to cellular ischaemia.[21] Furthermore, we provide proof that EPO-induced VEGF gene transcription is mediated via the JAK2/STAT3 signal transduction pathway. STAT3 has been identified as a crucial transcription factor for growth factor and hypoxia mediated VEGF production and an indispensable transcription factor for adaptive angiogenesis in cardiomyocytes.[22,23] The important role for STAT3 in EPO-mediated VEGF gene transcription therefore provides additional support for the restoration of the paracrine response of cardiomyocytes. The ischaemia-specific kinetics of EPO-induced VEGF production in cardiomyocytes might be explained by ischaemia-dependent EPOR upregulation.[24] Alternatively, this might indicate that other hypoxia sensing pathways are also operative in this signal. Further studies are required to delineate the mechanisms of the hypoxia-specific nature of EPO.

The finding that the angiogenic effects of EPO are at least partially mediated in a paracrine fashion rather than through direct stimulation of endothelial cells seems to contradict previous studies which showed that EPO markedly stimulates endothelial cell proliferation and vascular tubule formation in *in vitro* models of angiogenesis.[25] Indeed, we did observe significant stimulatory effects of EPO in the aortic sprouting assay. However, these effects of EPO were modest, whereas the angiogenic effects of EPO *in vivo* were more substantial and amenable to VEGF neutralisation, as evidenced by marked attenuation of PCNA-positive endothelial cells after VEGF inhibition. Our results confirm recent observations by Asaumi et al. whom also demonstrated that the angiogenic effects of EPO on top of VEGF were limited.[8] Thus, EPO elicits myocardial angiogenesis, more so than direct stimulation of endothelial cells. Finally, the fact that cardiac function is not altered by VEGF neutralization alone might seem counterintuitive, because VEGF has been identified as a main angiogenic factor in HF.[17] However, to avert intrinsic negative inotropic effects of VEGF neutralisation in our model, we only administered VEGF-neutralising antibodies in the first week after EPO administration.

With this, we established intermittent VEGF neutralisation, only during the pharmacological window of EPO (see supplementary data, figure S1).

EPO-increased VEGF expression facilitates myocardial incorporation of EPCs

EPO mobilises EPCs from the bone marrow and we and others have therefore postulated an important role for EPCs in EPO-induced neovascularisation.[26] We recently showed that EPO stimulates incorporation of EPCs into the myocardial microvasculature.[9] However, the present study indicates that EPC mobilisation alone does not necessarily augment neovascularisation. We previously demonstrated that EPCs incorporate into healthy and diseased tissues, whereas neovascularisation was specifically induced in the presence of VEGF upregulation and ischaemia.[12] Moreover, transplantation of normal bone marrow to EPOR null mice does not rescue VEGF expression nor does it restore neovascularisation.[27] One might therefore suggest that EPCs are dispensable for EPO-induced neovascularisation. However, attenuated expression of VEGF in EPOR null mice is also associated with impaired homing of EPCs,[4] which we also observed in our study. Thus, in addition to stimulation of local endothelial proliferation, VEGF seems to serve as an important chemotactic factor for EPO-mobilised EPCs.

Clinical implications

After several promising phase 2 studies,[28,29] a phase 3 clinical trial is currently evaluating the effects of EPO on outcome in HF patients with anaemia. From our present and previous data,[22] we postulate that a trial using low-dose EPO, with intermittent administration (circumventing unwanted and potentially damaging elevation of haematocrit levels) would be most likely to yield a positive outcome. Ideally, EPO-derivatives that specifically target the cardiomyocytic EPOR should be developed, as such agents might restore cardiac function without undesirable haematopoietic or pro-thrombotic side effects.[30] Possibly, (post)transcriptional cardiomyocyte-specific modification of the EPOR occurs that might enable us to design cardiac specific EPO derivatives. Alternative drug delivery approaches might include EPO-eluting hydrogels.[31] However, we cannot completely exclude that the beneficial effects of EPO are in part dependent on direct effects on endothelial cells or EPCs. Hence, efficacy of novel compounds should be compared in an experimental setting before embarking onto clinical trials.

Conclusion

EPO improves cardiac function in HF by stimulating VEGF production in cardiomyocytes. EPO fosters VEGF expression in cardiomyocytes which in turn stimulates myocardial endothelial proliferation and incorporation of EPCs. These findings provide new evidence for the non-haematopoietic, pro-angiogenic, and salutary effects of EPO in HF.

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Supplemental data to "Vascular endothelial growth factor is crucial for erythropoietin-induced improvement of cardiac function in heart failure"

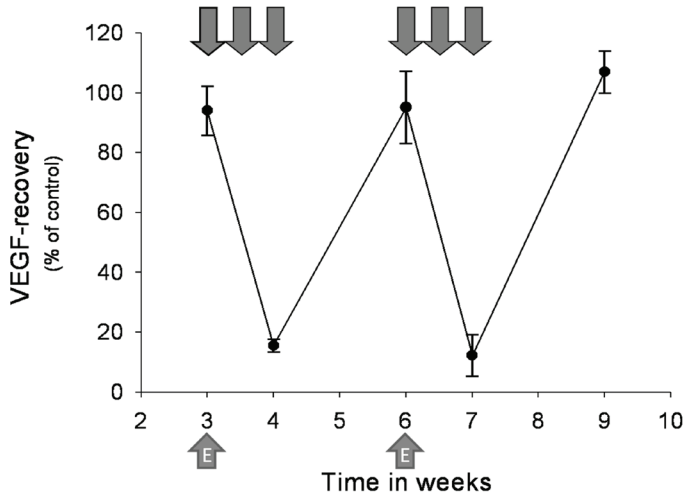

Figure 1

Temporal characteristics of VEGF neutralisation capacity of the plasma. Graphic representation of the percentage of VEGF-165 recovered from the plasma of rats treated with VEGF-neutralising antibodies, expressed as the percentage of VEGF-165 recovered from the plasma of control animals treated with control IgG. Red arrows indicate the intraperitoneal administration of antibodies. Blue arrows indicate the intraperitoneal administration of erythropoietin (EPO).

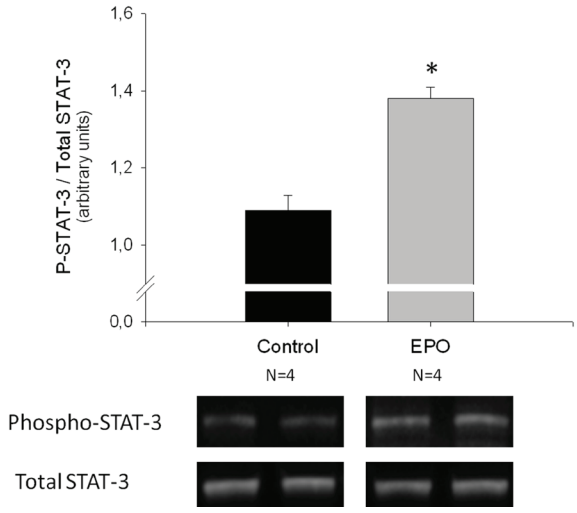

Figure 2

Effect of EPO on STAT3 phosphorylation in Neonatal Rat Ventricular Myocytes (NRVM). Deferoxamine pre-incubated NRVM were stimulated with EPO (10 IU/ml) or vehicle (Control). At baseline and after 30 minutes cells were lysated. Figure is a graphic representation of phospho-STAT3 normalised to total STAT3 and expressed as fold difference compared to the baseline sample. Below typical examples of Western Blot results shown. p-STAT3 = phosphorylated-signal transducers and activators of transcription-3. * $p<0.05$ vs. control.

4 CHAPTER

Erythropoietin receptor deficient mice have impaired exercise tolerance due to cardiac and muscle maladaptoin

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ABSTRACT

Background

We have demonstrated that erythropoietin (EPO) exerts cardioprotective effects via its receptor (EPOR) in pathophysiological hypertrophy (e.g. myocardial infarction and heart failure). Whether the EPO-EPOR signalling pathway exerts similar effects in physiological hypertrophy is, however, unknown.

Methods

We employed transgenic-rescued EPOR-null mutant mice ($EPOR^{-/-}$-rescued, EPOR) that express the EPOR exclusively in the haematopoietic cells. C57Bl/6 mice were used as controls (WT). Both groups had unlimited access to a running wheel for 28 days (ex). Furthermore, sedentary mice of both groups were studied (sed). We measured exercise performance: average distance, average daily speed and average running time. At sacrifice heart weights and haemodynamic parameters were measured. Furthermore, we measured myocyte size, capillary-to myocyte ratio and number of NADH-tetrazolium reductase (NADH-TR) positive cells.

Results

WT mice ran 8.0±0.4 km/day, whereas EPOR mice ran 5.5±0.6 km/day ($p<0.01$). This was mainly due to a lesser average speed (WT-ex 1.6±0.1 km/hrs vs. EPOR-ex 1.3±0.1 km/hrs, $p<0.01$). The percentage increase in cardiac mass was 26±4% in the WT-ex group compared to 10±2% in the EPOR-ex group ($p<0.01$). Indices of contractility and relaxation were increased after exercise in both groups. Myocyte size and capillary-to-myocyte ratio were increased in both the left ventricle and skeletal muscle in the WT-ex group as compared to the WT-sed group. The EPOR-ex group did not show any of these effects after exercise. The percentage of NADH-TR positive cells was increased in skeletal muscle of the WT-ex group as compared to the WT-sed group (78±2 vs. 54±2%, $p<0.01$). This increase was absent in the EPOR-ex group.

Conclusions

We conclude that the absence of EPO-EPOR signalling leads to a lower exercise capacity upon. $EPOR^{-/-}$-rescued mice appear to have impaired exercise tolerance due to cardiac and muscle maladaptation. We suggest that EPO-EPOR signalling is important for physiological adaptation during exercise.

INTRODUCTION

Physiologic hypertrophy is characterised by an increase in capillary density and an increase in myocyte size, so called hypertrophy, and is thought to be a mechanism to adapt to increased physiologic demands.[1-3] Physiologic hypertrophy may be caused by exercise training, such as in athletes and has both cardiovascular as non-cardiovascular effects.

In contrast to physiologic hypertrophy, pathophysiologic hypertrophy has been extensively researched in the context of cardiovascular disease. Maladaptive remodelling in cardiovascular disease is characterised by excessive myocyte hypertrophy and apoptosis, fibrosis and a decrease in the number of capillaries.[4] This is in contrast with physiologic hypertrophy, in which myocyte hypertrophy leads to an increase in the number of capillaries. Growth (in number and size) of capillaries, which theoretically would be needed to supply the hypertrophic left ventricle (LV) with oxygen and nutrients, is insufficient in pathophysiologic hypertrophy, thus creating an imbalance between the number of capillaries and cardiomyocytes. This imbalance is thought to further contribute to the remodelling process as it causes chronic hypoxia and less flow reserve, with further tissue damage as a result.[5]

The processes behind the angiogenic response in LV hypertrophy are not completely understood. Numerous growth factors and their receptors play a role, such as vascular endothelial growth factor (VEGF),[6,7] transforming growth factor (TGF),[8] the estrogen receptor,[9] and the erythropoietin (EPO)-EPO receptor (EPOR) system.

We and others have shown that administration of EPO in ischemic heart failure stimulates neovascularisation.[10-12] In pathological conditions, the endogenous EPO-EPOR system plays a pivotal role.[13] It plays a role in recruitment of endothelial progenitor cells and prevents the development of pulmonary hypertension during chronic hypoxia in mice,[14] it has a protective role against myocardial ischemia reperfusion injury,[15] and plays a protective role against pressure overload-induced cardiac dysfunction *in vivo*.[16] Furthermore, the EPO-EPOR system plays a role in angiogenesis in hind limb ischemia through upregulation of the VEGF-VEGFR by directly enhancing neovascularisation.[17] This notion is further supported by observations made in a mouse strain that expresses the EPOR exclusively in the haematopoietic cells (EPOR$^{-/-}$-rescued mice).[18] These mice have accelerated maladaptive remodelling upon various stimuli, because of an insufficient angiogenic response.[14-17]

Whether the EPO-EPOR system plays a role in angiogenesis seen in physiologic hypertrophy remains unknown. We hypothesized that EPO-EPOR signalling is crucial for angiogenesis. Therefore, we evaluated the role of EPO-EPOR signalling in a model of physiologic hypertrophy.

METHODS

Animals

We employed transgenic-rescued EPOR-null mutant mice (EPOR$^{-/-}$-rescued mice) that express EPOR exclusively in the haematopoietic cells. These mice have been previously described.[18] These mice have normal development without clear cardiac phenotype. The EPOR$^{-/-}$-rescued

mice have a C57Bl/6J genetic background and were bred and housed at the central animal facility of the University of Groningen. C57Bl/6J (Harlan, The Netherlands) mice were used as controls. All mice had *ad libitum* access to food and water. Mice were conventionally but individually caged with a 12 hr. day-night cycle. The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen.

Experimental protocol

Ten week old male EPOR$^{-/-}$-rescued mice (EPOR) and age and gender control mice (WT) were allowed to exercise in a model of voluntary wheel running (VWR); mice had unlimited access to a running wheel for four weeks (WT-ex, EPOR-ex). Furthermore, sedentary mice of all groups were studied (WT-sed, EPOR-ex). We tested two time periods: two weeks of VWR and four weeks of VWR. The current paper reports end-points of the four week period only; the two week outcomes were in concert with these data. The four week trial consisted of a total of 56 mice. The running wheel was equipped with sensor and a computer that recorded exercise performance: average distance, average daily speed, maximum speed and average running time. After four weeks, mice were anaesthetised and the right carotid artery was dissected. A microtip pressure-volume transducer was inserted (Millar Instr. Inc., Houston, Texas, USA) as described previously.[19] Systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), left ventricular end-diastolic pressure (LVEDP) and maximal rates of increase and decrease in left ventricular pressure (dP/dtmax and dP/dtmin) were measured. Thereafter, blood was drawn and hearts were rapidly excised and weighed. Myocardial tissue and muscle tissue (m. gastrocnemius) was dissected transversally and processed for immunohistochemistry or snap frozen for molecular analysis.

Echocardiography

Two days prior to sacrifice all mice were subdued to echocardiography (GE Vivid 7 Dimension, using a 13 MHz probe) under general anaesthesia (isoflurane 2%). We recorded parasternal long axis (PLAX), parasternal short axis (PSAX) and apical four chamber view (AP4CH). In PLAX, the left ventricular outflow tract diameter (LVOT) was measured. In PSAX, M-Mode was obtained to measure diameters of the septum and posterior wall in systole and diastole. PSAX was obtained at the height of the papillary muscles. Flow over the aortic valve was measured using colour Doppler and cardiac output (CO) was calculated. All echo measurements were obtained from the same researcher (RadB) to avoid inter observer variability.

Plasma EPO levels

Plasma EPO levels were analysed using a commercial Enzyme Linked Immunosorbent Assay according to the guidelines provided by the manufacturer (Mouse EPO Quantikine ELISA kit, #MEP00, R&D Systems Europe, Abingdon, UK).

Histology

Hearts and muscle were isolated, fixed with buffered 3.7% formalin and embedded in paraffin, or embedded in tissue tec and frozen in liquid nitrogen. Thereafter cut into three µm thick

sections on a microtome and sections were mounted on slides. Hearts were cut at the mid-papillary level and skeletal muscle was cut at the mid-belly.

Myocyte size

Deparaffinised thee µm thick sections were stained with a Gomori's silver staining. Myocyte hypertrophy was measured as the cross-sectional area (CSA) of transversally cut myocytes as previously described.[20] In each stained section, measurements were averaged from five different counting fields at 40x magnification.

Capillary density

To assess capillary density and neovascularisation, endothelial cells were stained with CD31. Cryosections were fixed in acetone and air dried. Next, endogenous peroxidases were blocked (#K4008, EnVision kit, 1:2, Dako, Glostrup, Denmark). Slides were incubated with purified rat anti-mouse CD31 antibody (#550274, 1:100, BD Biosciences, San Diego, CA, USA) for 1 hr. After washing with PBS, slides were incubated with secondary antibody (Unconjugated rabbit anti-rat IgG antibody, #AI-4001, 1:300, Vector Laboratories, Burlingame, CA, USA) for 1 hr. Slides were washed with PBS and incubated with a tertiary antibody (anti-rabbit polymer-HRP, #K4008, EnVision kit, Dako, Glostrup, Denmark) for 30 minutes. Next, sections were developed with AEC+ solution (#K4008, Envision kit, Dako, Glostrup, Denmark) and counter stained with Mayers Haematoxylin. Slides were mounted and stored in the dark. Number of capillaries was counted in eight random chosen fields at 40x magnification. A size criterion of ten µm was used to exclude small arterioles and venules. Capillary density is expressed as the number of capillaries per tissue area (mm^2). As a measure of neovascularisation, capillary-to-myocyte ratio was calculated dividing capillary with myocyte density, as previously described.[21]

Muscle fibre type

To assess whether exercise causes a shift in fibre type, we assessed the muscle fibre type in muscle tissue with NADH-tetrazolium reductase (NADH-TR) staining as previously described.[22] Type I muscle fibres have high NADH-dehydrogenase activity in mitochondria, therefore, type I muscle fibres are stained dark blue and type II muscle fibres remains unstained. In short, cryosections were fixed in acetone and incubated with incubation solution (NADH: #10107735001, Roche, Basel, Switzerland, 0.2 M phosphate buffer solution, Nitro-BT solution: #N-6876, Sigma-Aldrich, St. Louis, MO, USA, and H2O2) for 45 minutes at 37°C. Slides were washed with PBS and fixated with 4% paraformaldehyde for 10 minutes. Slides were mounted and stored in the dark. Number of type I and type II muscle fibres were counted in four random chosen fields at 40x magnification and expressed as percentage of total number of myocytes.

Quantitative real-time PCR

Total RNA from LV tissue and skeletal muscle was extracted using the Nucleospin RNA II kit, according to manufacturer's protocol (Machery-Nagel, Duren, Germany). cDNA synthesis and quantitative real time PCR (rtPCR) were performed as previously described with using 0.5 µg total RNA.[23] mRNA levels were expressed in relative units based on a standard curve obtained with serial dilutions of a calibrator cDNA mixture. To normalise expression data, multiple internal control genes were used as described.[24] Please see supplemental data for full protocol and list of primers.

Statistical analyses

Results are reported as mean ± standard error of the mean (SEM) or otherwise if stated. For reasons of clarity, we only provided statistics on the differences between the WT-sed and WT-ex groups and EPOR-sed and EPOR-ex groups. Statistical analysis among groups was performed by ANOVA with the Bonferroni post hoc test, if distributed normally or with the Kruskall-Wallis test followed by Mann-Whitney U test when skewed distributed. All p-values are two-tailed, and a p-value of less than 0.05 was considered significant. All analyses were performed using SPSS version 16.0 software (SPSS, Chicago, IL, USA).

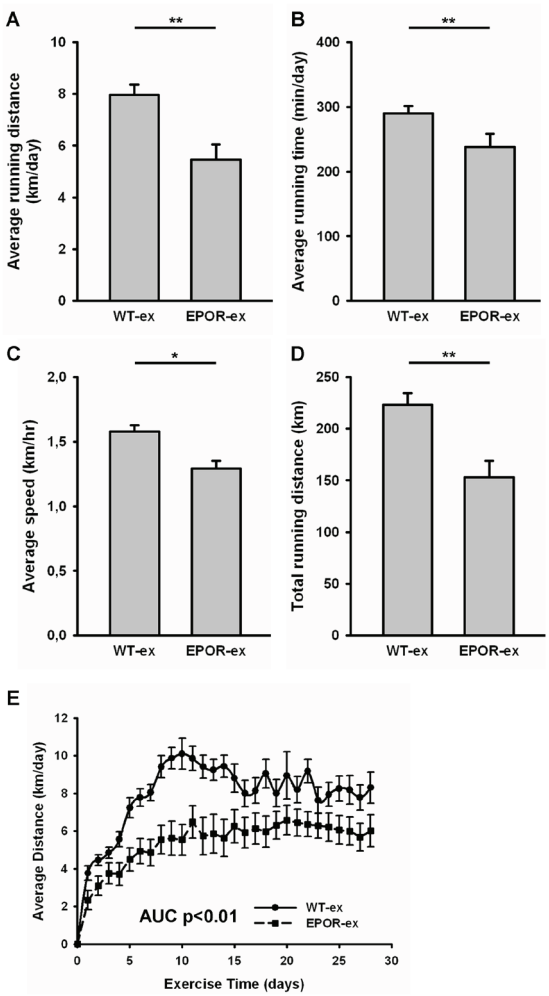

Figure 1. Voluntary wheel-running values for wild type and EPOR$^{-/-}$-rescued mice

A. Average running distance (in km/day) over the four week study period. B. Average running time (in min/day) mice voluntary spent in the cage-wheel over the four week study period. C. Average speed (in km/hr) mice achieved in the cage-wheel over the four week study period. D. Total distance (in km) mice ran over the four week study period. E. Average distance (in km/day) per day for every 24 hrs over the four weeks of voluntary exercise. AUC = area under the curve, WT = wild type mice, EPOR = EPOR$^{-/-}$-rescued mice, ex = exercise. * p<0.05, ** p<0.01.

RESULTS

EPOR$^{-/-}$-rescued mice have impaired exercise capacity as compared to wild type mice

Mice were allowed to exercise in a model of voluntary wheel-running for four weeks. Figure 1 shows the voluntary wheel-running values for WT and EPOR$^{-/-}$-rescued mice. Mice in the WT-ex group covered a larger distance per day compared to the EPOR-ex group (8.0±0.4 vs. 5.5±0.6 km/day, p<0.01, figure 1A). Furthermore, mice in the WT-ex group also spent more time in the cage-wheel (290±12 vs. 237±20 min/day, p<0.01, figure 1B), had a higher average speed (1.6±0.1 vs. 1.3±0.1 km/hr, p<0.05, figure 1C) and in total ran significantly more kilometres (223±11 vs. 153±16 km, p<0.01, figure 1D) as compared to the EPOR-ex group. Figure 1E shows the average running distances per every 24 hrs for the complete four weeks of voluntary wheel-running. Predominantly in the first two weeks, WT-ex mice ran more kilometres per day compared to the EPOR-ex mice (area under the curve, p<0.01).

Table 1. Clinical characteristics at sacrifice after four weeks of exercise

	WT-sed (n=10)	WT-ex (n=12)	EPOR-sed (n=13)	EPOR-ex (n=11)
HR (bpm)	495±21	484±22	532±38	486±25
SBP (mmHg)	102±2	101±2	109±4	110±3
DBP (mmHg)	63±2	61±2	65±4	69±3
MAP (mmHg)	75±2	74±2	80±4	82±2
LVEDP (mmHg)	5.8±0.9	5.1±1.1	5.7±1.2	7.9±1.2
dPdtmax (mmHg/s)	8969±293	10611±366 *	8901±585	11115±572 ‡
dPdtmin (mmHg/s)	-8318±484	-10057±467 *	-8278±413	-11192±660 ‡
HW (mg)	155±3.6	195±6.6 *	167±5.4	182±5.0
BW (gr)	27.1±0.4	27.1±0.4	28.1±0.8	26.6±0.4
Ht (%)	45±1	45±1	45±2	44±1
EPO (pg/ml)	107±6	111±10	154±8	177±13

Data is shown of exercise after four weeks. All values are mean±SEM. HR = heart rate, SBP = systolic blood pressure, DBP = diastolic blood pressure, MAP = mean arterial pressure, LVEDP = left ventricular end diastolic pressure, dPdtmax and dPdtmin = maximal rates of increase and decrease in left ventricular pressure, Ht = haematocrit, HW = heart weight, BW = body weight, WT = wild type mice, EPOR = EPOR$^{-/-}$-rescued mice, sed = sedentary, ex = exercise. * p<0.05 vs. WT-sed, ‡ p<0.05 vs. EPOR-sed.

Wild type mice show a stronger cardiac adaptation than EPOR$^{-/-}$-rescued mice

The effects of voluntary exercise are reflected by the haemodynamic parameters as shown in table 1. dPdtmax and dPdtmin are increased in both exercise groups (WT-ex and EPOR-ex). Other haemodynamic parameters are all similar between groups. LV pressures and blood pressures are comparable, as expected.

In figure 2, the heart weights are presented. We calculated 'cardiac adaptation' as a measure for the response of the heart on the performed exercise. Both the WT-ex as EPOR-ex group had a similar increased heart weight normalised for body weight as compared to WT-sed (5.7±0.1

vs. 7.1±0.2 mg/g, p<0.01) and EPOR-sed (5.9±0.2 vs. 6.8±0.2, p<0.01) respectively (figure 2A). To provide better insight in the magnitude of hypertrophy, we calculated the percentage increase in cardiac mass (figure 2B). The increased exercise capacity resulted in a larger cardiac adaptation in heart weight in the WT-ex group as compared to the EPOR-ex group (26±4 vs. 10±2%, p<0.01).

Echocardiography

Table 2 summarises the echocardiographic parameters. Only posterior wall thickness was increased in the WT-ex group (p<0.05) as compared to WT-sed group. This was not observed in the EPOR-ex group. Furthermore, CO and SV were both increased in the WT-ex group as compared to the WT-sed group, as expected in animals that exercise. This was not observed in the EPOR-ex group.

EPOR$^{-/-}$-rescued mice have increased EPO plasma levels but normal haematocrit values

To measure the influence of knock-out of the EPOR on EPO production, we measured EPO plasma levels and Ht (table 1). EPO plasma levels were significantly increased in EPOR$^{-/-}$r-rescued mice (both the EPOR-sed and EPOR-ex groups as compared to the WT controls, p<0.01). However, this increase did not cause an increase in Ht levels (p=NS in all groups). So, the lack of a functional EPO-EPOR system leads to a modest increase in EPO levels, without enhanced erythropoiesis.

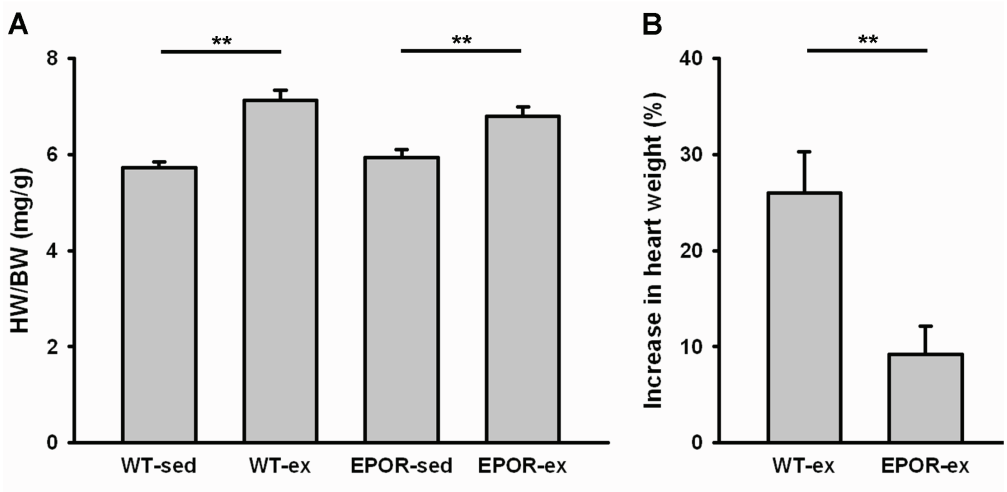

Figure 2. Cardiac adaptation in sedentary and exercised wild type and EPOR$^{-/-}$-rescued mice

A. Heart weight (HW) normalised for body weight (BW) determined from sedentary (sed) and exercised (ex) wild type (WT) and EPOR$^{-/-}$-rescued (EPOR) mice. B. Cardiac adaptation expressed as percentage change from mean sedentary HW in exercised groups. ** p<0.01.

Table 2. Echocardiographic parameters at sacrifice after four weeks

	WT-sed (n=10)	WT-ex (n=12)	EPOR-sed (n=13)	EPOR-ex (n=11)
LVEDD (mm)	4,09±0,48	4,09±0,46	4,11±0,30	3,89±0,25
LVESD (mm)	2.88±0.54	2.80±0.48	2.72±0.32	2.52±0.34
IVSd (mm)	0.77±0.08	0.78±0.14	0.79±0.13	0.85±0.10
LVPWd (mm)	0.69±0.14	0.85±0.09 *	0.63±0.20	0.69±0.28
FS (%)	29.97±7.49	31.68±7.24	33.67±5.36	35.52±6.80
CO (ml/min)	21.57±6.04	28.10±5.47 *	24.41±3.53	24.69±7.73
SV (µl)	62.5±12.9	74.0±12.0 *	65.6±10.0	69.3±19.9

Data is shown of exercise after four weeks. All values are mean±SEM. HR = heart rate, LVEDD = left ventricular end diastolic diameter, LVESD = left ventricular end systolic diameter, IVSd = thickness of the left ventricular septum during diastole, LVPWd = thickness of the left ventricular posterior wall during diastole, FS = fractional shortening, CO = cardiac output, SV = stroke volume. WT = wild type mice, EPOR = EPOR$^{-/-}$-rescued mice, sed = sedentary, ex = exercise. * p<0.05 vs. WT-sed.

WT mice have myocyte hypertrophy and adapted capillary-to-myocyte ratio after exercise

We measured CSA of both cardiomyocytes and skeletal muscle fibres. CSA was increased in WT-ex mice as compared to WT-sed mice in cardiomyocytes (216±6 vs. 280±10 µm^2, p<0.01, figure 3A), and in skeletal muscle myocytes (1366±45 vs. 1800±161 µm^2, p<0.05, figure 3B). CSA of both cardiomyocytes and skeletal muscle myocytes was not significantly increased in EPOR-ex mice as compared to EPOR-sed mice (cardiomyocyte CSA: 218±18 vs. 242±15 µm^2, p=NS, figure 3A; skeletal muscle: 1220±118 vs. 1509±201 µm^2, p=NS, figure 3B).

As a sign of neovascularisation, we calculated the capillary-to-myocyte ratio. After exercise, the capillary-to-myocyte ratio in the left ventricle is increased in the WT-ex group compared to the WT-ex group (1.2±0.03 vs. 1.6±0.06, p<0.01, figure 3C). The capillary-to-myocyte in the left ventricle was increased to a lesser degree in the EPOR-ex group as compared to the EPOR-sed group, and this did not reach statistical significance (1.1±0.06 vs. 1.3±0.11, p=NS, figure 3C). The capillary-to-myocyte ratio in skeletal muscle showed similar results (WT-sed vs. WT-ex: 0.9±0.11 vs. 1.4±0.16, p<0.05, figure 3D; EPOR-sed vs. EPOR-ex: 0.9±0.11 vs. 1.1±0.06, p=NS, figure 3D).

Skeletal muscle of exercised EPOR$^{-/-}$-rescued mice does not adapt to increased workload

To measure if skeletal muscle adapts to the increased workload with a shift in fibre type (from type II to type I), we measured the number of NADH-TR positive cells. Figure 3E shows the percentage of NADH-TR positive cells counted in skeletal muscle. Significantly more NADH-TR positive cells were found in the WT-ex group compared to the WT-sed group (78±2 vs. 54±2%, p<0.01). This was not observed in the EPOR-ex group compared to the EPOR-sed group (59±1 vs. 57±1%, p=NS), indicating that the EPOR$^{-/-}$-rescued mice have an impaired adaptive mechanism to increased workload.

mRNA expression of factors involved in structural, angiogenic and metabolic development

We measured expression of different genes involved in structural, angiogenic and metabolic development in both left ventricular tissue as in skeletal muscle (figure 4).

Structural

mRNA expression of atrial natriuretic peptide (ANP) in the ventricle was increased 1.6±0.1 times in the WT-ex as compared to the WT-sed group ($p<0.01$). ANP expression was also increased significantly in the EPOR-ex group (1.3±0.1 times vs. EPOR-sed, $p<0.05$), but to a lesser extent

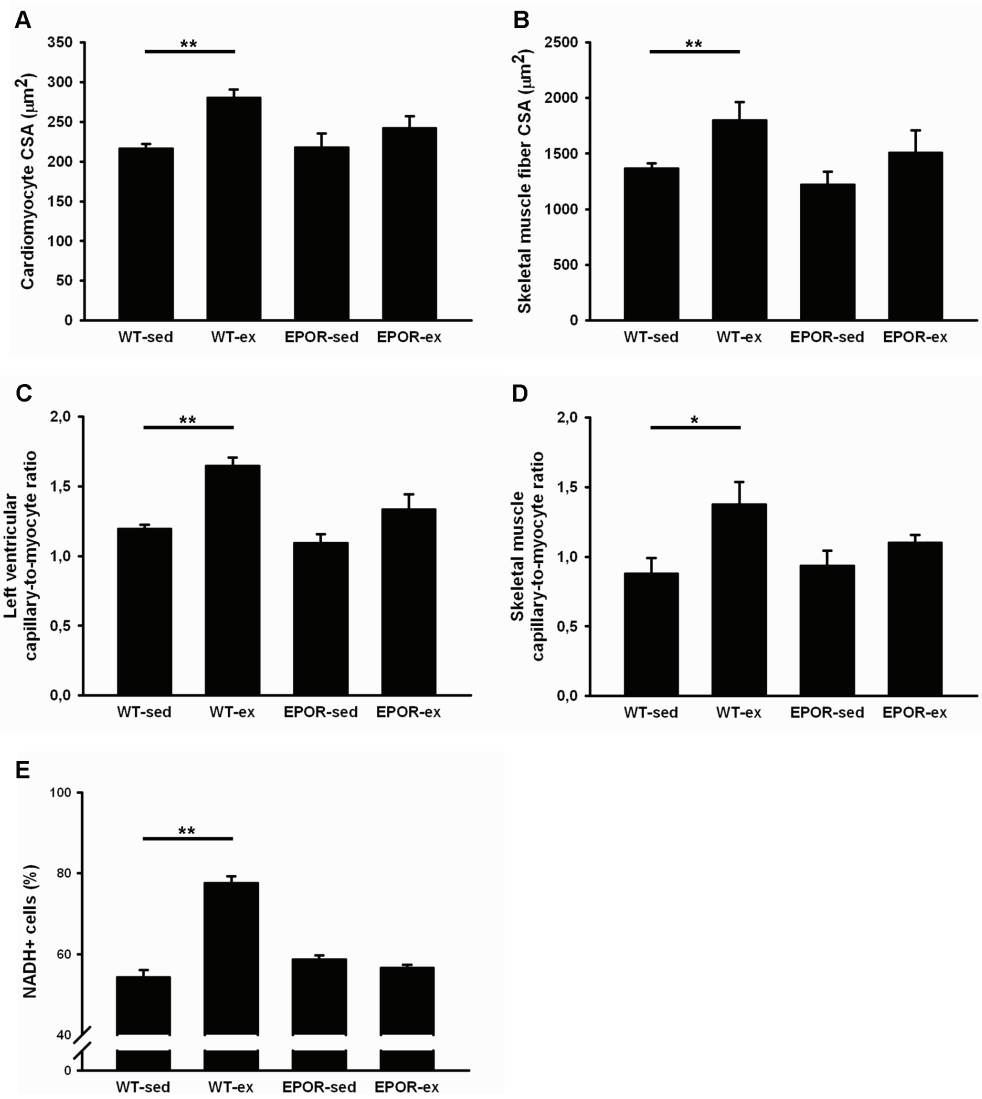

Figure 3. Expression of different genes involved in myocyte hypertrophy

A. cardiomyocyte cross sectional area (CSA) in sedentary and exercised mice. B. Skeletal muscle fibre CSA in sedentary and exercised mice. C. Capillary-to-myocyte ratio in left ventricle. D. Capillary-to-myocyte ratio in m. gastrocnemius. E. Percentage of NADH-TR positive cells in m. gastrocnemius. WT = wild type mice, EPOR = $EPOR^{-/-}$-rescued mice, ex = exercise. * $p<0.05$, ** $p<0.01$.

as in the WT-ex group. Actin, alpha-1, skeletal muscle (ACTA-1), is a conserved protein involved in cell motility, structure and integrity. Alpha actins are a major constituent of the contractile apparatus. ACTA-1 was increased in the ventricle of both the WT-ex (2.3±0.2 times vs. WT-sed, p<0.01) as EPOR-ex group (1.6±0.2 times vs. EPOR-sed, p<0.01), but not in skeletal muscle.

Angiogenic

We measured mRNA levels of VEGF-A as a marker of angiogenic development. VEGF-A was slightly, but significantly increased in the ventricle of the WT-ex group (1.4±0.1 times vs. WT-sed, p<0.01). This was not observed in the EPOR-ex group, and this effect was also absent in skeletal muscle.

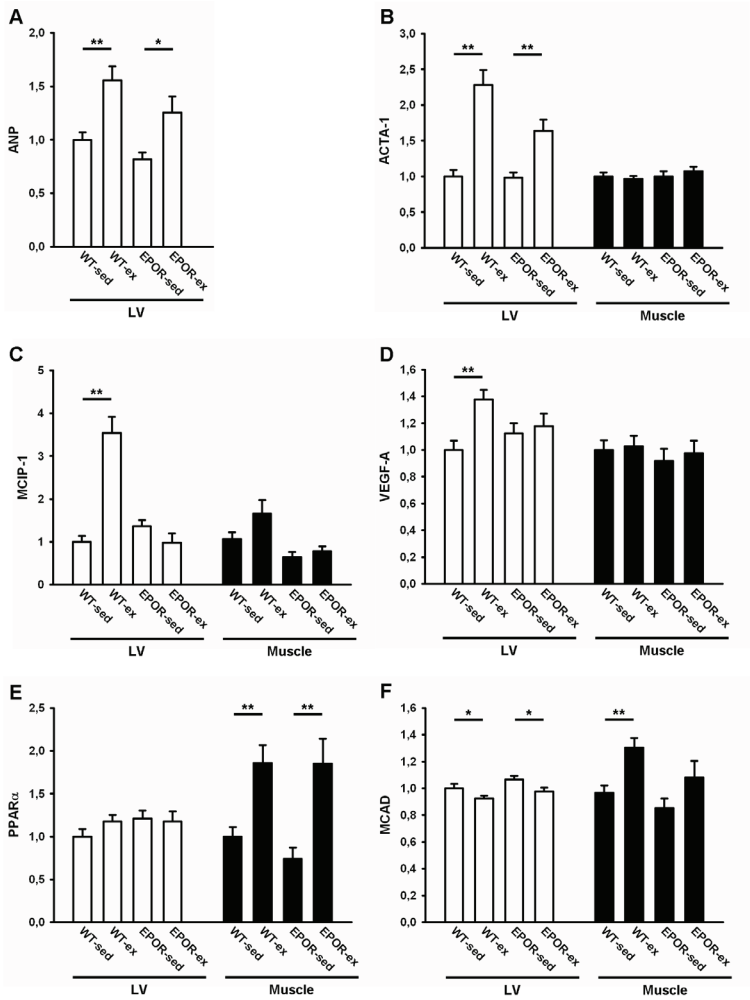

Figure 4 A. mRNA expression of atrial natriuretic peptide (ANP). B. mRNa expression of actin, alpha-1, skeletal muscle (ACTA-1). C. mRNA expression of myocyte-enriched calcineurin interacting protein-1 (MCIP-1). D. mRNA expression of vascular endothelial growth factor-A (VEGF-A). E. mRNA expression of peroxisome proliferator activated receptor alpha (PPARα). F. mRNA expression of acyl-CoA dehydrogenase (MCAD). WT = wild type mice, EPOR = EPOR$^{-/-}$-rescued mice, ex = exercise. * p<0.05, ** p<0.01.

Metabolic

Peroxisome proliferator activated receptor alpha (PPARα), involved in the regulation of cellular differentiation, development and metabolism, was not increased in the ventricle. In skeletal muscle on the other hand, PPARα was increased 1.9±0.2 times in the WT-ex group (vs. WT-sed, $p<0.01$). However, also in the EPOR-ex group, PPARα was increased (1.8±0.3 vs. EPOR-sed, $p<0.01$). mRNA expression of myocyte-enriched calcineurin interacting protein-1 (MCIP-1) was only increased in the ventricle of the WT-ex group (3.5±0.4 times vs. WT-sed, $p<0.01$) and not in the EPOR-ex group, and also not in skeletal muscle. mRNA expression of acyl-CoA dehydrogenase (MCAD) is decreased in the ventricle of both the WT-ex (with 8%, $p<0.05$) as the EPOR-ex group (with 4%, $p<0.05$). However in skeletal muscle, MCAD expression is increased 1.3±0.1 times in the WT-ex group (vs. WT-sed, $p<0.01$). This was not observed in the EPOR-ex group.

DISCUSSION

In a model of voluntary exercise, we show that $EPOR^{-/-}$-rescued mice have a reduced exercise capacity, which is associated with an impaired adaptation of cardiac and skeletal muscle. The increased exercise capacity seen in WT mice resulted in a larger cardiac adaptation in HW in the WT-ex mice (26%) as compared to the EPOR-ex mice (10%). This increased cardiac adaptation was accompanied by myocyte hypertrophy and neovascularisation as shown with the increase in CSA and capillary-to-myocyte ratio in both cardiomyocytes as skeletal muscle. $EPOR^{-/-}$-rescued did not show this adaptation to increased workload. Furthermore, exercised WT mice show a shift in muscle fibre type to adapt to the increased workload (from type II to type I), whereas $EPOR^{-/-}$-rescued mice do not show this adaptation al all.

The development of $EPOR^{-/-}$-rescued mice by the group of Yamamoto provided an interesting model to study the role of EPO-EPOR signalling.[18] Where EPO and its receptor are indispensable to erythropoiesis, knock-out of the EPOR is lethal in utero. Exploiting a GATA-1 minigene cassette with haematopoietic regulatory domains, the group of Yamamoto established $EPOR^{-/-}$-rescued mice expressing EPOR exclusively in the haematopoietic lineage, but without expression of the EPOR in any other organ. Despite the lack of EPOR expression in non-haematopoietic tissues, these mice develop normally and are fertile.[18] The group of Shimokawa showed that $EPOR^{-/-}$-rescued mice have an impaired angiogenic response in models of maladaptive hypertrophy, such as transverse aortic constriction and myocardial infarction.[15-17] In the current study, we show that also in adaptive hypertrophy these mice have an impaired angiogenic response. The capillary-to-myocyte ratio is not significantly increased in both the left ventricle and in skeletal muscle, indicating the impaired angiogenic response.

A functional 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, skeletal muscle and the central nervous system.[25] In WT mice, normal changes are observed in left ventricular and skeletal muscle as a response to exercise, such as the increase in CSA, capillary-to-myocyte ratio and, for skeletal muscle, a shift in fibre type. The diminished

responses in the EPOR$^{-/-}$-rescued mice could indicate a vulnerable state of cardiomyocytes and skeletal muscle myocytes in EPOR$^{-/-}$-rescued mice, due to the absence of EPO-EPOR signalling. Skeletal muscle fibres can be categorised to the degree of oxidative capacity of the fibre. Type I fibres appear red due to the high content of the oxygen binding protein myoglobin and mitochondrial cytochromes. These fibres are suited for endurance, because they use predominantly oxidative metabolism to generate ATP. Type II fibres are white and rely predominantly on glycolysis for energy production. These fibres are efficient for short bursts of speed and power and use both oxidative metabolism and anaerobic metabolism depending on the particular sub-type. These fibres cannot maintain contractions for longer periods. Increase in exercise, as in the WT-ex mice, causes a shift from type II fibres to more type I fibres, to adapt to the increased exercise. The absence of the EPOR signalling in skeletal muscle in the EPOR$^{-/-}$-rescued mice does not cause a shift in muscle fibre type, as determined by NADH-TR staining. This in clear contrast to the WT, in which we did observe a clear increase in type I fibres required for endurance exercise. It might well be possible that EPOR signalling plays a crucial role in this shift and that absence of this shift in fibre type causes the decreased exercise capacity as compared to the WT-ex mice.

As markers of hypertrophic response to exercise, we measured ANP and ACTA1. Both are increased in hypertrophy in the left ventricle.[22,26] The differences in ANP and ACTA1 mRNA expression show that the cardiac adaptive response to exercise is less pronounced in the EPOR-ex group than in the WT-ex group. This is in concert with the much larger cardiac adaptation in the WT-ex group. MCIP-1 is a downstream target of calcineurin and the nuclear factor of activated T-cells (NFAT) pathway. NFAT is involved in the development of cardiac and skeletal muscle.[27] Activation of MCIP-1 in left ventricular tissue, and les pronounced in the skeletal muscle myocytes, in the WT-ex group strongly suggests that the NFAT pathway is activated. MCIP-1 inhibits NFAT in a negative feedback loop and suggests a cross talk between EPO and NFAT pathways.

We recently have shown that VEGF is crucial for EPO-induced improvement of cardiac function.[6] VEGF neutralisation resulted in reduced neovascularisation in a model of experimental heart failure, despite EPO treatment, suggesting that EPO exerts its effects partially via VEGF. The absence of VEGF-A increase in the EPOR$^{-/-}$-rescued mice after exercise is in concert with these data.

Analyses of metabolic makers, PPARα and MCAD, show that exercise has effect on the metabolism, as expected.[28] However, no clear differences were observed between the WT and EPOR$^{-/-}$-rescued mice and might not be the limiting factor in adaptive hypertrophy.

EPO is one of the most scrutinised doping drugs in endurance sports these days. Numerous professional athletes, most notably professional cyclists, have been using EPO as a doping drug for years, to enhance their endurance. It is thought that EPO, by its main erythropoietic effect, increases the number of erythrocytes, hence increasing the oxygen carrying capability of the blood en thereby have increased oxygen supply to the organs during endurance sports. This would only be the case with (very) high haematocrit values. Nowadays, haematocrit values are critically monitored; not only absolute levels, but also variation (biological passport) and

athletes with suspicious haematocrit values are not allowed to enter competition. However, up to date, no clear scientific evidence exists if EPO usage is actually useful. Furthermore, athletes who use EPO (or CEPO or ARA), only take low amounts of EPO (1000-4000 IU), not enough to increase haematocrit levels significantly.[29] Current anti-doping strategies with the use of biometrical passports clearly do not exclude the use of low-dose EPO. From our data, we postulate that it might well be possible that the supposed effects of EPO as a doping drug might not be the exclusively erythropoietic but rather the non-erythropoietic properties of EPO.

As this is an animal model, always precaution is needed to translate the current findings to the clinical situation. Furthermore, this is a model of voluntary wheel-running. Mice still could run less, not because of less exercise capacity, but of other mechanisms. To investigate whether the absence of the EPO-EPOR signalling could lead to dysfunction heart and muscles, prolonged or forced exercise, for example swimming, should be used. We conclude that the absence of EPO-EPOR signalling leads to a lesser exercise capacity upon voluntary wheel-running. We suggest that EPO-EPOR signalling is important for physiological adaptation during exercise.

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Supplemental data to "Erythropoietin receptor deficient mice have impaired exercise tolerance due to cardiac and muscle maladaptation"

SUPPLEMENTAL METHODS

Plasma EPO levels

Plasma EPO levels were analysed using a commercial Enzyme Linked Immunosorbent Assay according to the guidelines provided by the manufacturer (Mouse EPO Quantikine ELISA kit, #MEP00, R&D Systems Europe, Abingdon, UK). Samples were 2 times diluted and incubated for 2 hrs at room temperature. After several wash steps, substrate solution was added and incubated for 30 minutes at room temperature in the dark. After incubation, the reaction was stopped with specific stop solution and the optical density was read 450 nm, with a wavelength correction of 540 nm (Microplate Spectrophotometer, Bio-Rad Laboratories, Hercules, CA, USA). Plasma EPO levels were determined against a standard curve using specific software (Microplate manager 5.2.1, Bio-Rad Laboratories).

Supplemental table 1. List of primers used for real-time PCR

	Real-time PCR primer, 5' to 3'	
Gene name	**Forward**	**Reverse**
Cyclo G	CTT CAT ACC AGC GAC GAT TC	ATG TGG AGG AGT CTC ACT TC
GAPDH	CAT CAA GAA GGT GGT GAA GC	ACC ACC CTG TTG CTG TAG
ANP	ATG GGC TCC TTC TCC ATC AC	TCT ACC GGC ATC TTC TCC TC
ACTA1	TGC CAT GTA TGT GGC TAT CCA	TCC CCA GAA TCC AAC ACG AT
MCIP1	GCT TGA CTG AGA GAG CGA GTC	CCA CAC AAG CAA TCA GGG AGC
VEGF-A	ACT GGA CCC TGG CTT TAC TG	CAG TAG CTT CGC TGG TAG AC
PPARα	TAT TCG GCT GAA GCT GGT GTA C	CTG GCA TTT GTT CCG GTT CT
MCAD	GCA GCC AAT GAT GTG TGC TTA C	CAC CCT TCT TCT CTG CTT TGG T

GAPDH = Glyceraldehyde 3-phosphate dehydrogenase, ANP = atrial natriuretic peptide, ACTA1 = actin, alpha-1, skeletal muscle, MCIP-1 = myocyte-enriched calcineurin interacting protein-1, VEGF-A = vascular endothelial growth factor-A, PPARα = peroxisome proliferator activated receptor alpha, MCAD = acyl-CoA dehydrogenase.

5 CHAPTER

Apoptosis during CABG surgery with the use of cardiopulmonary bypass is prominent in ventricular but not in atrial myocardium

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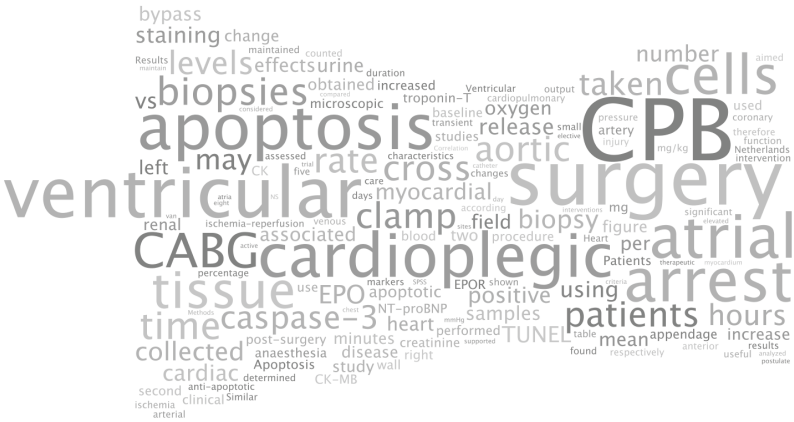

ABSTRACT

Objectives

We aimed to compare the rate of apoptosis after cardiopulmonary bypass (CPB) and cardioplegic arrest during coronary artery bypass grafting (CABG) surgery between atrial and ventricular tissue.

Methods

During CABG surgery with CPB and cardioplegic arrest, sequential biopsies were taken from the right atrial appendage and left ventricular anterior wall before CPB and after aortic cross clamp release. Change in number of apoptotic cells and biochemical markers of myocardial ischemia and renal dysfunction were assessed.

Results

CPB was associated with a transient small, but significant increase in CK (1091±374%), CK-MB (128±38%), troponin-T (102±13%) and NT-proBNP (1308±372%) levels (all: $p<0.05$). A higher number of apoptotic cells as assessed by caspase-3 staining was found in the ventricular biopsies taken after aortic cross clamp release compared to the biopsies taken before CPB (5.3±0.6 vs. 14.0±1.5 cells/microscopic field, $p<0.01$). The number of apoptotic cells in the atrial appendage was not altered during CPB. Correlation between the duration of aortic cross clamp time and the change in caspase-3 positive cells in the left ventricular wall was of borderline significance (r of 0.58, $p=0.08$). Similar results were obtained from TUNEL staining for apoptosis.

Conclusion

CABG surgery with CPB and cardioplegic arrest is associated with an elevated rate of apoptosis in ventricular but not in atrial myocardial tissue. Ventricular tissue may be more sensitive to detect changes than atrial tissue, and may be more useful to investigate the protective effects of therapeutic intervention.

INTRODUCTION

Coronary artery bypass grafting (CABG) surgery with cardiopulmonary bypass (CPB) and cardioplegic arrest is the most commonly used cardiac surgery procedure in the western world.[1] This procedure is associated with periods of oxygen deprivation and possibly a damaging effect to the heart. Restoration of coronary blood flow after release of the aortic cross clamp leads to ischemia-reperfusion injury. Subsequent production of reactive oxygen species result in cardiomyocyte death due to apoptosis.[2-4] These changes may cause (transient) cardiac dysfunction,[5,6] and therefore limiting the duration of cardioplegic arrest may reduce cell injury.

We hypothesised that CPB and cardioplegic arrest (in the setting of CABG) would cause considerable increase in number of apoptotic cells in the heart. We therefore studied the presence of apoptosis in hearts of patients undergoing CABG surgery with CPB and cardioplegic arrest and we aimed to compare the rate of apoptosis after CPB and cardioplegic arrest during CABG surgery between atrial and ventricular tissue.

METHODS

Patients

Patients were eligible for this study when aged between 18 and 80 years, when admitted for elective CABG surgery with CPB and cardioplegic arrest for the first time for two or more vessel coronary artery disease, and who met all the other inclusion and none of the exclusion criteria as listed in table 1. This study was approved by the Institutional Review Board of the University Medical Centre Groningen. Each participating patient has signed informed consent.

Anaesthesia

General anaesthesia was provided according to a fixed protocol.[7] Premedication consisted of oral diazepam 10-15 mg two hours preoperatively. After insertion of peripheral venous and arterial cannulae under local anaesthesia, general anaesthesia was induced with sufentanil 0.5-1 μg/kg and midazolam 0.05-0.1 mg/kg. Tracheal intubation was achieved with pancuronium 0.1 mg/kg and the lungs were ventilated with air and oxygen (fraction of inspired oxygen [FiO2] = 0.4). Anaesthesia was maintained with sufentanil, pancuronium and infusion of midazolam 0.1 mg/kg/hr. A flow-directed continuous cardiac output catheter was inserted in the right internal jugular vein, and an indwelling bladder catheter was used for urine collection. After induction of anaesthesia the patients received cefuroxim 1500 mg (clindamycin 600 mg when allergic to penicillin).

Fluid management: Hydroxyethyl starch 6% and saline were used to maintain mean arterial pressure >60 mmHg and to maintain filling pressure and cardiac output. Transfusion of packed RBC were administered at haemoglobin levels <5.0 mmol/l. According to standard care in our clinic intravenous insulin therapy was started at serum glucose levels >8.0 mmol/l. Before institution of CPB, activated clotting time of greater then 400 seconds was produced by administration of heparin (starting dose 3 mg/kg). Patient characteristics were recorded prospectively. Diuretics, mannitol and aprotinin were not used during the entire study period.

CPB: CPB was provided using a Stockert roller pump with an open reservoir, a hollow fibre oxygenator (Cobe optima), an arterial filter (Affinity, Medtronic) and an inline blood monitoring system (CDI500, Terumo). The CPB flow was maintained at 2.4 l/min/m^2 and the circuit was primed with 1000 ml Ringers-lactate, 500 ml Haes 200/0.5 10% and 50 mg heparin. Nasopharyngeal temperature was lowered to 32°C. MAP was maintained between 60 and 90 mmHg. Deviations were corrected with phenylephrin and nitroglycerin.

Surgery and biopsies

Surgery was performed by experienced cardiothoracic surgeons. All patients underwent CABG surgery with mild hypothermia (32-34°C). Cardioplegia in all patients was standardized to the crystalloid St. Thomas solution. The atrial biopsies were taken from the right atrial appendage and closed with prolene 4.0. Care was taken not to squeeze the biopsy places with forceps. Ventricular tissue samples were collected from the left anterior ventricular wall parallel to the left anterior descending artery, each from separate sites. Under an angle of approximately 30 degrees using a 24 Gauge tru-cut needle ventricular biopsies were taken. Part was put in 4% paraformaldehyde for 24 hrs, after which it was processed for paraffin embedding for immunohistological assessment. The second part was snap frozen in liquid nitrogen for RNA isolation. The ventricular biopsy site was closed using prolene 4.0 suture with teflon pledges. Before closure of the chest, biopsy sites were checked for bleeding.

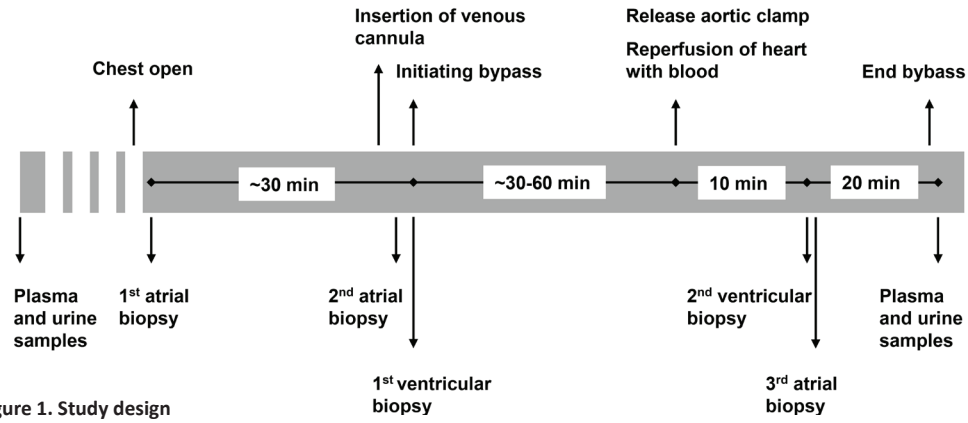

Figure 1. Study design

The figure shows the study design as described in the text.

Study design and mode of sampling biopsies

Tissue samples were collected at three different time points during surgery (figure 1). Immediately after opening the chest, a baseline atrial biopsy was collected. During placement of the venous cannula (20 to 40 minutes later) a second atrial biopsy was collected. Immediately after initiation of bypass the first ventricular biopsy was obtained. Ten minutes after aortic cross clamp release, the second ventricular biopsy was taken. Finally, a third atrial biopsy was obtained immediately after harvesting the second ventricular tissue sample. Additionally plasma and urine was collected for up to five days post-procedure to examine release of markers of both myocardial ischemia and of renal function.

Monitoring and measurements during surgery

Blood pressure, heart rate, electrocardiogram, cardiac output and mixed venous oxygen saturation were monitored according to routine clinical practice. Blood and urine samples were collected at different time point. One day before surgery, blood and urine was collected for screening. Furthermore, pre-surgery, post-surgery, and at 2, 5, 12, 24, 48, 72 and 120 hours after surgery, blood and urine samples were collected. Markers of myocardial ischemia (creatinine kinase (CK), CK-myoglobin (CK-MB), troponin-T, N-terminal-pro Brain Natriuretic Peptide (NT-proBNP)) and renal function (creatinine) were determined. In urine samples microalbiminuria and creatinine was determined. The estimated glomerular filtration rate (eGFR) was calculated using the modification of diet in renal disease (MDRD) formula. All markers were analysed in the hospital laboratory.

Histology

For apoptosis detection, we stained for caspase-3 and TUNEL. Formalin fixed paraffin sections were incubated with an antibody that specifically recognizes the active form of caspase-3 as previously reported.[8,9] TUNEL staining was performed as previously described.[8] For quantitative analysis, active caspase-3 positive cells or TUNEL-positive cells were counted in six random fields per section (80-120 cells per field) counted at high-power magnification (40x).

Statistics

Results are reported as mean ± standard error of the mean (SEM) or otherwise if stated. Because of small samples sizes, comparisons of differences between groups were analysed by Wilcoxon or Friedman test when appropriate. The correlation between aortic cross clamp time and the rate of apoptosis in ventricular tissue was assessed using Spearman coefficient. A p-value of less than 0.05 was considered significant. All analyses were performed using SPSS version 16.0 software (SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

We included ten patients, undergoing elective CABG surgery with CPB and cardioplegic arrest for 2- and 3-vessel disease. All patients were male, and their age ranged from 39 to 73 years. Two patients had 2-vessel disease, and eight patients had 3-vessel disease. Baseline characteristics are summarized in table 1. Mean duration of the complete procedure was 222±19 minutes with a mean perfusion time of 80±80 minutes and a mean aortic cross clamp time of 46±5 minutes. All patients recovered well from their surgery. No rethoracotomies for postoperative bleeding were performed. Post-operative atrial fibrillation was seen in two patients. Average stay after surgery at the intensive care unit was 1.2 days. Patients were discharged from the hospital at a mean of eight days after surgery.

Table 1. Inclusion and exclusion criteria

Inclusion criteria
Accepted for CABG surgery with CBP and cardioplegic arrest for 2- and 3-vessel coronary artery disease
Primary CABG-surgery
Haemoglobin concentration ≥7.2 mmol/l and ≤9.7 mmol/l
Man or woman
between 18 to 80 years of age
Exclusion criteria
Off-pump CABG surgery
Previous MI of the anterior wall
LVEF ≤40%
Chronic kidney disease (serum creatinine >106 mmol/ml for all females, >133 mmol/ml for black males, >115 mmol/ml for non-black males
Atrial fibrillation
Grand mal seizure within 1 year of enrolment
Malignant hypertension
Previous treatment with rhEPO
Blood transfusion <12 weeks before randomisation
Polycythaemia vera
Pregnancy/breast feeding
Severe valvular disease (including pulmonary and tricuspid) or left ventricular outflow obstruction, which requires surgery
Pulmonary hypertension
Concomitant inflammatory or malignant disease
Presence of other serious medical conditions
Participation in any investigational device or drug trial(s) or receiving other investigational agent(s) within 30 days prior to enrolment.

CABG = coronary artery bypass graft surgery, CPB = cardiopulmonary bypass, MI = myocardial infarction, LVEF = Left ventricular ejection fraction, rhEPO = recombinant human erythropoietin.

Apoptosis in atria and ventricles

Figure 2A and B show the rate of apoptosis in right atrial appendage and left ventricular wall as determined by caspase-3 and TUNEL staining. In the left ventricle, significantly more caspase-3 positive cells were counted in the biopsies taken after aortic cross clamp release compared to the number of caspase-3 positive cells found in the biopsies taken before the start of CPB (5.3±0.6 vs. 14.0±1.5 cells per microscopic field, $p<0.01$). Similar results were obtained from TUNEL staining (32.5±4.0 vs. 44.9±4.6 cells per microscopic field, $p<0.05$). In the atria, the number of caspase-3 positive cells was not altered during surgery (1.4±0.3 vs. 1.9±0.5 vs. 1.8±0.6 cells per microscopic field; p=NS). This was confirmed by TUNEL staining (23.1±4.1 vs. 22.2±2.1 vs. 25.3±3.8 cells per microscopic field; p=NS). Correlation between aortic cross clamp time and the change in ventricular caspase-3 positive cells showed a trend towards a positive correlation with an r of 0.58 (p=0.08; figure 2F). Similar results were obtained from the TUNEL staining (data not shown).

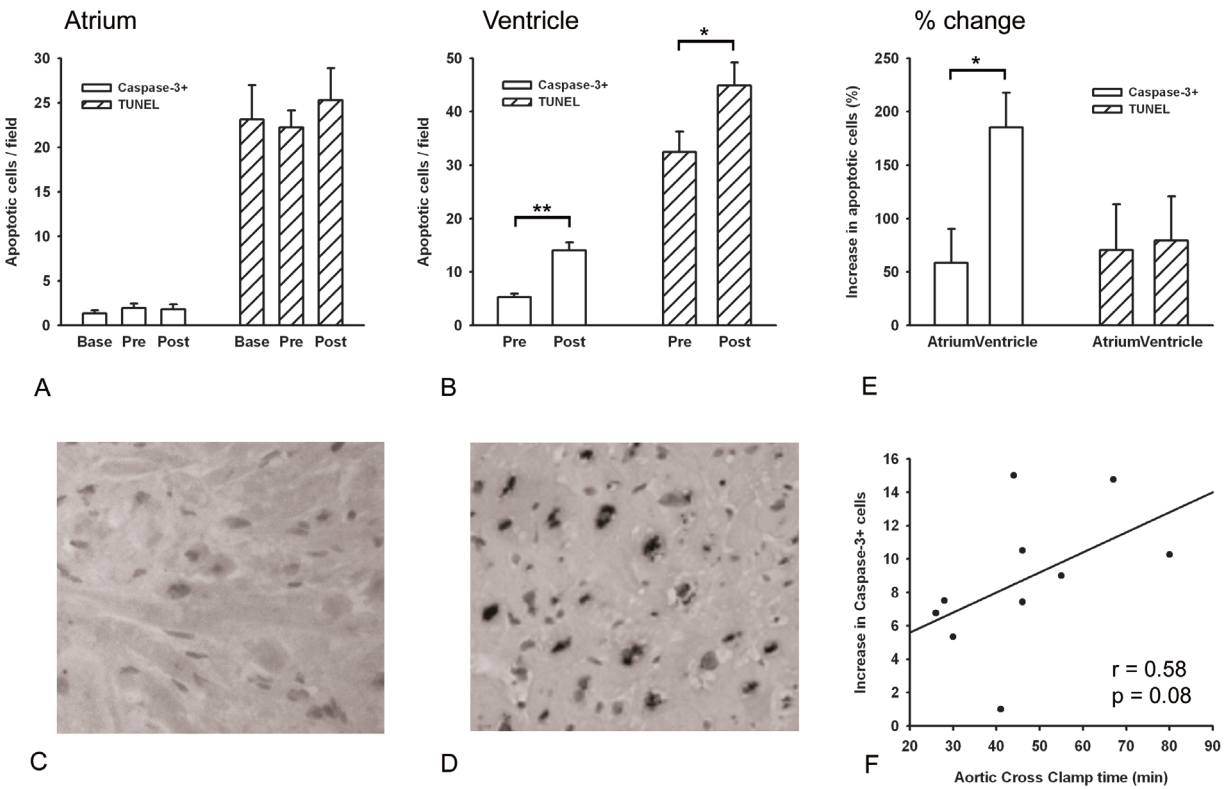

Figure 2. Number of apoptotic cells during cardiopulmonary bypass

A: number of caspase-3+ and TUNEL+ cells in the atria. B: number of caspase-3+ and TUNEL+ cells in the ventricles. C and D: immunohistological examples of caspase-3+ staining in atria (C) and ventricles (D). E: percentage change in caspase-3+ and TUNEL+ cells during cardiopulmonary bypass in atria and ventricles. F: linear regression of aortic cross clamp time and change in caspase-3+ cells in the ventricles. Base = baseline: atrial biopsy taken after opening chest, pre = biopsies taken during placement of the venous cannula (for atrial biopsy) and just after going on cardiopulmonary bypass (for ventricular biopsy), post = biopsies taken 10 minutes after aortic cross clamp release. * $p<0.05$, ** $p<0.01$. For colour figure, see supplement 1.

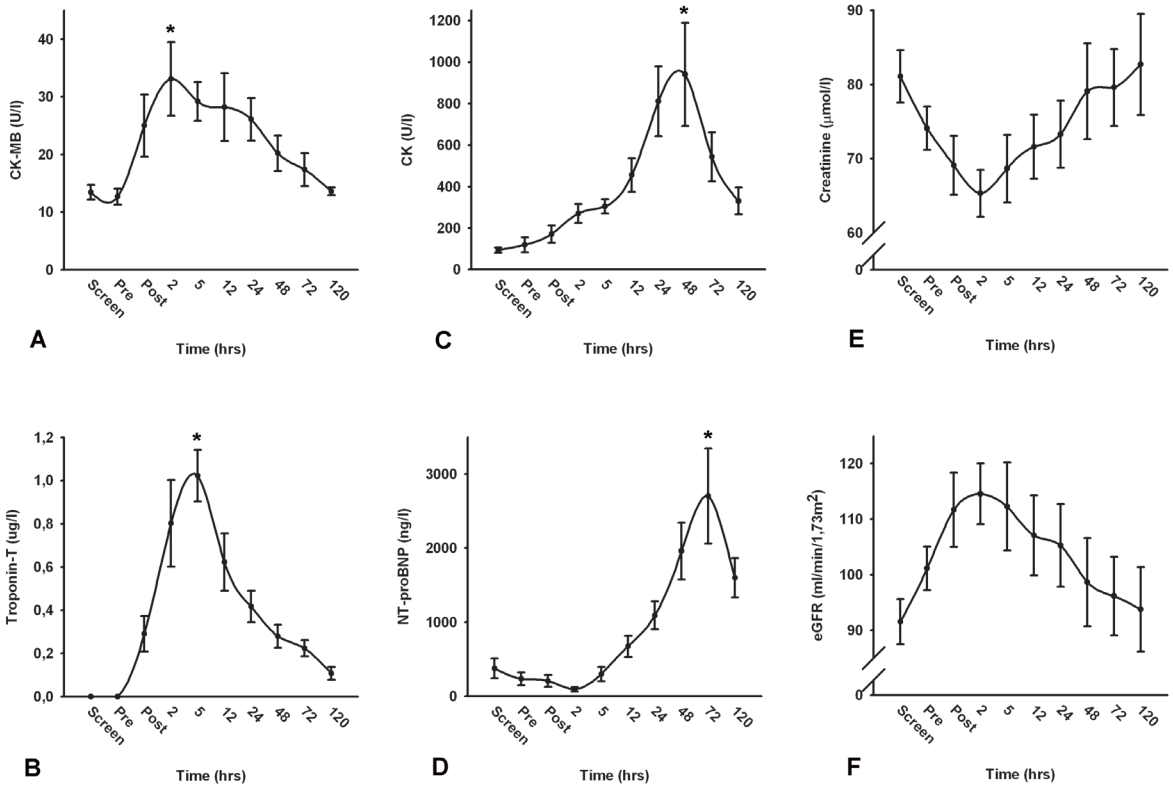

Figure 3. Time course of markers of myocardial ischemia and renal function

A: time course of creatinine kinase-myoglobin (CK-MB) pre- and post-surgery. B: time course of troponin-T pre- and post-surgery. C: time course of creatinine kinase (CK) pre- and post-surgery. D: development of NT-proBNP pre- and post-surgery. E: time course of creatinine pre- and post-surgery. F: time course of eGFR (estimated glomerular filtration rate) pre- and post-surgery. Screen: values during screening, pre = values directly before surgery, post = values during closing chest. * $p<0.05$ vs. screening values.

Cardiac enzymes and renal function

CPB was associated with a transient small, but significant increase in CK, CK-MB, troponin-T and NT-proBNP levels (all: $p<0.05$). CK-MB levels and troponin-T levels reached their maximum after two and five hours, respectively, and returned to baseline within 120 hours post-surgery (percentage change: 128±38% and 102±13%, respectively; figure 3A-B). CK levels peaked at 48 hours post-surgery (percentage change: 1091±374%). Maximum NT-proBNP levels were found at 72 hours post-CABG (percentage change: 1308±372%; figure 3C-D). Serum creatinine levels decreased shortly after the procedure and returned to baseline after 120 hours. Accordingly, eGFR levels were higher at two hours post-surgery with a return to baseline at day five (figure 3E-F). Other measurements showed no or very little fluctuation in time.

DISCUSSION

We demonstrate that CABG surgery with CPB and cardioplegic arrest causes an increased rate of apoptosis in ventricular but not in atrial myocardial tissue. Thus it seems that ventricular myocardium is more vulnerable to cardioplegic arrest. In addition, our data suggests that studies with pharmacological interventions aimed at salvaging apoptosis during cardioplegic arrest should use ventricular biopsies rather than atrial biopsies.

Different studies use either atrial or ventricular tissue to show apoptosis,[10-12] but no comparison has been made. Our study shows clear differences in the rate of apoptosis between ventricular and atrial myocardial tissue. Furthermore, manipulating myocardial tissue, for example with forceps, induces apoptosis. In our study, care was taken, to use only biopsies of non-manipulated tissue to avoid induction of apoptosis by manipulation.

Apoptosis has been considered as one of the mechanisms of cardiomyocyte loss during CPB and cardioplegic arrest, like in CABG surgery.[13,14] Apoptosis during CPB and cardioplegic arrest can be triggered by several mechanisms, including ischemia-reperfusion injury,[15] and release of cytokines and inflammatory factors.[16] Longer aortic cross clamp time correlates, as shown in this and other studies, with an increase in number of apoptotic cells.[17] This increased loss of cells might contribute to an impaired contractility of the heart.[18] Several strategies are being tested to ensure a lower rate of apoptosis, by reducing aortic cross clamp time as much as possible, or using cytoprotective agents against ischemia-reperfusion injury.[19,20]

Prevention of apoptosis during CPB: proposal for an intervention trial

We postulate that apoptosis during CABG surgery is a maladaptive effect of CPB and cardioplegic arrest and might be amendable for intervention. Under normal circumstances EPO and EPOR have a relatively low expression in non-haematopoietic tissue.[21] However, expression of EPO and EPOR is rapidly increased in response to hypoxia and increased reactive oxygen species.[22,23] Numerous studies have shown that EPO has cytoprotective effects,[24-26] and EPOR signalling pathways are associated with cell survival.[27-30] Since EPO reduces apoptosis, we postulate that EPO administration may have salutary effects on the heart in the setting of cardiac surgery employing CPB and cardioplegic arrest. We therefore started a prospective randomised clinical trial in which patients are randomised to EPO (60.000 IU) or placebo (clinical trials protocol ID

NCT00524901) before cardiac surgery employing CPB and cardioplegic arrest, which aims to prove the anti-apoptotic effects of EPO. Patients are included according to the inclusion and exclusion criteria summarised in table 1.

Conclusions

We conclude that CABG surgery with CPB and cardioplegic arrest is associated with an elevated rate of apoptosis in ventricular tissue. Ventricular tissue may be more sensitive to detect changes than atrial tissue, and may be more useful to investigate the protective effects of therapeutic interventions. We speculate that the observed increase in apoptosis may be amendable to ant-ischemic and anti-apoptotic agents. EPO may be useful in this respect, as it has established anti-apoptotic effects and the EPOR is expressed in the heart.

Acknowledgements

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Table 2. Baseline characteristics

	N=10 (ave±SEM)
General	
Age (yrs)	60±3.4
Sex (% male)	100
Race (Caucasian, %)	100
No. of diseased vessels	2.8±0.1
Height (cm)	177±1.8
Weight (kg)	85.1±2.7
BMI (kg/mm^2)	27±0.5
RR (mmHg)	146/73±6/3
HR (bpm)	70±5
Medical history	
Previous myocardial infarction (%)	20
Previous CABG surgery (%)	0
Previous PCI (%)	20
Diabetes mellitus (%)	40
History of hypertension (%)	70
Obesity (%)	80
History of hypercholesterolemia (%)	20
Smoking (%)	10
Family history [(a)]	40
Renal dysfunction	0
Medication	
Beta-blocker (%)	100
Ace-inhibitor (%)	20
Statin (%)	80
Diuretic (%)	10
Nitrate (%)	20
Calcium antagonist (%)	30
AT1-antagonist (%)	30
Acetylsalicylic acid (%)	100
Other anticoagulants (%)	10

Ave = average, SEM = standard error of the mean, BMI = body mass index, RR = blood pressure, HR = heart rate, CABG = coronary artery bypass graft surgery, PCI = percutaneous coronary intervention, AT1-antagonist = type I angiotensin II receptor antagonist, bpm = beats per minute.

(a) a positive family history was defined by the presence of a composite endpoint (myocardial infarction, revascularisation, chronic heart failure, stroke and death) in first degree family members before the age of 50 (males) or 60 (females).

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PART

2

Downstream pathways of erythropoietin:
Feasible targets for intervention

6 CHAPTER

Estradiol-induced, endothelial progenitor cell-mediated neovascularisation in male mice with hind limb ischemia

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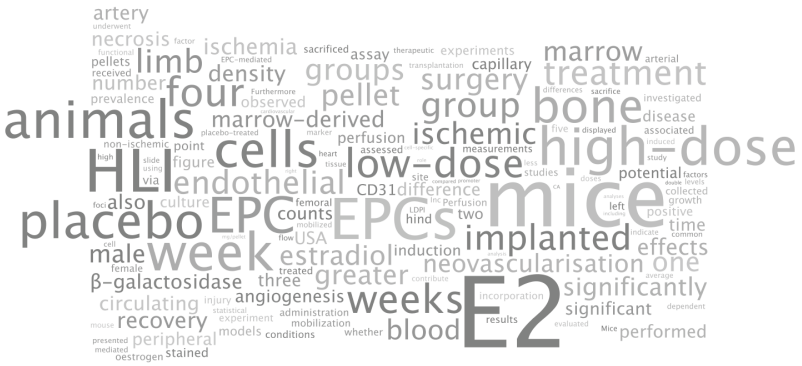

ABSTRACT

We investigated whether administration of estradiol to male mice augments mobilisation of bone marrow-derived endothelial progenitor cells (EPC) and incorporation into foci of neovascularisation after hind limb ischemia, thereby contributing to blood flow restoration. Mice were randomised and implanted with placebo pellets or pellets containing low-dose estradiol (0.39 mg) or high-dose estradiol (1.7 mg). Hind limb ischemia was induced by unilateral resection of the left femoral artery one week after pellet implantation, then EPC mobilisation and functional recovery was evaluated. EPC recruitment was assessed in mice transplanted with bone marrow from transgenic donors expressing β-galactosidase driven by the Tie-2 promoter. EPC culture assay performed two weeks after pellet implantation revealed a significantly greater ($p<0.05$) number of circulating EPCs in the high-dose estradiol group than in the low-dose estradiol and placebo groups. At three and four weeks after induction of hind limb ischemia, perfusion was significantly greater ($p<0.05$) in high-dose estradiol mice than in mice implanted with the low-dose estradiol or placebo pellets. At one and four weeks after hind limb ischemia surgery, more bone marrow-derived EPCs, identified as β-galactosidase-positive cells, were observed in ischemic regions from high-dose estradiol animals than in low-dose ($p < 0.05$) or placebo groups ($p<0.05$). These results indicate that estradiol dose-dependently increases the levels of EPCs in peripheral blood in male animals, improves the recovery of blood flow, and decreases limb necrosis after hind limb ischemia, and that this enhancement occurs, in part, through augmentation of EPC mobilisation and greater incorporation of bone marrow-derived EPCs into foci of neovascularisation.

INTRODUCTION

Since the discovery of endothelial progenitor cells (EPCs) in 1997,[1] many studies have investigated their potential angiogenic capacity.[2-6] The relationships between EPCs and various growth factors, cytokines, and hormones, including estrogen,[7] have been studied in an effort to identify their potential for therapeutic angiogenesis. The emerging consensus of these studies is that blood-vessel regeneration evolves not only from the migration and proliferation of endothelial cells adjacent to the site of injury (a process called angiogenesis), but that circulating EPCs also contribute to the formation of new blood vessels.[1,8-10] The mechanisms that regulate EPC-mediated neovascularisation are slowly being characterised.[5,11,12]

Oestrogen regulates angiogenesis by influencing the activity of endothelial cells. Under normal physiological conditions, angiogenesis in the uterus is associated with fluctuations of circulating estradiol (E2) and other sex steroids.[13-17] The well established link between oestrogen and breast tumour invasiveness also suggests that oestrogen exerts a pro-angiogenic effect because the formation of metastases is dependent on angiogenesis.[18] Studies in mice have revealed that E2 accelerates endothelial recovery after arterial injury,[19,20] and E2 has been shown to prevent endothelial cell apoptosis [5,21,22] and to exert other positive effects on mature endothelial cells.

To date, experiments in animal models that have investigated the effects of E2 on EPCs have been performed exclusively in female animals,[5,12,22] and it is not known whether these results can be extrapolated to males. Furthermore, the potential contribution of EPC-mediated neovascularisation to functional recovery at the ischemic site continues to be debated. This study sought to determine whether estrogen mobilises EPCs from the bone marrow after hind limb ischemia (HLI) in male animals, and whether the mobilised EPCs subsequently contribute to post-ischemic neovascularisation.

METHODS

Animal models

All experiments were conducted according to protocols approved by the Animal Care and Use Committee of St Elizabeth's Medical Centre, Boston, MA and conform the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health. Experiments were performed in male FVB wild type (WT) mice (Jackson Laboratory, Bar Harbor, ME, USA), aged six weeks. HLI was induced by unilateral resection of the left femoral artery from the proximal end of the femoral artery up to the distal portion of the saphenous vein. The femoral artery and all side-branches were dissected and excised, then the overlying skin was closed using a surgical stapler. After surgery, the mice were kept on a warm plate until fully awake. For bone marrow transplant experiments, mice were irradiated (16 G) to destroy endogenous bone marrow and transplanted with a minimum of 3×10^6 bone marrow cells from age-matched male transgenic donors that expressed β-galactosidase; β-galactosidase transcription was regulated by the endothelial cell-specific Tie-2 promoter. Mice were anaesthetised by intraperitoneal injection with a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg) and sacrificed with an overdose of the same anaesthetic. Animals were housed under standard conditions with *ad libitum* food and water. Over the duration of the experiments, the mortality rate was 6% for intact mice,

10% for mice that underwent HLI surgery alone, and 33% for mice that underwent both bone marrow transplantation and HLI surgery.

Design

Three experiments were performed. For each experiment, non-castrated WT male mice were implanted with 90-day release E2 pellets containing high (1.7 mg/pellet) or low (0.39 mg/pellet) doses of E2 (17β-estradiol), or placebo (Innovative Research of America, Sarasota, FL, USA). In a previous study of experimental carotid artery injury, serum E2 levels were approximately 300-fold greater in ovariectomised female mice implanted with the high-dose E2 pellet than in mice implanted with the placebo pellet.[5] Pellets were implanted subcutaneously as described elsewhere.[12]

The first experiment was performed with 60 mice (n=20 in each treatment group). Mice were implanted at week 0, and five mice from each group were sacrificed at weekly intervals over the next four weeks. Blood was collected by intracardiac puncture, and the number of circulating EPCs was assessed via EPC culture assay.

In the second experiment, 60 mice (n=20 in each treatment group) were implanted at week –1, HLI was induced at week 0, and five mice from each group were sacrificed at weekly intervals over the next four weeks. At sacrifice, all ischemic, non-necrotic tissue was collected and stained with fluorescent CD31 antibodies to assess capillary density; blood was collected by intracardiac puncture, and the number of circulating EPCs was assessed via EPC culture assay. Functional recovery was evaluated from Laser Doppler Perfusion Imaging (LDPI) measurements taken before HLI surgery and at sacrifice, and limb necrosis was assessed visually as previously described.[23]

For the third experiment, 42 mice underwent bone marrow transplantation surgery at week –5, and the bone marrow was allowed to regenerate over four weeks. Surviving mice (n=14 in each treatment group) were implanted at week –1, HLI surgery was performed at week 0, and mice were sacrificed at week one and week four. After sacrifice, all ischemic, non-necrotic tissue was collected and stained for expression of β-galactosidase (a specific marker for cells derived from donor bone marrow) and CD31 (a specific marker for endothelial cells) to identify bone marrow-derived EPCs at the sites of neovascularisation.

EPC culture assay

Total mononuclear cells were isolated from 1 ml of arterial blood by density gradient centrifugation using a Histopaque-1083 (Sigma-Aldrich Inc., St Louis, MO, USA), then cultured in phenol redfree endothelial cell basal medium (Clonetics Corp., San Diego, CA, USA) supplemented with 5% fetal bovine serum, antibiotics, and growth factors on four-well glass slides coated with rat plasma vitronectin (Sigma-Aldrich Inc.) in 0.5% gelatine solution. After four days, EPCs were recognized as attached spindle shaped cells and assayed by co-staining with acetylated LDL-DiI (Biomedical Technologies, Stoughton, MA, USA) and FITC-conjugated Bandeiraea simplicifolia lectin I (Vector Laboratories, Burlingame, CA, USA). Fifteen randomly selected fields on each slide were viewed with fluorescence microscopy at 20× magnification, and double-positive cells were identified as EPCs and counted;[24] EPC counts were presented as the average number of EPCs/mm^2.

Perfusion

Perfusion in both ischemic (left) and non-ischemic (right) limbs was quantified via LDPI (Moor Instruments Inc, Wilmington, DE, USA) before HLI surgery and at predetermined time points afterward. Three consecutive measurements were obtained over the affected region (leg and foot) for each time point. Colour-coded images were recorded, and the average perfusion in each (i.e. ischemic and non ischemic) foot was calculated. To account for variations in ambient light, temperature, and other conditions, perfusion was presented as the ratio of ischemic (left) to non-ischemic (right) measurements.

Capillary density

Tissue samples were mounted and stained for CD31 expression, and one slide for each mouse was examined with light microscopy at 20× magnification. Capillaries positively stained for CD31 (an endothelial cell-specific marker) were counted in five randomly chosen microscopic fields on each slide, and capillary density was presented as the average number of capillaries/ mm^2.

Statistics

Results are reported as mean ± standard error of the mean (SEM), and statistical analyses were performed with SPSS 12.0 for Windows. Comparisons between groups were analysed via ANOVA with Dunnett's correction, and a p-value of less than 0.05 was considered significant.

RESULTS

Circulating EPC counts

Within the low- and high-dose E2 treatment groups, mice with HLI displayed significantly greater EPC counts at all weeks than those measured in mice that did not undergo HLI surgery ($p<0.05$); EPC counts in animals implanted with the high-dose E2 pellet peaked at week two in both the presence and absence of HLI. In the absence of HLI, the number of circulating EPCs at weeks one and two was significantly higher in animals treated with the high-dose E2 pellet than in the low-dose ($p<0.05$) or placebo treatment groups ($p<0.05$) (figure 1A), and the difference between the high-dose group and the placebo group remained significant at week three ($p<0.05$); there was no significant difference between EPC counts in the low-dose and placebo groups at any time point. After induction of HLI, EPC counts were significantly greater in mice that had received the high-dose pellet than in placebo treated mice at week two ($p<0.05$ vs. placebo) and week three ($p<0.01$ for high-dose and $p<0.05$ for low-dose vs. placebo); EPC counts in the high-dose animals were greater than those in the low-dose group, but the difference was not statistically significant (figure 1B).

Perfusion and limb necrosis

At three and four weeks after HLI surgery, high-dose E2 treatment was associated with significantly greater restoration of perfusion than was observed in animals implanted with either the low-dose E2 or placebo pellets (figure 2B). The ratio of perfusion in the ischemic and non-ischemic limbs was 0.82±0.03 in week three and 0.88±0.11 in week four among animals

that received the high-dose E2 pellet, compared to 0.44±0.03 ($p<0.01$) and 0.47±0.01 ($p<0.05$), respectively, in animals that received the placebo pellet. The differences between low-dose E2 and placebo treatment were not significant at any time point. High-dose E2 treatment was also associated with a lower proportion of limb necrosis at week four (0%) than was observed in animals implanted with the placebo pellet (50%); limb necrosis in the low-dose E2 group was 22%.

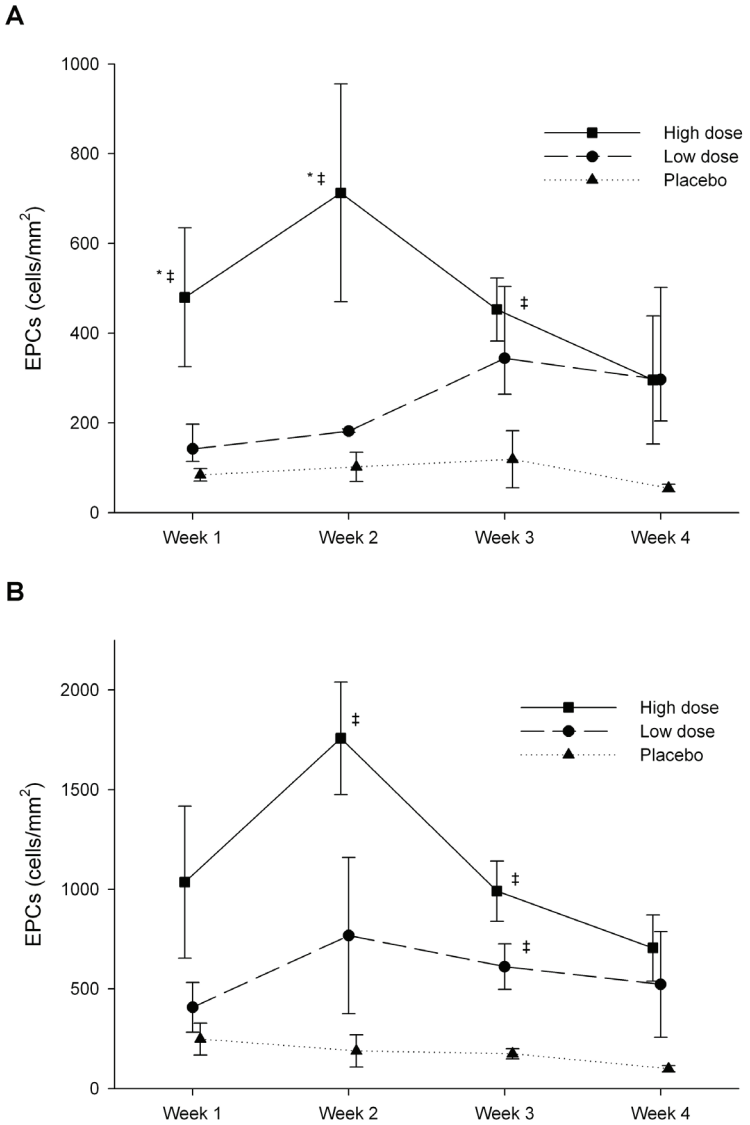

Figure 1. Endothelial progenitor cell (EPC) counts in mice implanted with pellets containing 1.7 mg estradiol (high dose), 0.39 mg estradiol (low dose), or placebo. (A) Intact mice were implanted at week 0 and EPC culture assay was performed one to four weeks later. (B) Mice were implanted at week –1, hind-limb ischemia was induced at week 0, and EPC culture assay was performed one to four weeks later. * $p<0.05$ vs. low-dose group, ‡ $p<0.05$ vs. placebo group.

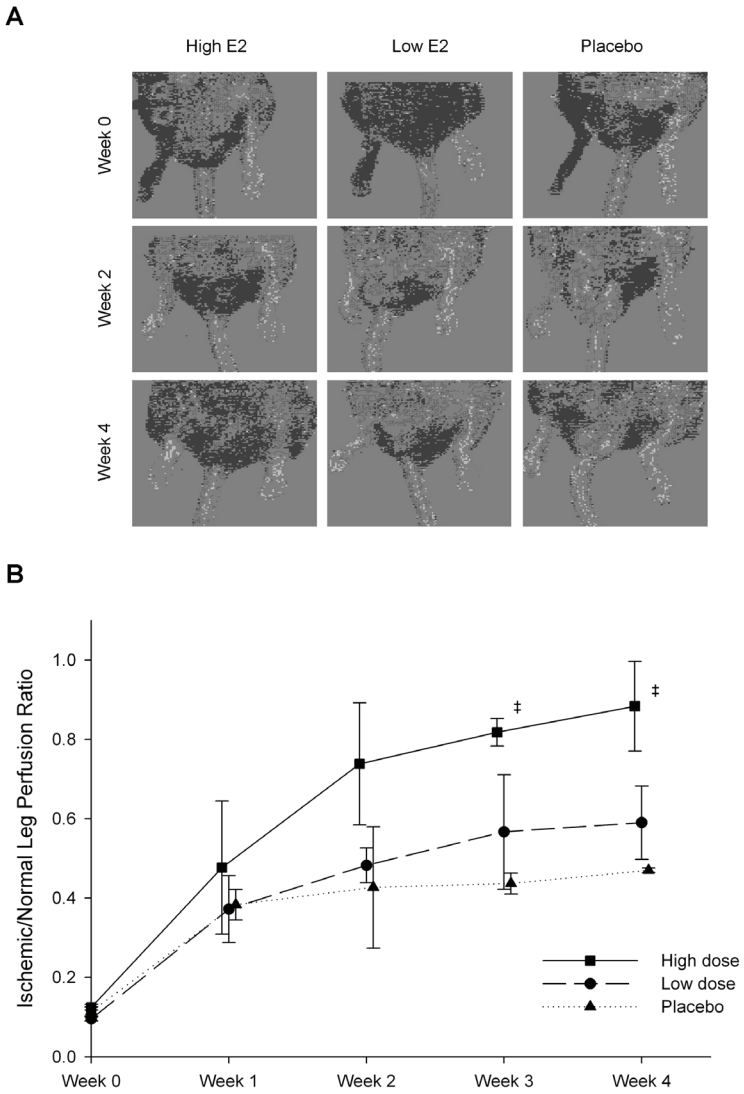

Figure 2. Recovery of perfusion in mice implanted with pellets containing 1.7 mg estradiol (high-dose), 0.39 mg estradiol (low-dose), or placebo. Mice were implanted at week -1, hind-limb ischemia was induced at week 0, and laser Doppler perfusion imaging (LDPI) was performed one to four weeks later. (A) Representative LDPI images for each treatment group at various time points; severely restricted limb blood flow is indicated in blue, and red indicates normal limb perfusion. (B) The ratio of perfusion recorded in the ischemic and normal limbs. ‡ $p<0.05$ vs. placebo group. For colour figure, see supplement 1.

Capillary density

At two, three and four weeks after induction of HLI, capillary density was significantly higher ($p<0.05$) in mice treated with a high-dose of E2 than in animals implanted with the placebo pellet (figure 3), and the differences between the high-dose and low-dose E2 treatment groups at weeks two and four were also significant ($p<0.05$).

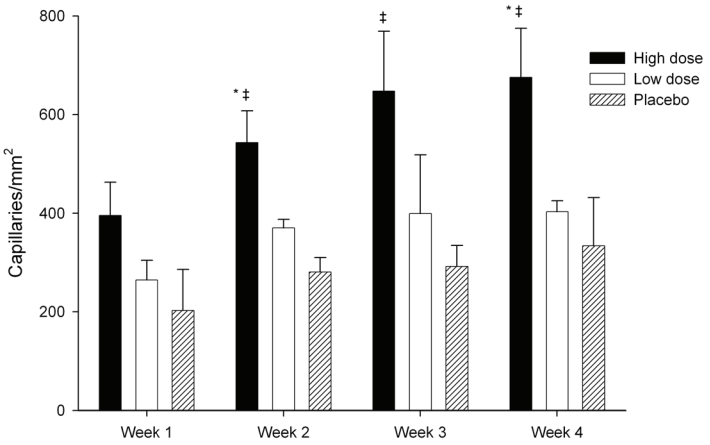

Figure 3. Capillary density in mice implanted with pellets containing 1.7 mg estradiol (high-dose), 0.39 mg estradiol (low-dose), or placebo. Mice were implanted at week –1, and hind-limb ischemia was induced at week 0. Tissue samples were obtained from mice sacrificed one to four weeks later and stained for expression of CD31 (an endothelial cell-specific marker). * $p<0.05$ vs. low-dose group, ‡ $p<0.05$ vs. placebo group.

Bone marrow-derived cells

At one and four weeks after induction of HLI in mice that had undergone bone marrow transplantation surgery, animals implanted with the high-dose E2 pellet displayed significantly more β-galactosidase positive (i.e. bone marrow-derived) cells at the site of ischemia than were observed in either the low-dose group or placebo-treated animals (figure 4A); the difference between the low-dose E2 and placebo-treatment groups was significant at week one. The number of β-galactosidase-positive cells at the site of ischemia declined between weeks one and four in all treatment groups.

Cells that stained positive for both β-galactosidase and CD31 (representative of bone marrow-derived capillary density) were significantly more common one week after induction of HLI in mice treated with a high-dose of E2 than in low-dose E2 or placebo-treated animals, but this difference abated by week four, when the prevalence of double positive cells was less than 25 cells/mm^2 in all groups (figure 4B). The prevalence of double positive cells did not differ significantly between the low-dose E2 and placebo groups.

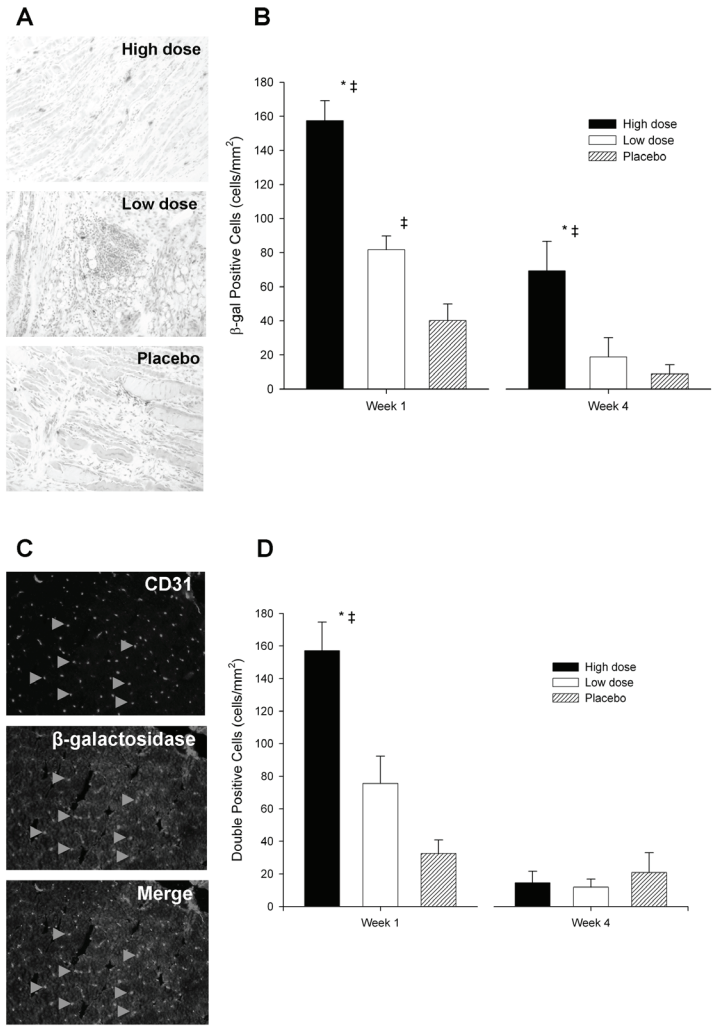

Figure 4. Incorporation of bone marrow-derived endothelial cells at the sites of neovascularisation in mice implanted with pellets containing 1.7 mg estradiol (high-dose), 0.39 mg estradiol (low-dose), or placebo. Mice underwent transplantation surgery at week –5, were implanted at week –1, and hind-limb ischemia was induced at week 0. Tissue samples were obtained from mice sacrificed one and four weeks later and stained for expression of CD31 (an endothelial cellspecific marker) and β-galactosidase (a specific marker for cells derived from donor bone marrow). (A) Representative slides quantitatively stained for β-galactosidase expression; positively stained cells are blue. (B) The prevalence of cells stained positive for β-galactosidase expression. (C) Representative slides quantitatively stained for CD31 and β-galactosidase expression; the three panels display (from top to bottom) endothelial cells (green), bone marrowderived cells (red), and bone marrow-derived endothelial cells (yellow). (D) The prevalence of cells stained positive for both CD31 and β-galactosidase expression. * $p<0.05$ vs. low-dose group, ‡ $p<0.05$ vs. placebo group. For colour figure, see supplement 1.

DISCUSSION

Peripheral artery disease affects 8-12 million people in the United States.[25,26] The prevalence of peripheral artery disease is lower in premenopausal women than in men,[27] and one factor believed to contribute to this difference is hormonal status. The potential protective role of E2 in peripheral and cardiovascular disease is implied by results in animal models and findings from extensive observational human studies.[28-30] Administration of E2 yields profound effects in both ischemia-reperfusion and chronic heart failure models,[31,32] and high doses of E2 attenuated the development of heart failure in a rat model of myocardial infarction.[33,34] The acute protective effects of E2 treatment likely evolve, at least in part, through the inhibition of apoptosis.[21,35]

To date, only female animals have been used in animal-model investigations of the potential beneficial effects of E2 for peripheral arterial disease, so we sought to extend these studies to male animals by administering E2 to male mice with HLI. Surgical induction of HLI is a common and reliable method for modelling peripheral arterial disease,[36-40] and because ischemia occurs in the limb, rather than in the heart, functional recovery can be easily monitored by using LDPI to measure blood flow. Functional data is much more difficult to obtain in models of cardiovascular diseases such as myocardial ischemia-reperfusion injury.

Our results indicate that the beneficial effects of E2 administration observed in female animals with HLI are reproducible in males. Four weeks after HLI surgery, mice administered high doses of E2 had recovered nearly 90% of non-ischemic perfusion and displayed no necrosis, compared to 47% recovery and 50% necrosis in placebo-treated animals; capillary density was also greater with high-dose E2 treatment than with placebo. Furthermore, the effects appeared to be mediated by EPCs; E2 administration dose dependently mobilised EPCs from the bone marrow, and the mobilised EPCs participated in neovascularisation. The prevalence of bone marrow-derived cells in the ischemic region was greater in animals that received the high-dose E2 pellet than in the placebo group at weeks one and four, and cells expressing both β-galactosidase and CD31 were more common in the high-dose group at week one. The difference in double-positive cell prevalence abated by week four because the activity of β-galactosidase decreased over time.

The involvement of EPCs in angiogenesis has been a popular topic of research, and many studies indicate that strategies involving the use of EPCs for therapeutic angiogenesis are feasible. Many growth factors, cytokines, and hormones can influence EPC mobilisation and homing,[41] including vascular endothelial growth factor,[4,24,42-44] stromal cell-derived factor 1,[45] statins,[46] and erythropoietin,[47-49] and the therapeutic potential of these agents for enhancing EPC mediated angiogenesis is being evaluated. The effects of E2 are dependent on EPC incorporation into the sites of neovascularisation and are mediated by endothelial nitric oxide synthase,[5] and matrix metalloproteinase-9.[12] Estradiol also reduces myocyte apoptosis *in vivo* and *in vitro* via oestrogen receptor- and phospho-inositide-3 kinase/akt dependent pathways.[21,34] Oestrogen receptors α and β have been extensively investigated and are vital for the incorporation of bone marrow-derived EPCs in ischemic tissue; the role of the α receptor appears to be more prominent.[11,50]

We acknowledge that the EPC culture assay does not completely exclude cells of a non-endothelial lineage, and that FACS analysis could provide additional specificity. However, the maximum amount of blood that can be taken from a mouse (1 ml) is not sufficient for both the EPC culture assay and FACS analysis, and this limitation can be overcome only by pooling blood samples from several mice, which would reduce the statistical power of subsequent analyses. The limited amount of blood obtainable from each mouse also precluded the measurement of circulating E2 levels. Finally, the small number of animals (five to seven per treatment group per time point) yielded a large distribution of measurements for each time point, which may have occasionally prevented the differences between treatment groups from reaching statistical significance.

In conclusion, our study shows that E2 dose dependently increases the level of circulating EPCs in the peripheral blood of both intact male mice and male mice with HLI, and that E2-mobilised EPCs home to the foci of neovascularisation during HLI to promote recovery. Furthermore, E2 was also associated with improved blood-flow recovery, greater capillary density, and less limb necrosis, with optimal effects observed two to three weeks after the onset of HLI. These findings suggest that systemic E2 administration may increase EPC-mediated neovascularisation under ischemic conditions caused by peripheral artery disease.

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7 CHAPTER

A pilot study on the HO-1 inducer heme arginate in non-ST-elevation myocardial infarction

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ABSTRACT

Background

In experimental models of ischemia-reperfusion injury, activation of HO-1 attenuates ischemia-reperfusion related damage. We conducted a pilot study to evaluate the safety and efficacy of an exogenous HO-1 inducer (heme arginate) in patients with non-ST-elevation myocardial infarction (NSTEMI).

Design

Patients with NSTEMI were open label randomised to a single dose of heme arginate (N=10) or placebo (N=5), infused within three hrs after hospital admission for NSTEMI. Blood, to assess different downstream markers of HO-1 activation and safety parameters (heart, kidney and liver function, electrolytes and general haematology), was taken at hospital admission and 3, 6 and 12 hrs and 1, 2, 3, and 7 days after administration of heme arginate.

Results

A single high dose of heme arginate after NSTEMI did not increase plasma ferritin and total, direct and indirect bilirubin concentrations or HO-1 mRNA expression and HO protein activity in peripheral blood mononuclear cells as compared to the placebo group. Safety parameters were all comparable between the two groups. Interestingly, NSTEMI itself did activate the HO-1 system. Median total bilirubin levels were increased with 60% after NSTEMI (peak compared to baseline, p=0.001). Also, median HO-1 mRNA (17.5 times compared to baseline, p=0.001) and median HO protein activity (peak compared to baseline, 780%, p=0.003) were increased after NSTEMI.

Conclusions

NSTEMI causes an endogenous HO-1 response, while adding the exogenous HO-1 inducer heme arginate does not further increase downstream HO-1 markers. Heme arginate administration is safe in patients with NSTEMI. Clinicaltrials.gov identifier: NCT00483587.

INTRODUCTION

The 'free radical hypothesis' for vascular dysfunction originally postulated that reactive oxygen and nitrogen species (ROS and RNS, respectively) led to non-specific modification of lipids, proteins and nucleic acids, which then contributed to the aetiology of cardiac diseases.[1,2] Especially ischemia-reperfusion (I/R) injury is associated with increased production of ROS and subsequent cell death.[3] Recently, degradation products of heme oxygenase-1 (HO-1) activity have been suggested to act as anti-oxidants.[4-7] The inducible isoform of HO, named HO-1, can be activated in virtually all tissues and represents the rate limiting step in the degradation of the pro-oxidant heme molecule into biliverdin, carbon monoxide (CO), and iron.[5] Biliverdin is then converted by biliverdin reductase (BVR) into bilirubin, whereas iron induces the expression of the iron sequestering molecule ferritin.[5,8-10] At physiological concentrations, bilirubin, CO and ferritin all exhibit high anti-oxidant and cytoprotective properties.[9,11-13] Moreover, during cardiovascular disorders, including acute myocardial infarction (MI), bilirubin undergoes oxidative modifications, probably due to the high levels of both ROS and RNS, originating specific products named biopyrrins. An increased concentration of biopyrrins was found in plasma and urine of subjects suffering from acute MI, coronary artery disease and chronic heart failure.[14-17]

Numerous animal studies in atherosclerotic, hypertensive, I/R-injury and MI models employing gene therapy or genetically engineered mouse models strongly suggested that HO-1 induction prevents cardiovascular damage.[18-20] However, this approach in the human setting is clearly not feasible.

Heme is both the substrate and a strong pharmacological inducer of HO-1 expression. Administration of hemin significantly decreased infarct size in experimental models of MI.[21-23] Furthermore, administration of hemin (up to 9 mg/kg/day, administered directly in the left atrium) attenuated myocardial stunning in dogs after ligation of the left ascending coronary artery.[24]

So, although a large body of evidence supports the beneficial effects of HO-1 activation, in particular by either hemin or heme arginate, in I/R-injury and other models, clinical data in cardiovascular patients are lacking to date. Therefore, we evaluated the safety and efficacy of a single dose of heme arginate in patients with a non-ST-elevation myocardial infarction (NSTEMI).

PATIENTS AND METHODS

Study design

This is a prospective, open label, placebo-controlled pilot study (EudraCT 2006-003689-40; NCT00483587). Figure 1A shows an outline of the study design. All patients gave written informed consent to the study procedures, which were approved by the Ethics Committee of the University Medical Centre Groningen, The Netherlands. This study conforms with the guidelines from the Declaration of Helsinki.

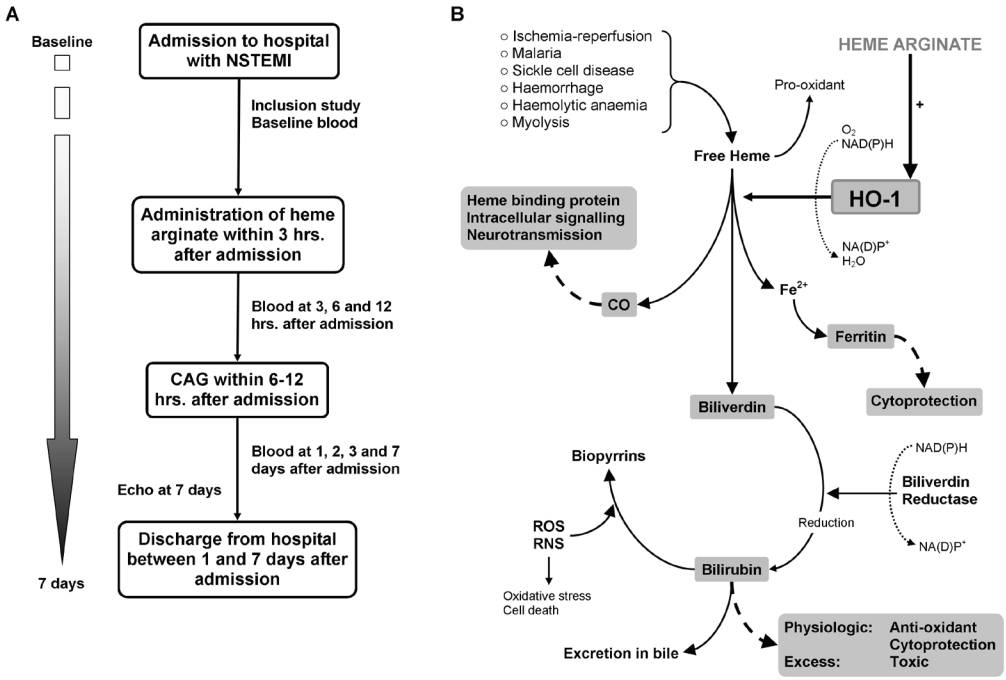

Figure 1. A. Flowchart of the study. B. Heme breakdown pathway. CAG = coronary angiogram, NSTEMI = non-ST-elevation myocardial infarction, HO-1 = heme oxygenase-1, CO = carbon monoxide, ROS = reactive oxygen species, RNS = reactive nitrogen species, Fe^{2+} = iron, NADP = nicotinamide adenine dinucleotide phosphate. For colour figure, see supplement 1.

Study patients

Fifteen (15) patients were recruited at the Department of Cardiology from the University Medical Centre Groningen, The Netherlands, between December 2007 and July 2008. Male and female patients were eligible for enrolment if they were admitted to the cardiac care unit with suspected diagnosis of NSTEMI confirmed by elevated creatinin kinase (CK) and/or troponin-T levels according to ESC guidelines for Management of acute coronary syndromes: acute coronary syndromes without persistent ST segment elevation.[25,26] Exclusion criteria were: ST-elevation on the electrocardiogram, a clinical history of chronic kidney disease, any known hepatic disease and any known hypersensitivity or allergic reactions to one of the constituents of heme arginate (hemin, L-arginine, propylene glycol, ethanol).

Study treatment

Patients were randomly allocated in order of admission and randomised with a ratio 1:2 to either placebo (0.9% sodium chloride, N=5) or heme arginate (Normosang®, Orphan Europe SARL, 250 mg intravenously in 15 minutes, N=10). This dose of heme arginate is considered safe and used in patients with acute porphyria (instructions of manufacturer). Heme arginate or placebo was administered within three hours after admission. Regardless of allocation, all patients received standard therapy according to national and international guidelines.[26] Blood

samples were drawn from the antecubital vein of the study subjects at baseline and 3, 6, 12 hrs and 1, 2, 3 and 7 days after admission. A coronary angiogram (CAG) was made 6 to 12 hrs after admission for diagnostic purposes. After CAG, an independent team of cardiologists and thoracic surgeons decided the optimal treatment for the NSTEMI (medical treatment only, percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG) surgery) and patients were treated accordingly. Seven days after inclusion in this study, an echocardiogram was made to assess left ventricular function, including left ventricular ejection fraction.

Endpoints

The primary efficacy endpoint was to evaluate the modification of the HO/BVR pathway upon administration of heme arginate, and the following markers were determined: mRNA levels of HO-1 in peripheral blood mononuclear cells (PBMCs), *in vitro* HO protein activity in PBMC lysates and plasma levels of ferritin and total, direct and indirect bilirubin. Key secondary endpoints were the overall changes in the HO/BVR expression and activity in patients who underwent NSTEMI. Key safety endpoints were heart function (CK, CK-MB, troponin-T, NT-proBNP and echocardiogram), kidney function (blood urea nitrogen, creatinin, estimated glomerular filtration fraction), liver function (aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase), electrolytes (sodium, potassium), regular haematology (haemoglobin, leucocytes, thrombocytes). Furthermore, specific markers of cardiac disease were analysed using ELISA kits according to manufacturers' protocol (IL-6: #D6050, Human IL-6 Quantikine HS ELISA Kit; IL-10: #D1000B, Human IL-10 Quantikine ELISA Kit; #TNF-α: DTA00C, Human TNF-α Quantikine ELISA Kit, VEGF: #DVE00, Human VEGF Quantikine ELISA Kit, all R&D Systems Europe, Abingdon, UK). All samples were analysed at the general laboratory of the hospital.

Analytical assays

Isolation of mononuclear cells

PBMCs were isolated from eight ml of whole blood using a PBMC preparation tube (#362761, BD, New Jersey, USA) according to manufacturer's protocol. Cells were kept at -80°C till further use.

RNA isolation, cDNA synthesis and Quantitative rt-PCR

Total RNA was isolated from snap frozen PBMCs using QIAschredder and RNeasy kits (QIAGEN) according to manufacturer's recommendations. cDNA was created according to the Omniscript protocol (QIAGEN) using 1 µg RNA and random primers (Invitrogen). Subsequently, quantitative real time rt-PCR was conducted using Taqman Universal PCR Master Mix protocol and primer probe sets (all from Applied Biosystems) specific to detect human GAPDH (Hs99999905_m1) and HO-1 (Hs00157965_m1). For each experimental group, the Ct values of GAPDH were subtracted from the Ct values of the gene of interest (Delta Ct) and the average Delta Ct values of the duplicates were taken. A Delta Ct difference between the two independent PCR experiments of up to 0.6 was tolerated. See supplemental data for extended protocol.

HO protein activity assay

To determine whether the induced HO protein was active, we performed an HO-activity

assay. Briefly, 100.000 cells were resuspended in lysis buffer and subsequently lysed by three freeze-thaw cycles. To analyse the amount for bilirubin that was formed, 2 mM heme (Frontier scientific) was added and samples were incubated for 60 min. at 37°C in the dark. Next, methanol was added, samples were mixed well and centrifuged at 13.000 x g. Samples were run on a HPLC (Spectra-Physics Analytical, Spectrasystem SCM400) equipped with a 5 µm Discovery C18 column and a Discovery C18 Superguard Cartridge 5 µm particle size precolumn (both Sigma-Aldrich). Separation was done by a mixture of 80% solvent A (40% 100 mM NH4Ac, pH 5.5, 5% methoxy-ethanol and 50% methanol (all Sigma-Aldrich) and 20% solvent B (5% methoxy-ethanol and 95% methanol) over a 20 minute time interval at a flow rate of 1 ml/min. Biliverdin and bilirubin were detected at a wavelength of 377 and 450 nm, respectively. Standards of biliverdin and bilirubin (both from Frontier Scientific) were also run in order to quantify the amount of biliverdin/bilirubin found in the samples. The HO protein activity is expressed as µmol/mg total protein/hour. See supplemental data for extended protocol.

Statistics

Summaries of quantitative continuous variables are presented as median and inter quartile ranges (IQR) or otherwise if appropriate. HO-1 mRNA expression and HO protein activity are presented as percentage change during the study (between baseline and peak values, peak is 12 hrs for mRNA expression and one day for protein activity). Because of small samples sizes, comparisons of differences between groups were analysed by Wilcoxon signed-rank test and comparisons within groups were analysed by Mann-Whitney U test. All tests were done two-sided. To assess if NSTEMI upregulates markers of the HO-1 pathway, groups were pooled. A p-value of less than 0.05 was considered significant. All analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL, USA).

RESULTS

Patients

Clinical characteristics of the study patients are summarised in table 1. Median age of the heme arginate group was 63 (51-72) years, where the placebo group was slightly older (median 77 (61-84) years, p=0.13). Predominantly male patients participated in this study (heme arginate group 80%, placebo group 60%, p=0.43. Medical history, medical treatment at admission and discharge, and echo parameters did not differ significantly between the two groups. Furthermore, we did not observe any differences in heart rate, blood pressure or cholesterol levels between the two groups. Patients in the heme arginate group had significantly more often hypertension (90% vs. 40%, p=0.046).

Coronary angiography was performed 6 to 12 hrs after admission and afterwards, an independent team of cardiologists and thoracic surgeons decided the optimal treatment (see supplemental data for table with diagnoses after CAG and the chosen treatments). All patients in the placebo group were treated with PCI, where 30% of the patients in the heme arginate group underwent CABG surgery, all these patients being diagnosed with 3-vessel disease. Patients in the heme arginate group tended to stay longer in the hospital than those in the placebo group (3 (2-4) vs. 6.5 (2-13.3) days, p=0.24), which can be largely ascribed to the chosen treatment after CAG (more often CABG).

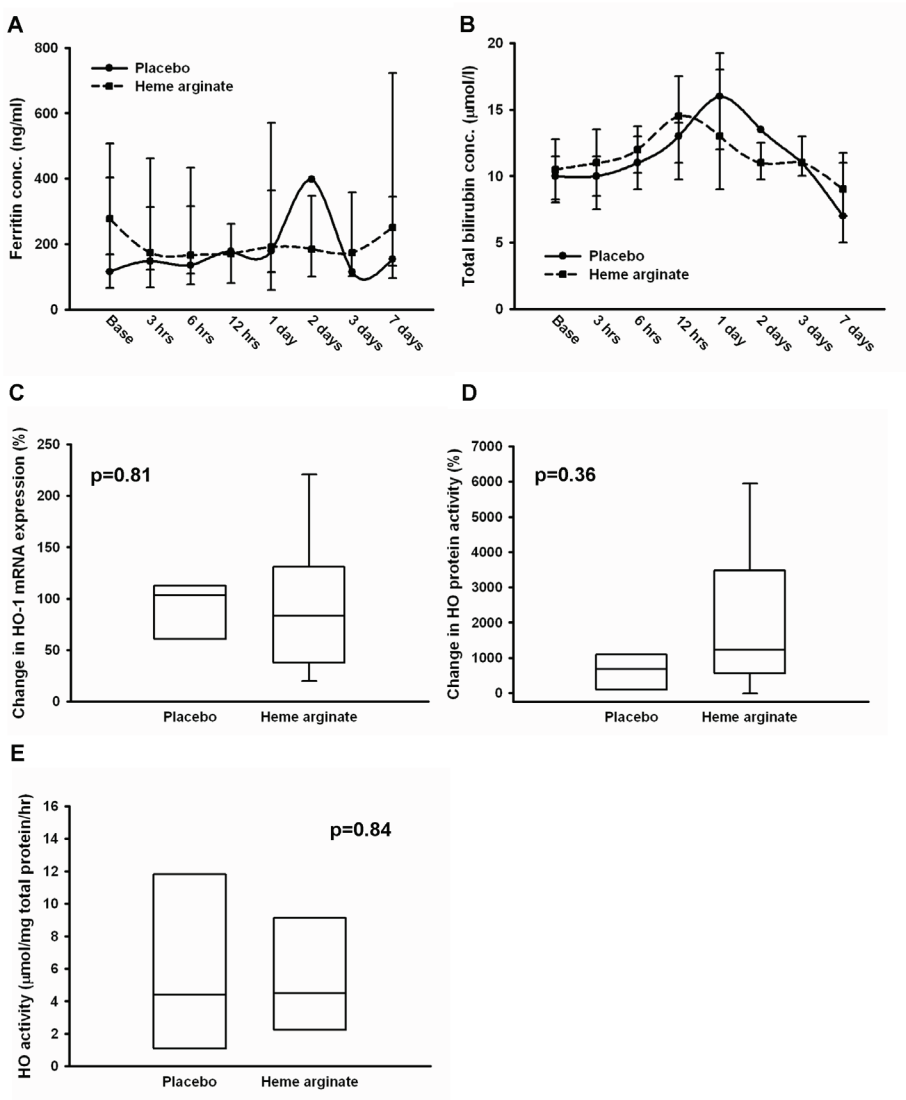

Figure 2.

A. Ferritin plasma concentration during the study. B. Total bilirubin plasma concentration during the study. C. Change of HO-1 mRNA expression, change between baseline and peak values (12 hrs after admission). D. Change in HO protein activity, change between baseline and peak values (1 day after admission). F. HO protein activity 1 day after admission. Base = baseline, hr = hour.

Table 1. Clinical characteristics

	Placebo (N=5)	Heme arginate (N=10)	p
Age (years)	77 (61-84)	63 (51-72)	0.13
Male (N, %)	3 (60)	8 (80)	0.43
Days in hospital	3 (2-4)	6.5 (2-13.3)	0.24
Medical history			
None (N, %)	1 (20)	1 (10)	0.60
PCI (N, %)	0	1 (10)	0.48
CABG (N)	0	0	1.00
Other (N, %)	4 (80)	8 (80)	1.00
Risk factors			
Hypertension (N, %)	2 (40)	9 (90)	0.046
Family (N, %)	1 (20)	5 (50)	0.28
Smoking (N, %)	3 (60)	5 (50)	0.72
DM (N, %)	1 (20)	1 (10)	0.60
Obesity (N, %)	1 (20)	1 (10)	0.60
Hypercholesterolemia (N, %)	1 (20)	3 (30)	
Medication at admission			
Beta-blocker (N, %)	0	1 (10)	0.48
ACEi (N, %)	0	3 (30)	0.19
Diuretics (N, %)	1 (20)	5 (50)	0.28
ARB (N, %)	0	2 (20)	0.30
Anti-coagulants (N, %)	1 (20)	2 (20)	1.00
Lipid lowering drugs (N, %)	1 (20)	1 (10)	0.60
Nitrates (N, %)	0	1 (10)	0.48
Ca-antagonist (N, %)	0	2 (20)	0.30
Other (N, %)	3 (60)	4 (40)	0.48
Medication at discharge			
Beta-blocker (N, %)	5 (100)	8 (80)	0.30
ACEi (N, %)	3 (60)	6 (60)	1.00
Diuretics (N, %)	1 (20)	4 (40)	0.45
ARB (N, %)	0	2 (20)	0.30
Anti-coagulants (N, %)	5 (100)	9 (90)	0.48
Lipid lowering drugs (N, %)	4 (80)	9 (90)	0.60
Nitrates (N, %)	1 (20)	2 (20)	1.00
Ca-antagonist (N, %)	0	1 (10)	0.48
Other (N, %)	3 (60)	7 (70)	0.71
HR admission (bpm)	84 (61-92)	87 (81-94)	0.46
HR discharge (bpm)	67 (62-75)	68 (62-82)	0.76
Systolic BP (mmHg)	160 (144-177)	147 (129-152)	0.10
Diastolic BP (mmHg)	80 (75-90)	94 (75-102)	0.24

Table 1 cont'd.

	Placebo (N=5)	Heme arginate (N=10)	p
Echocardiography			
IVSd (mm)	10 (9-15)	10 (10-12)	0.92
LVPWd (mm)	10 (8-11)	9 (9-10)	0.49
LVEDD (mm)	44 (36-46)	52 (47-53)	0.05
LVESD (mm)	26 (21-32)	37 (33-38)	0.032
LVEF (%)	53 (45-60)	45 (45-60)	0.64
Cholesterol			
Triglycerides (mmol/l)	2.0 (1.1-3.3)	1.5 (1.1-2.4)	0.71
Total cholesterol (mmol/l)	4.6 (4.2-6.2)	5.1 (4.4-6.0)	0.67
HDL-cholesterol (mmol/l)	1.2 (0.9-1.8)	1.3 (1.2-1.5)	0.66
LDL-cholesterol (mmol/l)	3.1 (2.5-4.0)	3.6 (2.9-4.1)	0.50

Table 1 shows the clinical characteristics of the two groups. Data is presented as median (IQR). PCI = percutaneous coronary intervention, CABG = coronary artery bypass graft, DM = diabetes mellitus, ACEi = ACE-inhibitor, ARB = angiotensin II receptor blockers, HR = heart rate, BP = blood pressure, IVSd = inter ventricular septum thickness in diastole, LVPWd = left ventricular posterior wall thickness in diastole, LVESD = left ventricular end systolic diameter, LVEDD = left ventricular end diastolic diameter, LVEF = left ventricular ejection fraction, HDL-cholesterol = high density lipoprotein cholesterol, LDL-cholesterol = low density lipoprotein cholesterol.

Efficacy endpoints

Blood ferritin levels (figure 2A) and total bilirubin concentrations (figure 2B) were measured, to assess downstream markers of HO-1 activation. Furthermore, changes in mRNA levels of HO-1 (figure 2C), changes in HO protein activity (figure 2D) and HO protein activity one day after admission (figure 2E) were analysed in PBMCs. None of the markers are upregulated after heme arginate administration as compared to placebo group in this study (median change in HO-1 mRNA expression: 103 (61-113)% vs. 83 (38-131)%, p=0.81, median change in HO protein activity: 680 (91-1086)% vs. 1221 (555-3481)%, p=0.36).

Because heme arginate administration did not upregulate HO-1 pathway markers, we combined all patients and assessed whether NSTEMI did activate the HO-1 pathway. As shown in table 3, NSTEMI is associated with upregulation of some of the aforementioned markers. Total, direct and indirect bilirubin levels, HO-1 mRNA expression and HO protein activity were all significantly upregulated after NSTEMI.

Safety endpoints

To monitor patient safety after heme arginate administration, we evaluated different markers of heart function, kidney function, liver function, electrolyte status, regular haematology and specific markers of cardiac disease (table 2, only baseline values and values after one and seven days are shown). None of the measured markers differed significantly at any time point within the study between the two groups.

Table 2. Clinical characteristics

	Placebo (N=5)			Heme arginate (N=10)		
	Baseline	1 day	7 days	Baseline	1 day	7 days
Heart function						
CK (U/l)	302 (138-488)	313 (131-916)	86 (43-116)	141 (103-230)	145 (77-190)	85 (37-190)
CK-MB (U/l)	30 (19-51)	26 (84)	11 (12)	17 (13-33)	15 (9-22)	13 (10-21)
Troponin T (µg/l)	0.28 (0.11-0.65)	0.44 (0.11-0.94)	0.01 (0.00-0.02)	0.07 (0.04-0.17)	0.80 (0.20-0.22)	0.00 (0.00-0.10)
NT-proBNP (ng/l)	407 (104-955)	601 (321-3088)	178 (79-238)	400 (201-745)	745 (147-1680)	159 (88-1063)
Kidney function						
BUN (mmol/l)	5.3 (5.1-6.7)	6.0 (5.2-8.0)	6.3 (6.7-6.7)	6.0 (4.1-7.6)	6.2 (4.7-8.6)	6.1 (5.2-8.8)
Creatinin (µmol/l)	67 (61-81)	79 (75-81)	77 (56-86)	83 (55-101)	85 (68-115)	76 (67-106)
eGFR (ml/min*1.73m^2)	90 (80-106)	88 (69-93)	82 (75-102)	86 (60-139)	72 (57-107)	87 (65-114)
Liver function						
AST (U/l)	40 (20-62)	49 (37-109)	23 (18-28)	37 (26-46)	33 (30-47)	42 (27-64)
ALT (U/l)	25 (19-50)	27 (15-57)	25 (15-40)	32 (20-41)	33 (18-39)	55 (32-95)
LDH (U/l)	182 (142-254)	209 (164-312)	175 (157-199)	195 (172-241)	209 (158-231)	208 (182-340)
AP (U/l)	72 (67-76)	68 (44-84)	77 (73-08)	66 (66-79)	61 (56-64)	67 (62-98)
Electrolytes						
Na^+ (mmol/l)	139 (137-141)	134 (133-138)	141 (136-143)	140 (139-143)	139 (137-143)	141 (137-143)
K^+ (mmol/l)	4.2 (3.5-4.7)	4.4 (3.3-5.8)	3.9 (3.6-4.4)	4.0 (3.7-4.5)	4.0 (3.8-4.2)	4.3 (4.0-4.6)
Haematology						
Hb (mmol/l)	9.7 (7.9-9.7)	8.2 (7.3-9.2)	8.3 (7.8-8.4)	9.0 (8.3-9.3)	8.6 (7.9-8.9)	8.4 (7.0-9.5)
Leuco (10^9/l)	8.6 (7.4-10.4)	10.5 (8.2-16.8)	7.4 (7.0-8.3)	8.6 (7.0-10.3)	8.0 (6.0-10.2)	7.1 (5.9-8.2)
Thrombo (10^9/l)	209 (196-244)	194 (185-231)	261 (214-267)	203 (191-233)	196 (184-233)	249 (231-291)
Markers						
hsCRP (mg/l)	4.6 (1.5-19.9)	18.6 (7.8-30.4)	1.5 (1.0-4.2)	8.8 (6.1-28.6)	10.2 (7.0-24.4)	5.9 (1.6-10.0)
IL-6 (pg/ml)	2.8 (1.5-4.7)	6.3 (4.2-30.8)	2.2 (0.3-2.3)	5.5 (2.6-9.6)	7.6 (4.9-8.8)	2.7 (1.0-14.7)
IL-10 (pg/ml)	12.6 (8.8-19.6)	15.4 (11.9-22.1)	9.9 (6.4-12.0)	15.5 (12.0-37.2)	23.3 (17.9-29.6)	18.4 (10.9-21.1)
TNF-α (pg/ml)	1.7 (0.0-16.0)	0.0 (0.0-7.0)	0.0 (0.0-4.3)	0.9 (0.0-4.9)	0.0 (0.0-5.2)	0.0 (0.0-2.1)
VEGF (pg/ml)	35 (22-145)	90 (39-102)	42 (38-111)	101 (62-132)	47 (36-89)	92 (43-220)

Data is presented as median (IQR). Only baseline values and values after 1 and 7 days are shown. We compared the values of the placebo patients with the heme arginate treated patients for each time point; none of the comparisons were statistically significant different (p-values not shown). CK = creatinin kinase, NT-proBNP = N-terminal pro-brain natriuretic peptide, BUN = blood urea nitrogen, eGFR = estimated glomerular filtration fraction calculated with the modification of diet in renal disease (MDRD) formula, AST = aspartate aminotransferase, ALT = alanine aminotransferase, LDH = lactate dehydrogenase, AP = alkaline phosphatase, Na+ = sodium, K+ = potassium, Hb = haemoglobin, hsCRP = high sensitive C-reactive protein, IL = interleukin, TNF = tumour necrosis factor, VEGF = vascular endothelial growth factor.

Table 3. Effects of NSTEMI on HO-1 pathway and its breakdown products

	All (N=15)		p
	Baseline	Peak	
Total bilirubin (μmol/l)	10.0 (9.0-12.0)	16.0 (12.0-18.0)	0.001
Direct bilirubin (μmol/l)	0.0 (0.0-1.0)	2.0 (1.0-3.0)	0.005
Indirect bilirubin (μmol/l)	9.0 (7.0-10.0)	14.0 (12.0-16.0)	0.001
HO-1 mRNA expression (Δ/ΔCt)	0.2 (1.0-2.1)	3.5 (3.2-4.3)	0.001
HO protein activity (μmol/mg total protein/hour)	0.5 (0.2-1.8)	3.9 (2.2-14.2)	0.003

Data is presented as median (IQR). Groups are pooled. Total bilirubin, direct bilirubin and indirect bilirubin are measured in plasma, time to peak was 1 day. HO-1 mRNA expression and HO protein activity were measured in peripheral blood mononuclear cells; time to peak was 12 hrs and 1 day respectively. HO = heme oxygenase.

DISCUSSION

This is the first study that describes the use of an exogenous HO-1 activator, such as heme arginate, to attenuate I/R-injury in human I/R-injury (here: NSTEMI). We show that NSTEMI itself is associated with an activation of the HO-1 pathway. Single dose administration of heme arginate did not further activate this pathway.

Indeed, heme and its derivatives (heme arginate and heme lysinate) act all as strong inducers and substrates for HO-1, the rate limiting enzyme in the breakdown of free heme. As an effect of HO-1 activation and heme degradation, an increase in CO and biliverdin/bilirubin occurs as well as the upregulation of the iron storing molecule ferritin. All these molecules have both cytoprotective and cytotoxic effects depending on their amount and the redox status of the cell (figure 1B).[5,8-10,21]

In this pilot study, a single (high) dose of heme arginate after NSTEMI did not increase plasma ferritin and total, direct and indirect bilirubin concentrations, as well as HO-1 mRNA expression and HO protein activity in PBMCs as compared to the placebo group. However, our data demonstrate that heme arginate administration is safe in patients with NSTEMI: no significant adverse effects on heart, kidney and liver along with abnormalities related to main biochemical parameters (electrolytes, blood tests, markers of inflammation) appeared in these patients. These findings resemble and confirm previous studies showing that heme arginate is well tolerated and the infusion does not induce adverse effects on haemostasis in healthy volunteers.[27,28]

An important corollary of our study was the observation, for the first time, of a systemic activation of the HO-1/BVR system in NSTEMI as demonstrated by the significant increase in plasma total, direct and indirect bilirubin, HO-1 mRNA expression and protein activity in PBMCs. This is in good agreement with previous studies which demonstrated that the activation of the HO-1/BVR system in free radical-induced diseases, such as Alzheimer disease and type II diabetes mellitus, is not confined in target organs but becomes systemic and affects also immune cells.[20,29] Taking into consideration previous studies showing a free radical-induced degradation of HO-1/BVR-derived bilirubin in acute MI, it is plausible to hypothesize that

the degree of activation of the HO-1/BVR system in NSTEMI is even higher than found in our study.[14-17]

MI resembles a state with high levels of cellular and oxidative stress and in several animal models of cardiovascular diseases HO-1 induction was shown to prevent cardiovascular damage.[18,30,31] HO-1 induction was thoroughly tested in animal MI models.[21-24] Genetically engineered mice overexpressing HO-1 are protected against MI, whereas on the other hand HO-1 null mice have severe cardiac dilatation and enhanced infarct size.[19,30] The group of Victor Dzau developed a model of gene therapy overexpressing HO-1, using adeno-associated virus-2 as the delivery vector, in an MI model in mice. In the acute setting, overexpression of HO-1 reduced apoptosis, prevented myocardial wall thinning, inflammation, fibrosis and deterioration of cardiac function.[32] Also, there was a significant reduction in apoptosis. In the setting of post-MI remodelling and function, overexpression of HO-1 markedly reduced fibrosis and ventricular remodelling and restored left ventricular function and chamber dimensions after MI.[33]

Although these pre-clinical lines of evidence lend support to the therapeutic potential of HO-1 upregulation in cardiovascular disease, our data in humans demonstrated inefficacy of a single dose of 'exogenous' heme arginate to further increase HO-1 in NSTEMI. It is established that in the presence of excess heme (a condition mimicked by the administration of heme arginate) and/or perturbations of the redox intracellular milieu as during I/R-injury, HO-1 undergoes transcriptional repression, rather than upregulation. This could be a possible explanation for our findings.[34,35] Another explanation could be that the dose was too low to elicit further or sustained activation of the HO-1 pathway. Alternatively, single dose is not the preferred regimen and one should rather administer repeated dosages of heme arginate or another heme derivative.

Our study has several limitations. Firstly, the sample size only allows for a first insight in the use of exogenous HO-1 inducers in patients with MI. Secondly, we only administered a single dose of heme arginate. Multiple infusions with heme arginate could well induce the downstream HO-1 markers. Thirdly, the dose administered is derived from the dose administered in patients with acute porphyria, which is considered safe. A dose-finding study should be carried out, in order to determine the optimal dose for patients with MI.[36] Furthermore, in experimental models, HO activity was maximal at the time of infarction. In our study, we measured HO activity hours after onset of complaints.

In conclusion, this is the first study that describes the use of an exogenous HO-1 inducer in patients with cardiovascular disease. We conclude that NSTEMI induces HO-1, as shown by the increase of HO-1 mRNA expression, HO-1 protein activity and increase of total, direct and indirect bilirubin levels. A single high dose of heme arginate does not affect these parameters. So, although there is an endogenous HO-1 response to NSTEMI, adding an exogenous HO-1 inducer does not increase downstream HO-1 markers further. Heme arginate administration seems safe in patients with NSTEMI.

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Heart failure-associated anaemia: Bone marrow dysfunction and response to erythropoietin

CHAPTER 8

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ABSTRACT

Heart failure (HF)-associated anaemia is common and has a poor outcome. Because bone marrow (BM) dysfunction may contribute to HF-associated anaemia, we first investigated mechanisms of BM dysfunction in an established model of HF, the transgenic REN2 rat, which is characterised by severe hypertrophy and ventricular dilatation and SD rats as controls. Secondly, we investigated whether stimulation of haematopoiesis with erythropoietin (EPO) could restore anaemia and BM dysfunction. After sacrifice, erythropoietic precursors (BFU-E) were isolated from the BM and cultured for ten days. BFU-E were quantified and transcript abundance of genes involved in erythropoiesis were assayed. Number of BFU-E were severely decreased in BM of REN2 rats compared to SD rats (50±6.2 vs. 6.4±1.7, $p<0.01$). EPO treatment increased haematocrit in the SD-EPO group (after six weeks: 49±1 vs. 58±1%, $p<0.01$), however in the mildly anaemic REN2 rats, there was no effect (43±1 vs. 44±1%). This was parallelled by a 67% decrease in BFU-E in BM of REN2 rats compared to SD ($p<0.01$). EPO significantly improved BFU-E in both SD and REN2, but could not restore this to control levels in the REN2 rats. Expression of several genes involved in differentiation (LMO2), mobilisation (SDF-1) and iron incorporation (transferrin receptor) of the BM were differentially expressed in REN2 rats compared to SD rats, and EPO did not normalise this. Altogether, these results suggest that BM dysfunction is an important contributor to HF-associated anaemia and that EPO is not an effective agent to treat HF-associated anaemia.

INTRODUCTION

Anaemia as a co-morbidity in heart failure (HF) is associated with a poor outcome.[1,2] The prevalence of anaemia in HF is reported to be over 40% in patients with advanced HF.[3] The causes of anaemia in HF are only partially understood, although several mechanisms have been implicated,[4] including treatment with ACE-inhibitors,[5] a blunted erythropoietin (EPO) production due to renal dysfunction,[6] congestion,[7] and iron deficiency.[8] However, these causes only partially explain the severity of anaemia and are difficult to target.

Recent publications pointed towards bone marrow (BM) dysfunction as a potential contributing factor to anaemia in HF. Iversen et al. showed decreased haematopoiesis in BM in an experimental (murine) model of post myocardial infarction HF.[9] Our group recently provided the first clinical proof for the existence of BM dysfunction in human ischemic HF.[10] We showed that HF is associated with a profound and general BM dysfunction, simultaneously affecting multiple haematopoietic lineages. Very recently, the group of Iversen provided further evidence that HF is associated with general BM dysfunction by showing that anaemia occurs in both ischemic and non-ischemic murine HF models.[11] Apparently, the mechanisms of anaemia in HF are dependent of aetiology and are possibly caused by general HF factors, like overproduction of neurohormones or hypoperfusion.[11] Importantly, data as to whether pro-erythropoietic therapy could restore anaemia and BM function in HF is lacking.

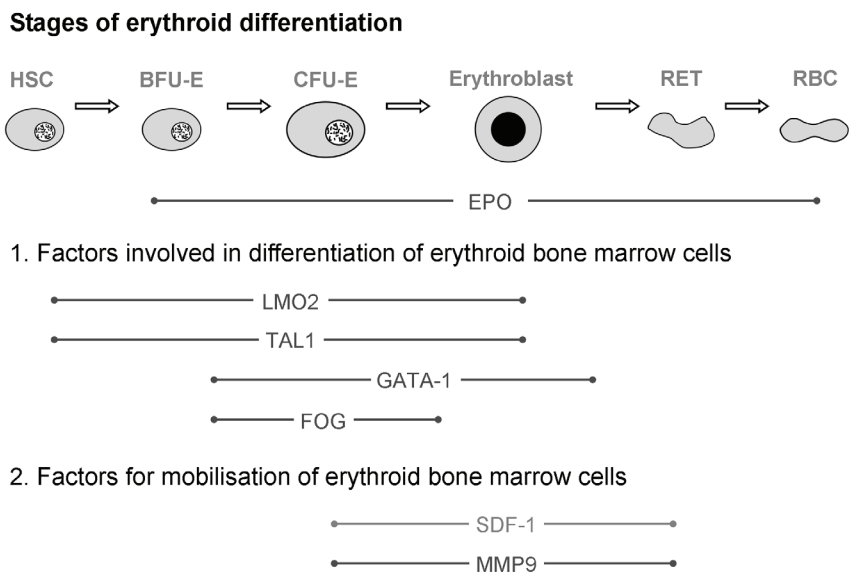

Figure 1. Overview of different factors involved in differentiation and mobilisation during different stages of erythropoiesis, which expression was measured (results shown in figure 5). Adapted from Koury et al,[12] according to Hanson et al,[29] Ohneda et al,[31] and Jin et al.[32] Factors highlighted in green are positive regulators, factor highlighted in red is a negative regulator. HSC = haematopoietic stem cell, BFU-E = burst forming unit-erythroid, CFU-E = colony forming unit-erythroid, RET = reticulocyte, RBC = red blood cell, LMO = LIM domain only 2 protein, TAL = T-cell acute lymphocytic leukemia protein, GATA = GATA binding protein, FOG = friend of GATA, MMP = matrix metallopeptidase, SDF = stromal cell-derived factor. For colour figure, see supplement 1.

Therefore, we evaluated the severity and mechanisms of anaemia and BM dysfunction in an established experimental model of hypertensive HF, the transgenic TGR(mREN2)27 rat. Furthermore, we investigated if stimulation of haematopoiesis with EPO administration could restore, at least in part, HF-associated anaemia and BM function. Finally, we studied in detail well established factors of erythroid differentiation (LMO2, GATA-1) and mobilisation (MMP9, SDF-1) (figure 1, adapted from Koury et al.[12]). LMO2 and GATA-1 are both differentiation factors, where MMP9 mobilises erythroid precursors from the BM into the peripheral blood and SDF-1 is a negative regulator of erythroid mobilisation. Furthermore, we analysed factors involved in iron incorporation in the BM (transferrin receptor and ferroportin, both involved in maintaining cellular iron homeostasis) and studied if expression of these factors is changed during EPO administration.

MATERIALS AND METHODS

Animal model

We employed six week old, male, homozygous TGR(mREN2)27 rats.[13] See supplemental data for detailed description. Rats were fed with *ad libitum* regular chow that contains sufficient amounts of vitamin B and iron (RMH-B 2181, AB Diets, The Netherlands). Over the course of the experiment, the mortality rate of REN2 rats was 20% (as reported; mortality was 0% for control (Sprague Dawley, SD) rats). The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen, The Netherlands, and conducted in accordance with existing guidelines for the care and use of laboratory animals.

Experimental protocol

To stimulate erythropoiesis, rats were randomised to treatment with the long-acting EPO analogue darbepoetin alpha (40 µg/kg, Aranesp®, Amgen Inc., Thousand Oaks, CA, USA) administered subcutaneously every three weeks (baseline, three and six weeks) or saline (used as control). The dose of EPO was based on our extensive experience with this compound and previous data that this dose effectively raises haematocrit (Ht) in SD rats.[14-19] Rats were sacrificed at week seven of the experimental protocol (when aged 13 weeks). In total four groups were studied: SD-PL (placebo; n=12), REN2-PL (n=12), SD-EPO (n=12), REN2-EPO (n=15). At baseline, three weeks and six weeks, blood was drawn from the tail vein for a full blood count. At sacrifice, we analysed serum iron status. Blood pressure via tail cuff and haemodynamic data at sacrifice were measured as described.[20,21] Furthermore, to monitor the development of HF, echocardiography was performed at baseline, three weeks and six weeks. For details, see supplemental data.

Bone marrow isolation and MACs sorting

BM was isolated from tibia and femurs as previously described.[22] C-kit positive (c-kit+) cells were isolated as described in the supplemental data. Briefly, the cell pellet was washed twice with PBS to remove platelets. Cells were labelled with polyclonal rabbit anti c-kit antibody, followed by goat anti-rabbit IgG Microbeads. Anti c-kit-labelled cells were sorted by using mini-

magnetic activated cell sorting columns in a magnetic field. Effluent was collected in chilled IMEM medium. C-kit+ cells were collected with 1 ml chilled IMEM medium and cell number was counted.

Cell proliferation assay

After MACs column sorting, 1×10^4 c-kit+ BM cells were plated in 1 ml semi-solid medium (MethoCult, Stem Cell Technologies, Köln, Germany) in 35 mm Petri dishes in duplicate. The dishes were incubated at 37°C with 5% CO^2 and 95% humidity for two weeks. Colonies were scored using an inverted phase-controlled microscope (Leica 090-135002, Leica Microsystems, CMS GmbH, Germany). Burst-forming units-erythroid (BFU-E) were defined as consisting of 30 or more cells. Colony-forming units (CFU) were defined as consisting of 50 or more cells.

FACS analysis

BM mononuclear cells were harvested from tibia and femur of experimental animals and frozen in liquid nitrogen with 10% DMSO in medium in assay. FACS analysis was performed on an LSRII cytometer (BD Biosciences, San Jose, CA, USA). For phenotypic analysis by flow cytometry, 2-5 × 10^7 cells were dissolved in 10 ml cold DMEM with 10% FBS. Cell pellet were resuspended in 80 µl PBS/0.5% BSA. Samples were subsequently stained with rabbit polyclonal anti c-kit antibody (1 µg/10^6 cells, Santa Cruz Biotechnology, Heidelberg, Germany) on ice for 30 min. After washing twice with 2 ml PBS/0.5% BSA, cells were incubated with Alexa Fluor® 647 (1 µg/10^6 cells, Invitrogen, OR, USA) and FITC rat anti-mouse IgG (1 µg/10^6 cells, BD Biosciences Pharmingen, Breda, The Netherlands) as secondary antibodies. Cells were then suspended in 0.5 ml PBS/0.5% BSA and analysed within one hour. Unstained cells and cells stained with second antibody only were used as a negative control. Data were analysed by FlowJo software (V7.5.5, Ashland, OR, USA).

Quantitative real-time PCR

Total RNA from c-kit+ cells and left ventricular (LV) tissue was extracted using the Nucleospin RNA II kit, according to manufacturer's protocol (Machery-Nagel, Düren, Germany). cDNA synthesis and quantitative real time PCR (RT-qPCR) were performed as previously described with using 0.5 µg total RNA.[23] mRNA levels were expressed in relative units based on a standard curve obtained with serial dilutions of a calibrator cDNA mixture. To normalise expression data, multiple reference genes were used as described.[24] Please see supplemental data for full protocol and list of primers.

Statistical analyses

Results are reported as means ± standard errors of the mean (SEM). Number of BFU-E, CFU and c-kit+ cells were normalised to SD-PL group. Statistical analysis among groups was performed by ANOVA with the Bonferroni post hoc test if distributed normally, or with the Kruskall-Wallis test followed by Mann-Whitney U test when skewed distributed. All p-values are two-tailed, and a p-value of less than 0.05 was considered significant. All analyses were performed using SPSS version 16.0 software (SPSS, Chicago, IL, USA).

RESULTS

Hypertensive REN2 rats develop heart failure

REN2 and SD rats were treated with or without EPO and monitored for six weeks and sacrificed at an age of 13 weeks. As expected, REN2 rats suffered from severe hypertension (figure 2), providing the substrate for HF development. Heart weight (adjusted for tibia length), LV end diastolic pressure (LVEDP) and atrial natriuretic peptide (ANP) mRNA expression were all significantly increased in the REN2-PL and REN2-EPO groups, indicative for HF (figure 2). Table 1 shows the haemodynamic data at sacrifice. Indices of maximal contraction and relaxation of the LV (dPdtmax and dPdtmin) were both significantly decreased in the REN2 groups, compared to the SD groups, indicating development of HF. Cardiac output (CO), assessed with echo Doppler prior to sacrifice, is decreased in both REN2 groups compared to the SD groups. Echocardiographic data during the experiment confirmed development of LV hypertrophy and LV dilatation in REN2 rats (shown in supplemental data).

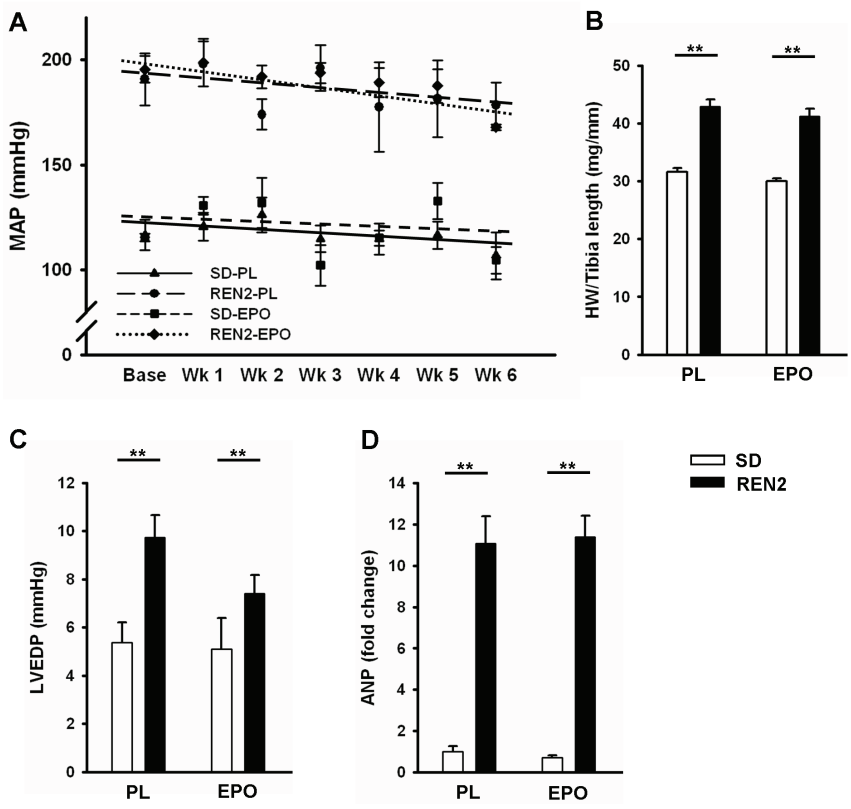

Figure 2. Figure 2 shows that the used animal model is a model of hypertensive heart failure. A. Mean arterial pressure (MAP) during the experiment. MAP is significantly higher at all time points for REN2 groups compared to SD groups. B. Heart weight normalised to tibia length. C. Left ventricular end diastolic pressure (LVEDP) at sacrifice. D. mRNA expression of atrial natriuretic peptide (ANP) in left ventricular tissue. ANP is expressed as fold change. HW = heart weight, Base = baseline, Wk = week, PL = placebo. * p<0.05, ** p<0.01.

Table 1. Data at sacrifice

	SD-PL (n=12)	REN2-PL (n=12)	SD-EPO (n=12)	REN2-EPO (n=15)
LV weight (mg)	841±15	1105±44 *	809±17	1157±36 ‡
TL (mm)	38.3±0.3	38.1±0.2	38.6±0.2	38.2±0.3
Body weight (g)	367±6	309±7 *	360±8	316±9 ‡
Heart rate (bpm)	352±17	347±12	352±12	350±10
dPdtmax (mmHg/s)	7993±290	5625±356 *	8676±618	5785±295 ‡
dPdtmin (mmHg/s)	-9211±666	-5730±335 *	-10249±711	-6010±367 ‡
CO (ml/min)	145±4	112±10 *	138±6	121±11 ‡

Table shows weights and haemodynamic data at sacrifice. Cardiac output (CO) was assessed with echo Doppler at sacrifice in anesthetized animals. LV = left ventricle, TL = tibia length, PL = placebo. * p<0.05 vs. SD-PL, ‡ p<0.05 vs. SD-EPO.

REN2 rats are anaemic and have a limited response to EPO

Table 2 shows the full blood count for all experimental groups at sacrifice. As hypothesized, REN2 rats were mildly anaemic, and had mild to moderate reductions of other haematologic parameters as well (except platelet and reticulocyte count, which were increased). Administration of EPO increased Ht levels significantly in the SD-EPO group compared to the SD-PL group (57.8±1.1 vs. 49.3±0.8%, p<0.01), however EPO administration did only marginally increase Ht in the REN2-EPO group (44.0±0.6 vs. 42.7±0.5%, compared to REN2-PL, p=NS, figure 3). An identical pattern was observed for haemoglobin levels. Furthermore, RBC, MCV and MCH were all significantly decreased in both the REN2-PL and the REN2-EPO groups, providing further evidence of anaemia. We observed an increased reticulocyte count in the REN2 groups, which is commonly seen in anaemia. EPO plasma levels increased significantly in all EPO treated groups (measured with ELISA as before,[17] see supplemental data). Deficiencies of iron, vitamin B12 and folic acid were ruled out and did not differ between SD and REN2 groups (see supplemental data).

Bone marrow dysfunction in the REN2 rats

To provide mechanistic insight into the causes of anaemia, we studied the number of BFU-E and CFU colonies with a cell proliferation assay (figure 3B and 3C). REN2 rats (treated with placebo) had a severely decreased number of BFU-E colonies, compared to SD-PL (0.33±0.11 fold, normalised to SD-PL, p<0.05). This indicates either a reduced number or a limited proliferation capacity of erythroid precursors in the BM. EPO administration increased the number of BFU-E colonies, both in the SD-EPO group (1.92±0.33 fold vs. SD-PL, p<0.05) as well as in the REN2-EPO group, albeit much less (0.88±0.21 fold vs. SD-PL, p<0.05).

To dissect abnormalities in the erythroid precursors only from general abnormalities in the BM, we also investigated number of CFU colonies. Number of CFU is only slightly, but significantly decreased in the REN2-PL group compared to SD-PL group.

Low levels of haematopoietic stem cells in REN2 rats

To confirm the findings in the cell proliferation assay, we performed FACS analysis of c-kit+ cells in BM (figure 4). Compared to SD-PL, the number of c-kit+ cells is reduced in REN2-PL (with 43%, p<0.05). In line with the BFU-E cell proliferation assay number, EPO treatment increased the number of c-kit+ cells measured by FACS in both the SD-EPO group (1.6 fold, p<0.05 vs. SD-PL) and the REN2-EPO group (to baseline, vs. SD-PL, p=NS). However, the response to EPO was less strong in the REN2 rats.

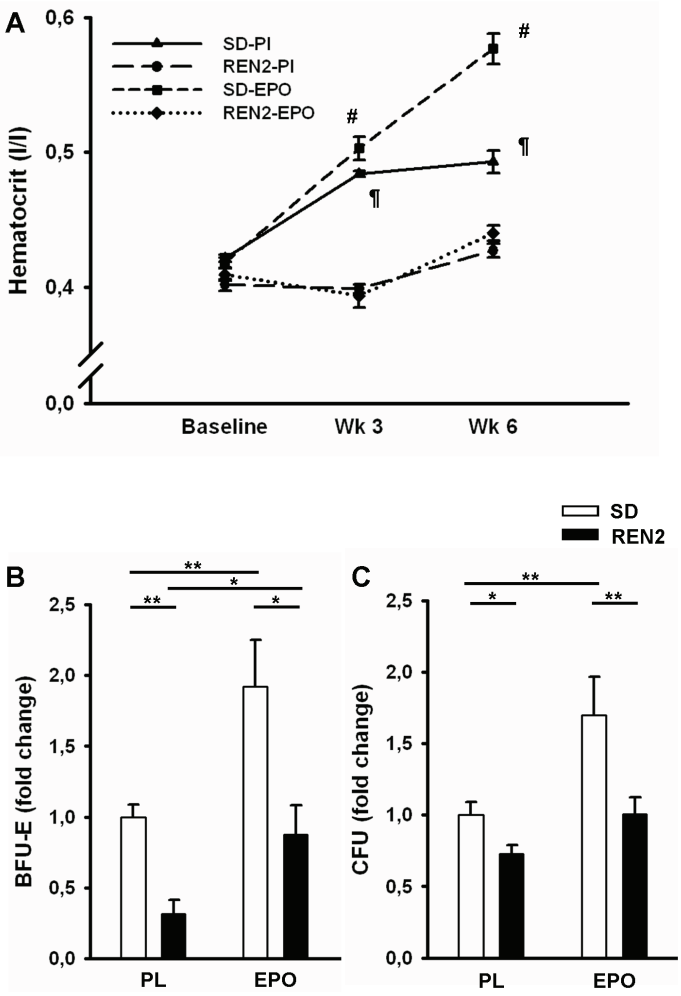

Figure 3. A. Change in haematocrit (Ht) levels during experiment. EPO administration cannot increase Ht levels in REN2 rats. B. Number of burst forming units-erythroid (BFU-E, normalised to SD-PL). C. Number of colony forming units (CFU, normalised to SD-PL). PL = placebo, BM = bone marrow, Wk = week, # p<0.05 vs. SD-PL, REN2-PL and REN2-EPO, ¶ p<0.05 vs. REN2-PL and REN2-EPO, * p<0.05, ** p<0.01.

mRNA expression of factors involved in erythroid differentiation, mobilisation and iron incorporation (figure 5)

LMO2 was significantly reduced in the REN2-PL group (34% vs. SD-PL, p<0.05). EPO treatment reduced the LMO2 expression in the SD-EPO group, but did not increase the LMO2 expression in the REN2-EPO group. Vice versa, expression of GATA-1 was 2.3 fold increased in the REN2-PL group compared to the SD-PL group (p<0.01). Treatment with EPO increased the GATA-1 expression in the SD-EPO group, but did not affect the GATA-1 expression in the REN2-EPO group. These data suggest that differentiation of erythrocyte-like BM precursors is inhibited in HF and that EPO treatment partially restores this.

Expression of MMP9 was reduced with 60% in the REN2-PL group compared to the SD-PL group (p<0.05). Treatment with EPO did not exert substantial effects on MMP9 expression in neither SD nor REN-2 rats. In contrast, SDF-1 expression was significantly increased in the REN2-PL group (3.5 fold vs. SD-PL, p<0.05). Treatment with EPO further increased the expression of SDF-1 (5.6 fold vs. SD-EPO, p<0.01). These data suggest that mobilisation of erythrocyte-like BM precursors is inhibited in HF and that this cannot be restored by EPO treatment.

mRNA expression of ferroportin was not statistically different between the four groups, albeit that ferroportin is slightly less expressed in both REN2 groups. mRNA expression of transferrin receptor was comparable between the SD-PL vs. REN2-PL groups (1.6±0.2 fold vs. SD-PL, p=NS). However, after treatment with EPO, the expression of transferrin receptor was increased in the SD-EPO group (2.8±0.7 fold vs. SD-PL, p<0.05), where the expression was not increased in the REN2-EPO group, indicating a reduction in iron incorporation in the erythroid cells in REN2 rats after EPO administration.

Table 2. Haematology

	SD-PL (n=12)	REN2-PL (n=12)	SD-EPO (n=12)	REN2-EPO (n=15)
Hb (mmol/l)	9.6±0.8	9.0±0.1	12.4±0.2 *	9.3±0.1 ‡
Ht (%)	49.3±0.8	42.7±0.5 *	57.8±1.1 *	44.0±0.6 ‡
WBC (x10^9/l)	14.6±0.5	11.4±0.3 *	14.8±0.6	11.6±0.5 ‡
RBC (x10^{12}/l)	8.8±0.2	8.0±0.1 *	9.8±0.2 *	8.0±0.1 ‡
PLT (x10^9/l)	522±32	744±33 *	485±36	711±25 ‡
RET (promille)	27.5±6.1	31.1±2.4 *	7.0±1.4 *	33.2±2.7 ‡
MCV (fl)	57.0±0.7	53.2±0.3 *	58.2±1.2	54.8±0.4 †‡
MCH (atto mol)	1204±8	1127±12 *	1247±20 *	1082±83 ‡
MCHC (mmol/l)	21.1±0.2	21.2±0.2	21.5±0.2	22.2±1.0

Table shows full blood count at sacrifice. Hb = haemoglobin, Ht = haematocrit, WBC = white blood cell count, RBC = red blood cell count, PLT = platelet count, RET = reticulocytes, MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean corpuscular haemoglobin concentration, PL = placebo. * p<0.05 vs. SD-PL, † p<0.05 vs. REN2-PL, ‡ p<0.05 vs. SD-EPO.

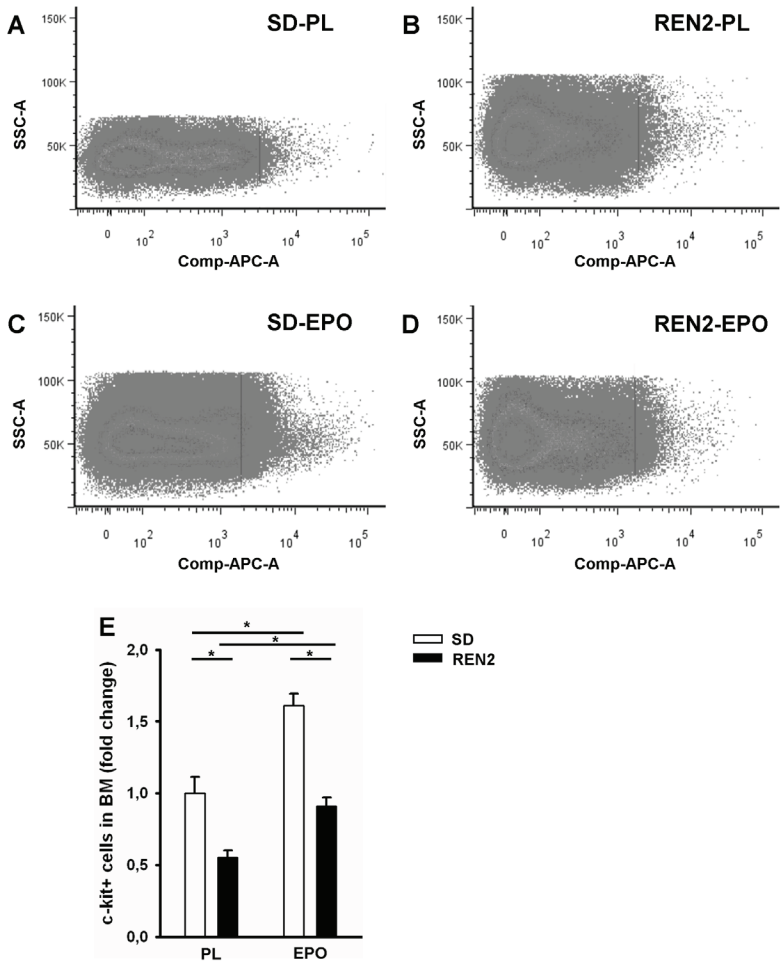

Figure 4. A-D. Typical examples of FACS analysis. Every dot is a single cells count. The x-axis (Comp-APC-A) is the gate for fluorescent cells, the y-axis (SSC-A) is the cell size. Red color is high density of cell count; blue color is lower density of cell count. E. Number of the erythroid c-kit+ cells in bone marrow. PL = placebo, BM = bone marrow. * $p<0.05$, ** $p<0.01$. For colour figure, see supplement 1.

DISCUSSION

In hypertensive REN2 rats with progressive HF we observed mild anaemia and a profound dysfunction of the erythroid lineage in the BM. Treatment with EPO with the aim to rescue this anaemia did not diminish the anaemia. Apparently, HF-associated BM dysfunction is a rather complex phenomenon that may require other treatment options. In concert with this, we observed that along the entire line of maturation of the erythroid lineage, multiple genes associated with differentiation, mobilisation and iron incorporation were abnormally expressed in BM from HF rats. Figure 6 provides an overview of the mechanisms of BM dysfunction involved in HF-associated anaemia.

We used a monogenetic rat model with overexpression of the renin gene, resulting in hypertension and concomitant HF development. These rats also develop mild anaemia, which is in line with the observations of Iversen et al., who described for the first time the existence of HF-associated anaemia in mice.[9] Our results extend this observation of Iversen to another species (rat) and moreover show that this also occurs in animals in which hypertension is the sole source for HF development.

Since HF-associated anaemia has a dramatic impact on prognosis in humans,[1-3,25] we tried to restore this by EPO treatment. As expected, EPO treatment resulted in a clear increase in Ht

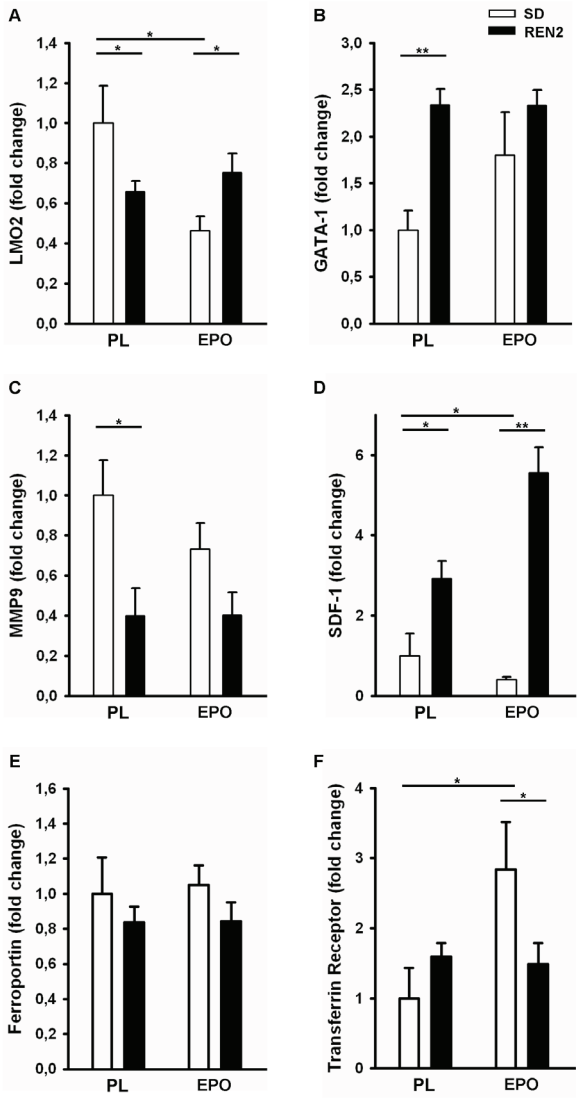

Figure 5. mRNA expression of different factors involved in differentiation (A+B) and mobilisation (C+D) of c-kit+ cells. mRNA expression of different factors involved in iron metabolism (E+F). LMO2 = LIM domain only 2 protein, GATA-1 = GATA binding protein, MMP9 = matrix metallopeptidase, SDF-1 = stromal cell-derived factor, PL = placebo. * $p<0.05$, ** $p<0.01$.

in the control rats. Surprisingly, however, EPO did not rescue anaemia in the REN2 rats and only showed marginal effects on Ht values. Since the main action of EPO is the regulation of erythropoiesis, this is apparently not the single culprit in HF-associated anaemia. Several clinical observations from our group suggested that EPO levels in HF are disconcordant with the (expected) levels of haemoglobin,[26-28] and our current observations that EPO cannot rescue HF-associated anaemia are in concert with this. This dysbalance between EPO and haemoglobin levels is related to an increased mortality risk. Mechanistically, this may be explained by a diminished response of the BM to high plasma EPO levels in HF, a phenomenon we confirm

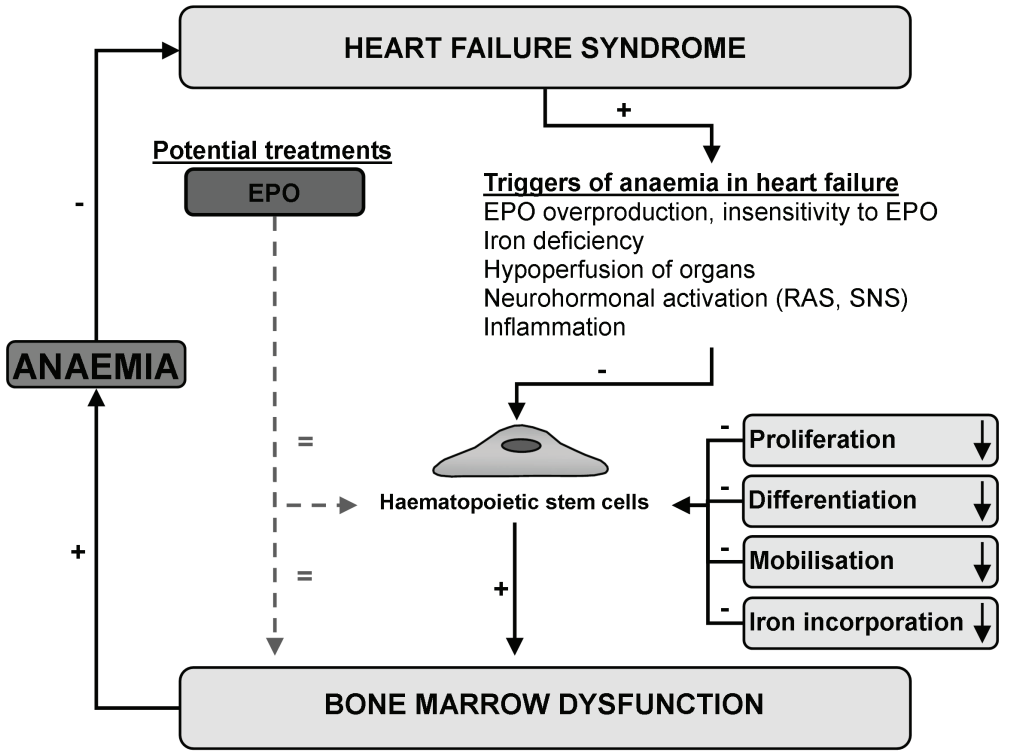

Figure 6. Schematic overview of the mechanisms of bone marrow dysfunction involved in HF-associated anaemia. Different triggers of anaemia in heart failure such as insensitivity of EPO, iron deficiency and neurohormonal activation have a negative effect on the haematopoietic stem cells in the bone marrow. Furthermore, impaired proliferation, differentiation, mobilisation and iron incorporation in haematopoietic stem cells contribute to the bone marrow dysfunction, causing anaemia and adding to the heart failure syndrome, which in turn triggers anaemia in heart failure, etc. EPO treatment does not rescue the dysfunction of the erythroid lineage and does not improve heart failure-associated anaemia. SNS = sympathetic nervous system, RAS = renin-angiotensin system. For colour figure, see supplement 1.

in our present study. Together, this indicates that in HF-associated anaemia other treatment options will be needed.

To obtain further insight in the cause of this anaemia we analysed the BM. We observed a profound (43%) decrease in BM haematopoietic progenitor cells (c-kit+ cells) in REN2 rats as compared to control animals. This is in line with the observation of Iversen et al. who observed a reduction of up to 60% of similar CD34+ cells in mice with HF.[9] Although not investigated in our rat model, they suggested that the mechanisms of HF-associated anaemia may depend on the etiology of the cardiac disease, where inflammation seems to be dominant in ischemic HF, while neurohormonal factors seem the player in non-ischemic HF.[11]

To further characterize BM dysfunction and the irresponsiveness to EPO in the REN2 rat model of HF, we performed RT-qPCR analysis to measure the expression of markers of differentiation (LMO2, GATA-1) and mobilisation (MMP9, SDF-1) of the erythroid lineage and incorporation of iron in these cells (ferroportin, transferrin receptor) (figure 5). LMO2, a positive regulator of erythroid differentiation,[29] was downregulated in the REN2-PL group, which may contribute to the decreased number of BFU-E. GATA-1 is a transcription factor playing a key role in gene regulation during erythroid differentiation.[30] This factor is strictly controlled during development and differentiation of erythroid cells and essential for the survival of erythroid progenitors.[31] GATA-1 mRNA expression is 2.3 fold increased in our experiments in the REN2-PL group, indicating that survival of the erythroid progenitors is promoted in this weakened erythropoietic system.

Furthermore, impaired mobilisation of erythroid progenitors from the BM could contribute to the lack of increase in Ht upon EPO stimulation. Degradation of BM SDF-1 by MMP9 contributes to increased mobilisation of erythroid progenitors.[32] So, decreased levels of MMP9, which result in increased levels of BM SDF-1 could in turn lead to impaired mobilisation of erythroid progenitors from the BM in REN2 rats.

Analysis of factors involved in iron transportation yielded some interesting results. mRNA expression of ferroportin, involved in iron transportation from inside of a cell to the outer surface, was not different between any group. mRNA expression of transferrin receptor, involved in maintaining cellular iron homeostatis, was comparable between the SD-PL vs. REN2-PL groups. However, after treatment with EPO, expression was increased in the SD-EPO group, where expression was not increased in the REN2-EPO group. We speculate that the lower expression of transferrin receptor in REN2 rats treated with EPO is associated with lesser iron incorporation in erythroid cells, which may explain in part the lesser response to EPO. In general, we observed a changed expression profile of genes involved in differentiation, mobilisation and incorporation of iron in BM.

Besides defects in the erythropoietic lineage of the BM (reduction in c-kit+ cells and BFU-E), we also observed a reduction in number of CFU (with 27%) compared to control animals. EPO treatment did not improve these values in the REN2 rats. We recently showed that human HF is associated with a profound and general BM dysfunction, simultaneously affecting multiple haematopoietic lineages.[10] Since all these patients obtained optimal HF treatment and were obviously more heterogeneous, our confirmation in this monogenetic rat model further confirms these observations in a human population and show that it is treatment independent.

Moreover, HF-associated anaemia appears to be a general phenomenon in mammals.
dPdtmax, dPdtmin and CO were all significantly decreased in the REN2 groups, compared to the SD groups indicating development of HF. Hypoperfusion of the bone marrow by reduced CO could potentially affect erythropoiesis in HF.[8] However, we believe that hypoperfusion is not (yet) a critical factor, as we have sacrificed the REN2 rats before overt (decompensated) HF had ensued.
Because the REN2 transgene is expressed ubiquitously and thus also in the haematopoietic cells, we cannot exclude that the effects of the transgene mediate the phenotype at least to some extent. However, the sequel of events in the development of HF in the REN2 rat (hypertrophy, diastolic dysfunction, systolic dysfunction, overt HF, death) are very much alike the human situation. Furthermore, the mild anaemia and bone marrow dysfunction associated with HF in the REN2 rat are also comparable to what we observed in earlier human studies. So, although this model (like any rodent model) has limitations, the phenotype we study herein is, at least to a large extent, comparable to the human situation.
In conclusion, in REN2 rats with progressive HF there is mild anaemia and a profound dysfunction of the erythroid lineage of the BM. Response to EPO is diminished in REN2 rats and does not rescue the dysfunction of the erythroid lineage. Multiple genes appear to be involved in this process. This does, however, not exclude EPO as a treatment option in HF, because we and others have shown that EPO has also direct positive effects on the heart, including anti-apoptotic, pro-angiogenic and anti-ischemic actions.[14-19] Therefore, EPO can be beneficial in HF, but does not improve HF-associated anaemia. The latter, probably needs a more complex treatment regimen.

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Supplemental data to "Heart failure-associated anaemia: Bone marrow dysfunction and response to erythropoietin"

SUPPLEMENTAL METHODS

Animal model

We employed 6 week old, male, homozygous TGR(mREN2)27 rats. These rats over express the mouse renin gene (ren-2d), and have a phenotype of severe hypertension and left ventricular (LV) hypertrophy, which culminates into heart failure (HF) over the course of 12-16 weeks.[1] The rats were obtained from the Max Delbrück Centre for Molecular Medicine, Berlin Buch, Germany (Prof. Dr. M. Bader). Age-matched Sprague Dawley (SD) rats were used as controls (Harlan, The Netherlands). Animals were housed under standard condition.

Experimental protocol

At baseline, three weeks and six weeks, blood was drawn from the tail vein for a full blood count: haemoglobin (Hb), haematocrit (Ht), white blood cell count (WBC), red blood cell count (RBC), red blood cell indices [mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC)], platelet count (PLT) and reticulocytes (RET) were measured. In a subset of rats, we analysed serum iron status at sacrifice to rule out deficiencies. To assess hypertension, each week systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in conscious rats using a noninvasive blood pressure monitoring system (CODA-6, Kent Scientific Corporation, Torrington, CT) as described before.[2] Mean arterial pressure (MAP) was calculated ((2xDSP+1xSBP)/3). Rats were trained for blood pressure measurement two weeks prior to the start of the experiment. Prior to sacrifice haemodynamic function was assessed invasively by introducing a 2-French microtip pressure-volume transducer (Millar Instr. Inc., Houston, TX, USA) in the right carotid artery and the left ventricle (LV).[3] Heart rate (HR), LV end diastolic pressure (LVEDP) and maximal indices of contraction and relaxation of the LV (dPdtmax and dPdtmin) were measured. Thereafter, blood was drawn, and hearts were rapidly excised and weighed. Heart tissue was processed for analysis by snap-freezing in liquid nitrogen at -80°C.

Cardiac function was prospectively assessed by echocardiography at baseline, three weeks and six weeks (Vivid 7, GE Healthcare, Chalfont St Giles, UK; equipped with a 10-MHz phase array linear transducer). The echocardiographic measurements were performed under general anaesthesia with 2% isoflurane. Both 2-dimensional (2D) images in parasternal long-axis and short-axis view and 2-D guided M-mode tracing were obtained. Long-axis views were obtained, ensuring that the mitral and aortic valves and the apex were visualized. Short-axis views were recorded at the level of mid-papillary muscles. LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) were measured from the M-mode. LV fractional shortening was (automatically) calculated as (LVEDD − LVESD)/LVEDD × 100%. Cardiac output was obtained by echo Doppler measurements over the aortic valve.

EPO plasma levels prior to sacrifice were measured with ELISA according to protocol (Quantikine EPO Immunoassay MEP00, R&D Systems Inc, Minneapolis, MN, USA).

Bone marrow isolation and MACs sorting[4]

BM was isolated from tibia and femurs of REN2 and SD rats by flushing bone marrow cavity with Dulbecco's phosphate buffered saline (PBS). Mononuclear cell fraction was obtained by density gradient centrifugation on lympholyte-rat (CEDARLANE, Sanbio BV, Canada). C-kit positive (c-kit+) cells were isolated according to protocol. Briefly, the cell pellet was washed twice with PBS containing 2 mM EDTA and 1% fetal bovine serum (FBS) to remove platelets. Cells were labelled with polyclonal rabbit anti-c kit antibody (Santa Cruz Biotechnology, CA, USA; SC-5535; 1:25 dilution) followed by goat anti-rabbit IgG Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany, 1:5 dilution) for 30 min. at room temperature. The anti-c-kit-labelled cells were sorted by using Mini-magnetic activated cell sorting columns (MS-MACs, Miltenyi Biotech, Bergisch Gladbach, Germany) in a magnetic field. The effluent was collected in chilled IMEM medium and combined with effluents from three washes. C-kit+ cells were collected into a 5 ml polystyrene round-bottom tube with 1 ml chilled IMEM medium and cell number was counted.

Quantitative real-time PCR[5]

Total RNA from c-kit+ cells and LV tissue was extracted using the Nucleospin RNA II kit, according to manufacturer's protocol (Machery-Nagel, Düren, Germany). cDNA synthesis was performed with 0.5 µg total RNA using a specific cDNA synthesis kit according to manufacturer's protocol (Quantitect Rev. Transcriptase kit, Qiagen, Venlo, The Netherlands). Quantitative real-time PCR (RT-qPCR) was performed using SYBR Green mix according to protocol (Absolute SYBR Green ROX mix, Thermo Scientific, Breda, The Netherlands, on C1000 Thermal Cycler CFX384 Real-Time PCR Detection System, Bio-Rad Laboratories, Veenendaal, The Netherlands). All targets were evaluated under the same reaction conditions: 95°C for 15 minutes, then 36 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Samples were analysed by quantification software (Bio-Rad CFX Manager 1.6). mRNA levels were expressed in relative units based on a standard curve obtained with serial dilutions of a calibrator cDNA mixture. To normalise expression data, multiple reference genes were used as described by Vandesompele et al.[6] Reference genes were chosen with little sample-to-sample variability (B2M and GAPDH). Please table S2 for a list of primers used for RT-qPCR.

S1. Iron status

	SD-PL (n=8)	REN2-PL (n=5)
Iron (µmol/l)	30.9±1.5	27.2±1.7
Folic acid (nmol/l)	>45	>45
Vitamin B12 (pmol/l)	>1476	>1476

Table shows serum iron levels at sacrifice. Data are expressed as mean±SEM. PL = placebo. Folic acid and vitamin B12 serum levels were both above upper detection limit. There was no insufficiency in iron, folic acid or vitamin B12 status.

S2. List of primers used for RT-qPCR

	RT-qPCR primer, 5' to 3'	
Gene name	Forward	Reverse
B2M	CTC GGT GAC CGT GAT CTT TC	AGT TGG GCT TCC CAT TCT CC
GAPDH	CAT CAA GAA GGT GGT GAA GC	ACC ACC CTG TTG CTG TAG
ANP	ATG GGC TCC TTC TCC ATC AC	TCT ACC GGC ATC TTC TCC TC
LMO2	TGC AGG CGA GAC TAT CTC AGG	CGC GCA TCG TCA TCT CAT AGG
GATA-1	ATG CCT GCG GCC TCT ACT AC	CAG ATG CCT TGC GGT TCC TC
MMP9	CGG GAA CGT ATC TGG AAA TTC G	CATG GCA GAA ATA GGC CTT GTC
SDF-1	CCG ATT CTT TGA GAG CCA TGT C	TTC GGG TCA ATG CAC ACT TGT C
Ferroportin	GCT GTT TGC AGG AGT CAT TG	TGG AGT TCT GCA CAC CAT TG
TFR	CTT CCG TGC TAC TTC TAG AC	ACA TAG GGT GAC AGG AAG TG

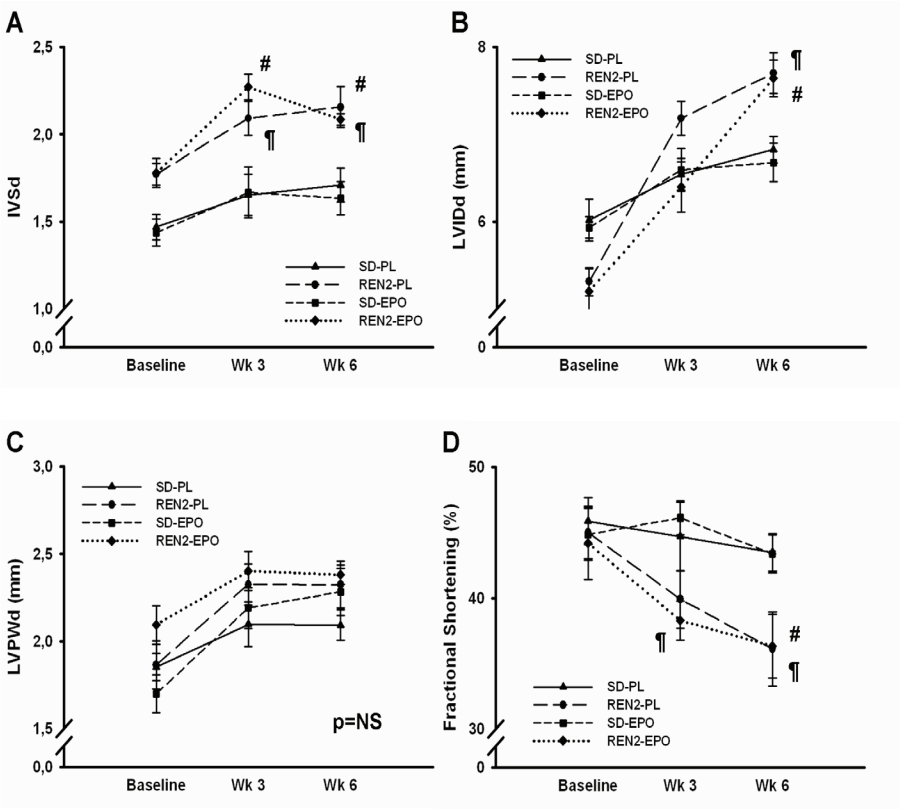
B2M = β2-microglobulin, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, ANP = atrial natriuretic peptide, LMO = lim-domain partner of TAL, GATA = zinc finger factor that binds GATA sequences, MMP = matrix metalloproteinase, SDF = stromal cell-derived factor, TFR = transferrin receptor.

S3. Plasma EPO levels

	SD-PL (n=12)	REN2-PL (n=12)	SD-EPO (n=12)	REN2-EPO (n=15)
EPO levels (pg/ml)	13±3	10±4	44±18 *	35±5 †

Table shows the plasma EPO levels. PL = placebo. * $p<0.05$ vs. SD-PL, † $p<0.05$ vs. REN2-PL. Data is expressed as mean±SEM.

S4. Echocardiographic data during experiment



IVSd = thickness of the interventricular septum in diastole (A). LVIDd = left ventricular internal diameter in diastole (B), LVPWd = thickness of the left ventricular posterior wall in diastole (C), Fractional shortening (D). PL = placebo, Wk = week. # $p<0.05$ REN2-PL vs. SD-PL and SD-EPO, ¶ $p<0.05$ REN2-EPO vs. SD-PL and SD-EPO. Data is expressed as mean±SEM.

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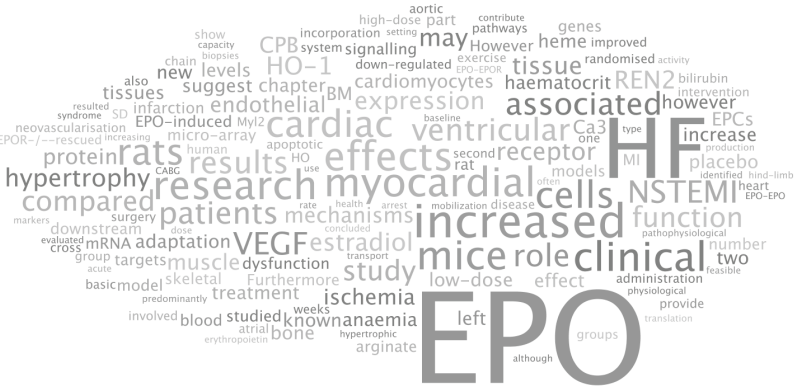
9 CHAPTER

Summary and future perspectives



SUMMARY

Heart failure (HF) is a clinical syndrome, involving not only the heart, but many other vital organs as well, including the brain, muscles, kidney and bone marrow.[1] HF is associated with an increased serious risk of hospitalisation and mortality, and the economic burden of HF for health care systems and society is enormous.[2] Atherosclerotic coronary disease is an important risk factor for the development of HF. New treatment options to preserve left ventricular function after myocardial infarction are therefore warranted. Furthermore, by increasing our understanding of the pathophysiological processes involved in HF, we may be able to improve pharmacological treatment options. Therefore, we studied the extra-haematopoietic mechanisms of erythropoietin (EPO) and the EPO-EPO receptor system in HF. Furthermore, we studied downstream pathways of erythropoietin and potential targets for intervention, to gain further insight in the pathophysiological processes involved in HF.

Extra-haematopoietic mechanisms of EPO and the EPO-EPO Receptor system

In the first part of this thesis, we explored the extra-haematopoietic mechanisms of EPO and the role of the EPO-EPO receptor system in HF. We intended to delineate the mechanisms of EPO-induced cardiac vascular endothelial growth factor (VEGF) production. Furthermore, we established if VEGF is crucial for EPO-induced improvement of cardiac performance (**chapter 3**). We studied the effects of EPO on VEGF expression in cultured endothelial cells and cardiomyocytes and EPO-treated hearts. The role of VEGF in EPO-induced neovascularisation was studied with two distinct VEGF-neutralising antibodies (or irrelevant control IgG) in an aortic sprouting assay and in rats with HF after myocardial infarction (MI) treated with EPO. EPO stimulated VEGF mRNA expression through the STAT3 signal transduction pathway in neonatal rat cardiomyocytes, but not in endothelial cells or fibroblasts. Similarly, the direct effects of EPO on endothelial sprouting were modest and VEGF independent. In rats with HF, EPO increased VEGF protein expression predominantly in cardiomyocytes, associated with a 37% increase in capillary density and improved cardiac performance. Administration of VEGF-neutralising antibodies abrogated the salutary effects of EPO on myocardial microvascularisation and function. VEGF neutralisation attenuated EPO-induced proliferation of myocardial endothelial cells and reduced myocardial incorporation of endothelial progenitor cells (EPCs) in rats with alkaline phosphatase-labelled bone marrow cells. We conclude that VEGF is crucial for EPO-induced improvement of cardiac function in HF. EPO fosters VEGF expression predominantly in cardiomyocytes, which in turn stimulates myocardial endothelial proliferation and incorporation of EPCs.

The role of the EPO receptor in physiologic hypertrophy was studied next (**chapter 4**). We have demonstrated that EPO via its receptor exerts cardioprotective effects in pathophysiological hypertrophy (e.g. MI and HF).[3,4] It is unknown however if the EPO-EPOR signalling pathway exerts similar results in physiological hypertrophy. EPO receptor rescued knock-out mice ($EPOR^{-/-}$-rescued mice) are mice that express the EPO receptor exclusively in the haematopoietic cells.[5] These mice have accelerated maladaptive remodelling upon various stimuli, because of an insufficient angiogenic response.[6-9] Mice had unlimited access to a running wheel for

four weeks. We show that EPOR$^{-/-}$-rescued mice have a reduced exercise capacity, which is associated with an impaired adaptation of cardiac and skeletal muscle. The increased exercise capacity seen in WT mice resulted in a larger cardiac adaptation in HW in the WT-ex mice (26%) as compared to the EPOR-ex mice (10%). This increased cardiac adaptation was accompanied by myocyte hypertrophy and neovascularisation as shown with the increase in cross sectional area and capillary-to-myocyte ratio in both cardiomyocytes as skeletal muscle. EPOR$^{-/-}$-rescued did not show this adaptation to increased workload. Furthermore, exercised WT mice show a shift in muscle fibre type to adapt to the increased workload (type II to type I), where EPOR$^{-/-}$-rescued mice do not show this adaptation al all. We concluded that the absence of EPO-EPOR signalling leads to a lesser exercise capacity upon voluntary wheel-running. We suggest that EPO-EPOR signalling is important for physiological adaptation during exercise.

Studies in animal models of acute myocardial infarction suggest that ischemia-reperfusion (I/R) injury accounts for up to 50% of the final size of an MI, and in I/R models, a number of strategies have been shown to ameliorate lethal reperfusion injury.[10] Of note, the translation of these beneficial effects into the clinical setting has been disappointing.[11] To corroborate our data we collected in animal studies, we evaluated a human model of I/R injury and compared the rate of apoptosis after cardiopulmonary bypass (CPB) and cardioplegic arrest during coronary artery bypass grafting (CABG) surgery between atrial and ventricular tissue (**chapter 5**). During CABG surgery with CPB and cardioplegic arrest, sequential biopsies were taken from the right atrial appendage and left ventricular anterior wall before CPB and after aortic cross clamp release. We assessed the change in number of apoptotic cells and biochemical markers of myocardial ischemia and renal dysfunction. CPB was associated with a transient small, but significant increase in CK, CK-MB, troponin-T and NT-proBNP levels. A higher number of apoptotic cells as assessed by caspase-3 staining was found in the ventricular biopsies taken after aortic cross clamp release compared with the biopsies taken before CPB. The number of apoptotic cells in the atrial appendage was not altered during CPB. Correlation between the duration of aortic cross clamp time and the change in caspase-3 positive cells in the left ventricular wall was of borderline significance. We concluded that CABG surgery with CPB and cardioplegic arrest is associated with an elevated rate of apoptosis predominantly in ventricular tissue. Ventricular tissue may be more sensitive to detect changes than atrial tissue, and may be more useful to investigate the protective effects of therapeutic intervention.

Downstream pathways of erythropoietin: feasible targets for intervention

In the second part of this thesis, we focused on feasible targets for intervention to ameliorate the effects of ischemia in the heart. In **chapter 6**, we investigated whether administration of estradiol to male mice augments mobilisation of bone marrow-derived EPC and incorporation into foci of neovascularisation after hind-limb ischemia, thereby contributing to blood flow restoration. EPC culture assay performed two weeks after estradiol pellet implantation revealed a significantly greater number of circulating EPCs in the high-dose estradiol group than in the low-dose estradiol and placebo groups. Three and four weeks after induction of hind limb ischemia, perfusion was significantly greater in high-dose estradiol mice than in low-dose estradiol or placebo treated mice. At one and four weeks after hind-limb ischemia

surgery, more bone marrow-derived EPCs, identified as β-galactosidase-positive cells, were observed in ischemic regions from high-dose estradiol animals than in low-dose or placebo groups. These results indicate that estradiol dose-dependently increases the levels of EPCs in peripheral blood in male animals, improves the recovery of blood flow, and decreases limb necrosis after hind-limb ischemia, and that this enhancement occurs, in part, through augmentation of EPC mobilisation and greater incorporation of bone marrow-derived EPCs into foci of neovascularisation.

I/R-injury, for example in acute myocardial infarction, is accompanied by an severely increased production of reactive oxygen species and subsequent cell death, contributing to the loss of viable myocardial tissue after sustained MI.[10] In **chapter 7** we evaluated the safety and efficacy of an exogenous heme oxygenase (HO)-1 inducer (heme arginate) in patients with a non-ST-elevation myocardial infarction (NSTEMI) to scavenge these reactive oxygen species. Degradation products of HO-1 activity have been suggested to act as anti-oxidants.[12-15] The inducible isoform of HO, named HO-1, can be induced in virtually all tissues and represents the rate limiting step in the degradation of the pro-oxidant heme molecule into biliverdin, carbon monoxide (CO), and iron.[12] Biliverdin is then converted by biliverdin reductase (BVR) into bilirubin, whereas iron induces the expression of the iron sequestering molecule ferritin.[12,16-18] At physiological concentrations, bilirubin, CO and ferritin all exhibit high ant-oxidant and cytoprotective properties.[18-21] In our study, patients with NSTEMI were open label randomised to a single dose of heme arginate or placebo. Heme arginate or placebo was infused within three hours after hospital admission for NSTEMI. A single high dose of heme arginate after NSTEMI did not increase plasma ferritin and total, direct and indirect bilirubin concentrations, HO-1 mRNA expression and HO protein activity in peripheral blood mononuclear cells as compared to the placebo group. Safety parameters were all comparable between the two groups. However, NSTEMI itself did activate the HO-1 system. Median total bilirubin levels were increased with 60% after NSTEMI. Also, median HO-1 mRNA (17.5 times compared to baseline) and median HO protein activity (780% compared to baseline) were increased after NSTEMI. We concluded from this study that although there is an endogenous HO-1 response to NSTEMI, adding an exogenous HO-1 inducer does not increase downstream HO-1 markers further, however, heme arginate administration is safe in patients with NSTEMI. We suggest that before embarking on larger sized trials, a dose-finding study should be carried out, in order to determine the optimal dose for patients with MI (NSTEMI of STEMI).

One of the common co-morbidities in HF Is anaemia, which presence is associated with a particularly poor outcome.[22-24] The causes of anaemia in HF are only partially understood, although several mechanisms have been implicated.[25-29] Because bone marrow dysfunction may contribute to HF-associated anaemia, we investigated mechanisms of BM dysfunction in an established model of HF (**chapter 8**), the transgenic REN2 rat, which is characterized by severe hypertrophy and ventricular dilatation and Sprague Dawley (SD) rats as controls. Secondly, we investigated whether stimulation of haematopoiesis with EPO could restore anaemia and BM dysfunction. Number of burst forming units-erythroid (BFU-E) was severely decreased in BM of REN2 rats compared to SD rats. EPO treatment increased haematocrit in the SD-EPO group, however in the mildly anaemic REN2 rats, there was no effect. This was paralleled by a 67%

decrease in BFU-E in BM of REN2 rats compared to SD. EPO significantly improved BFU-E in both SD and REN2, but could not restore this to control levels in the REN2 rats. Expression of several genes involved in differentiation and mobilisation of the BM is deregulated in REN2 rats, and EPO did not normalise this. Altogether, these results suggest that BM dysfunction is an important contributor to HF-associated anaemia in the hypertensive REN2 rat and that EPO is not an effective agent to treat HF-associated anaemia, at least in this model.

TRANSLATIONAL RESEARCH

Before discussing what future experiments could help to unravel the pathways of EPO and the potential clinical applicability of EPO or its derivatives, I would like to share some thoughts on translational research.

To improve human health, scientific discoveries must be translated into practical applications. Such discoveries typically begin at 'the bench' with basic research - in which scientists study disease at a molecular or cellular level - then progress to the clinical level, or the patient's 'bedside.' Often, preclinical researchers are doing the 'why and how', where clinical researchers are looking for the best ways to treat their patients. Knock-out and overexpression models are used to unravel the mechanisms behind diseases. However, this approach in the human setting is clearly not feasible. Scientists are however increasingly aware that this bench-to-bedside approach to translational research is really a two-way street. Basic scientists provide clinicians with new tools for use in patients and for assessment of their impact, and clinical researchers make novel observations about the nature and progression of disease that often stimulate basic investigations. Translational research has proven to be a powerful process that drives the clinical research engine. Unfortunately, as is common in translation research, results found in experimental models, are not necessarily translated to the human setting. Preclinical and clinical research are two distinct worlds and often there is a disappointing consistency between these two worlds. Growing barriers between clinical and basic research, along with the ever the increasing complexities involved in conducting clinical research, are making it more difficult to translate new knowledge to the clinic - and back again to the bench.

However, a stronger research infrastructure could strengthen and accelerate this critical part of the clinical research enterprise. Of pivotal importance is the doctor-scientist (MD, PhD), who is able to bridge the gap between basic and clinical science. Their increasing awareness that not only the 'why and how' is of major importance, but also the feasibility of these new findings for their patients should bring health care forward.

FUTURE PERSPECTIVES

Now the results of the HEBE III are published,[30] two more large-scale randomised EPO trials still have to reveal their results. First, the results of second study evaluating the effect of EPO in first ST-segment elevation MI (REVEAL) are awaited in the fourth quartile of 2010.[31] Second, the results of first large randomised study to evaluate the effects of EPO in HF (RED-HF) are awaited in 2011.[32] Results of both should give a definitive answer on the use of EPO in cardiovascular disease. However, regardless of these results, the search for cellular protection against ischemia and I/R-injury continues.

The dosing regimens used in previous HF 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.[33] Therefore, our group 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.[3] 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 carbamylated EPO,[34-39] and other non-

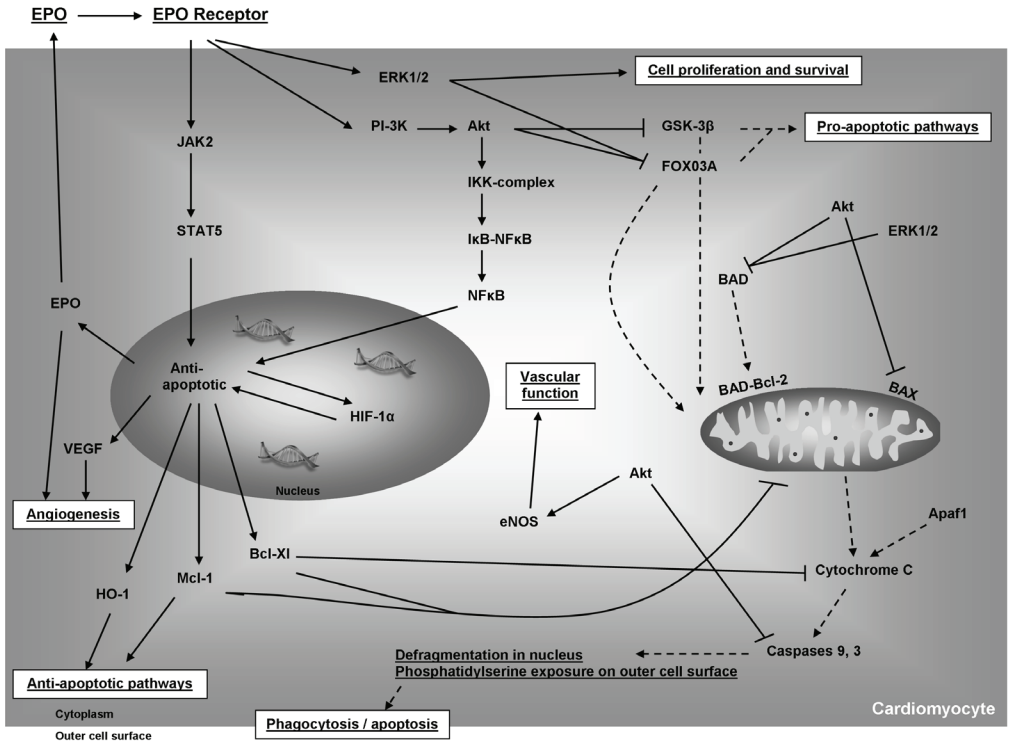


Figure 1. Overview of the potential mechanisms of cytoprotection of erythropoietin (EPO). Activation of the EPO receptor by EPO leads to downstream activation of intracellular pathways, such as PI3K/akt, ERK1/2, and STAT. These pathways originate with the binding of EPO to the EPO receptor to activate JAK2. Subsequently, PI3K and Akt are activated by downstream phosphorylation. Activation of STAT can regulate EPO-mediated cell protection and protect against apoptosis. EPO maintains cellular integrity and prevents apoptosis through a number of pathways, such as the modulation of apoptosis protease activating factor-1, the release of cytochrome c, and the prevention of activation of caspases 9 and 3. Dotted lines, suppressed pathways; solid lines, activated pathways. For colour figure, see supplement 1.

Table 1. Baseline characteristics

	Con-PL (n=5)	Con-EPO (n=4)	MI-PL (n=5)	MI-EPO (n=5)
BW (gr)	359±7	433±22	397±9	403±9
HW (mg)	1181±37	1650±172*	1959±297*	1598±89*
HW/BW (mg/gr)	3.28±0.11	3.82±0.23	4.91±0.70*	3.97±0.20*‡
MAP (mmHg)	90.8±3.8	97.0±3.1	91.0±4.5	89.6±2.9
HR (bpm)	384±36	321±13	325±13	333±9
dPdtmax (mmHg/s)	9900±764	10556±976	6225±1126*	9518±247
dPdtmin (mmHg/s)	-10775±462	-9753±743	-5737±830*	-8456±274
LVEDP (mmHg)	2.8±0.5	2.7±1.8	19.8±7.1*	12.8±2.5*‡
Ht (%)	51±1.9	63±5.2*	47±1.9	59±3.5*‡
ANP (fold change)	1.00±0.12	1.78±0.40	7.65±1.23*	5.17±1.06*‡

Table shows the baseline characteristics of the groups used in the micro-array. Sprague Dawley rats were used. HF was induced by permanent ligation of the left ascending coronary artery to produce a myocardial infarction (MI) and control rats received a sham procedure. Three weeks after MI, rats were randomly assigned to treatment with the long-acting erythropoietin darbepoetin alfa (EPO, 40 μg/kg) or placebo (PL), given once every three weeks subcutaneous. Con = control rats, BW = body weight, HW = heart weight, MAP = mean arterial pressure, HR = heart rate, dPdtmax and dPdtmin are indices of maximal contraction and relaxation, LVEDP = left ventricular end diastolic pressure, Ht = haematocrit. To determine if true heart failure existed, we measured atrial natriuretic peptide (ANP, shown as fold change vs. SD). Data is presented as mean±SEM. *p<0.05 vs. Con-PL, ‡ p<0.05 vs. MI-PL

erythropoietic derivates of EPO,[40,41] retaining the tissue protective properties, without the undesired effect on erythropoiesis.[42] Two independent studies have demonstrated that these non-erythropoietic EPOs retain their cardioprotective potential in models of acute MI.[43,44] It is however uncertain whether these new EPOs will improve cardiac function in chronic HF. Large randomised trials have to be conducted to answer this question.

Another possibility is to better understand downstream EPO signalling (figure 1). We conducted a micro-array to elucidate the transcriptomic changes that occur after EPO treatment in post-MI HF. For this micro-array, we applied ventricular hypertrophic mRNA from tissue obtained from a post-MI HF model as previous described.[4,45,46] A micro-array is a multiplex technology used in molecular biology. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, known as probes. Probe-target hybridisation is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labelled targets to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a micro-array experiment can accomplish many genetic tests in parallel, in our study, the entire genome of a rat. We attempted to identify a set of key genes which are differentially regulated by EPO and can provide us with new possible targets for research.

Table 1 shows the baseline characteristics of the groups that were analysed with the micro-array technique. Among other things, we compared the transcriptome of rats that had ischemic HF

by permanent ligation of the left ascending coronary artery vs. rats with ischemic HF treated with EPO. Seven genes were identified that were up- or downregulated after EPO treatment.

Upregulated genes

Upregulated during skeletal muscle growth 5 (Usmg5), also known as diabetes-associated protein in insulin-sensitive tissues (Dapit), is a protein that is expressed in skeletal muscle. One study found it is downregulated in insulin-sensitive tissues in streptozotocin-induced diabetes.[47] It is also associated with ATP synthase in heart mitochondria.[48,49] Little is known about the exact function of Usmg5. Upregulation in response to active stretching of skeletal muscle and downregulation of Dapit mRNA in insulin-sensitive rat tissues upon induction of streptozotocin-induced diabetes may point to a role in the energy metabolism of cells, in glucose metabolism, and/or in oxidative phosphorylation.

The second upregulated gene, myosin regulatory light chain 2, ventricular/cardiac muscle isoform, or Myl2, encodes the regulatory light chain associated with cardiac myosin beta heavy chain. Calcium triggers the phosphorylation of regulatory light chain that in turn triggers contraction. Mutations in Myl2 are associated with left ventricular hypertrophic cardiomyopathy.[50-53] Upregulation by EPO could mean that Myl2 has a role in preventing left ventricular hypertrophy, however not much is known about the role of Myl2 in HF.

Downregulated genes

Carbonic anhydrase 3 (Ca3) is part of a family of enzymes that catalyze the rapid conversion of CO2 and H2O to bicarbonate and protons, a reaction that occurs rather slowly in the absence of a catalyst. The primary function of Ca3 in animals is to maintain acid-base balance in blood and other tissues, and to help transport CO2 out of tissues. Only one study describes a role for Ca3 in the emergence of cardiac hypertrophy.[54] Apparently, activation of the plasma membrane Na^+-H^+ exchanger (NHE1) and Cl^- -$HCO3^-$ exchanger (AE3) has emerged as a central point in the hypertrophic cascade. Both NHE1 and AE3 bind carbonic anhydrase (Ca3), which activates their transport flux, by providing H^+ and $HCO3^-$, their respective transport substrates. Treatment of cardiomyocytes *in vitro* with phenylephrine to induce hypertrophy, increased the expression of Ca3, where a specific inhibitor of Ca3 prevented the phenylephrine-induced hypertrophy. Possibly, the expression of Ca3 is downregulated in our *in vivo* model as a protective mechanism.

The second downregulated gene, Eukaryotic translation elongation factor 1 alpha 1, Eef1α1, encodes a ribosomal protein and plays a role in inflammation. For example, it is identified as an auto-antigen in 66% of patients with Felty syndrome, a syndrome characterised by the combination of rheumatoid arthritis, splenomegaly and neutropenia. The role of Eef1α1 in HF is not known and calls for speculation. HF is often characterised by increases in circulating pro-inflammatory cytokines (TNFα, interleukin (IL)-6, IL-1, and IL-2) and their soluble receptor or receptor antagonists that become more pronounced as myocardial function deteriorates.[55-60] In addition, increased production of pro-inflammatory cytokines and other inflammatory markers may identify patients at increased risk of developing HF in the future.[61,62] Furthermore, a cause-and-effect relationship may contribute to the development of HF, as excess TNFα and IL-6 have a deleterious effects on cardiac function. Eef1α1 might play a role in the downstream effects of inflammatory cytokines.

Some limitations to this approach apply. First, we do not see any upregulation of known factors associated with the apoptotic and neovascularising properties of EPO, such as STAT3 and VEGF.[8,45,63,64] However, one of the limitations of a micro-array is that the association with protein levels remains unknown. Second, the tissue was harvested one week after the last administration of EPO. We cannot rule out that the effects of this last administration are not picked up at a transcriptional level.
Nevertheless, these factors provide additional insight in the downstream pathways of EPO signalling and could contribute towards new therapeutic possibilities.

Table 2. Up- and downregulated genes in MI compared to MI-EPO

Upregulated			
Description	**Fold change**	**p**	**Symbol**
Upregulated during skeletal muscle growth 5	2,4	0,03	Usmg5
Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	1,7	0,03	Myl2
Ribosomal protein L22 like 1	1,5	0,04	RPL22L1
Thioesterase superfamily member 2	1,5	0,04	Them2
Downregulated			
Description	**Fold change**	**p-value**	**Symbol**
Carbonic anhydrase 3	8,1	0,02	Ca3
Eukaryotic translation elongation factor 1 alpha 1	1,6	0,01	Eef1a1
WW domain binding protein 5	1,5	0,003	Wbp5

Table describes the genes found in the micro-array. Depicted are the genes found comparing MI vs. MI-EPO, >1.5 fold change, $p<0.05$.

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POPULAR SUMMARY IN DUTCH (NEDERLANDSE SAMENVATTING)

INLEIDING

Hartfalen is een klinisch syndroom. Dit syndroom omvat niet alleen het hart, maar treft ook vele andere vitale organen, inclusief de hersenen, spieren, nieren en het beenmerg. Hartfalen is geassocieerd met een sterk verhoogd risico op ziekenhuisopnames en een verhoogd risico op overlijden. De economische last van hartfalen op de gezondheidszorg en de samenleving is enorm groot. Coronairlijden is een belangrijke risicofactor voor het ontstaan van hartfalen. Nieuwe behandelopties ter behoud van de hartfunctie na een hartinfarct zijn daarom nodig. Daarnaast, door uitbreiding van onze kennis over de pathofysiologische processen die ten grondslag liggen aan hartfalen, zijn we in staat betere farmacologische behandelopties voor hartfalen te ontwikkelen. In dit proefschrift worden twee doelen beschreven. Als eerste hebben wij de extra-hematopoietische effecten van erythropoietine (EPO) en het EPO-EPO receptor systeem in hartfalen bestudeerd. Als tweede hebben wij de verschillende processen waarmee EPO zijn werkingsmechanisme tot stand brengt onderzocht en hebben we potentiële targets voor interventie onderzocht, om meer inzicht te krijgen in de pathofysiologische processen die een rol spelen in hartfalen.

Extra-hematopoietische effecten van EPO en het EPO receptor systeem

In het eerste deel van dit proefschrift, hebben we de extra-hematopoietische effecten van EPO en de rol van het EPO receptor systeem in hartfalen bestudeerd. De intentie was om de achterliggende mechanismes van vascular endothial growth factor (VEGF) productie door EPO te ontrafelen. Verder hebben we vastgesteld dat VEGF van groot belang is voor de verbetering van de hartfunctie door EPO (**hoofdstuk 3**). We hebben de effecten van EPO op VEGF expressie onderzocht in gekweekte endotheliale cellen, in cardiomyocyten en harten behandeld met EPO. De rol van VEGF in neovascularisatie door behandeling met EPO werd bestudeerd met behulp van twee afzonderlijke VEGF neutraliserende antilichamen (en irrelevante controle) in een aorta sprouting essay en in ratten met ischemisch hartfalen behandeld met EPO. EPO stimuleerde VEGF mRNA expressie in neonatale cardiomyocyten van de rat via de STAT3 signaal transductie route, echter dit was niet het geval in endotheel cellen of fibroblasten. Vergelijkbaar hiermee waren ook de directe effecten van EPO op endotheliale sprouting bescheiden en onafhankelijk van VEGF. In ratten met hartfalen verhoogde EPO de VEGF eiwit expressie voornamelijk in cardiomyocyten, wat geassocieerd was met een 37% vergroting van de capillaire dichtheid en een verbeterde hartfunctie. Toediening van VEGF neutraliserende antilichamen deed de effecten van EPO op de myocardiale microvascularisatie en hartfunctie teniet. VEGF neutralisatie verzwakte de EPO geïnduceerde proliferatie van myocardiale endotheel cellen en verminderde de incorporatie van endotheel stamcellen (EPCs) in het hart van ratten met alkaline fosfatase gelabelde beenmerg cellen. We concluderen dat VEGF een belangrijke rol speelt in de verbetering van de hartfunctie door EPO in hartfalen. EPO bevordert de VEGF expressie voornamelijk in cardiomyocyten, wat vervolgens de proliferatie van de endotheel cellen en de incorporatie van EPCs in het hart stimuleert.

De rol van de EPO receptor in fysiologische hypertrofie werd vervolgens bestudeerd (**hoofdstuk 4**). We hebben laten zien dat EPO, via zijn receptor, beschermende effecten heeft op het hart

in pathofysiologische hypertrofie (bijvoorbeeld na een hartinfarct en in hartfalen). Het is onbekend of EPO-EPO receptor activatie dezelfde effecten heeft in fysiologische hypertrofie. EPO receptor knock-out muizen ($EPOR^{-/-}$-rescued muizen) zijn muizen die de EPO receptor exclusief in de hematopoietische cellen tot uiting brengen. Deze muizen hebben een versnelde maladaptieve aanpassing op verschillende stimuli, door een insufficiënte angiogenetische respons. Muizen hadden vrije toegang tot een loop wiel voor vier weken. We laten zien dat $EPOR^{-/-}$-rescued muizen een verminderde inspanningscapaciteit hebben, wat geassocieerd is met een verminderde adaptatie van zowel hart als spierweefsel. De verhoogde inspanningscapaciteit die gevonden werd in de controle muizen, resulteerde in een grotere aanpassing in hartgewicht in de controle muizen met inspanning (26%), vergeleken met de $EPOR^{-/-}$-rescued muizen met inspanning (10%). Deze verhoogde aanpassing van het hart ging samen met hypertrofie van de spiercellen en neovascularisatie, zoals aangetoond door een vergroot spiercel oppervlakte en een verhoogde ratio in het aantal capillairen ten opzichte van het aantal spiercellen in zowel het hart als skelet spieren. $EPOR^{-/-}$-rescued muizen lieten deze aanpassing bij inspanning niet zien. Daarnaast lieten de controle muizen een verschuiving zien in spiercel type om beter te kunnen voldoen aan de verhoogde werklast (type II naar type I), een verandering die $EPOR^{-/-}$-rescued muizen niet lieten zien. We hebben geconcludeerd dat de afwezigheid van EPO-EPO receptor activatie leidt tot een verminderde inspanningscapaciteit bij vrijwillige inspanning en suggereren dat EPO-EPO receptor activatie belangrijk is fysiologische aanpassing tijdens inspanning.

Studies in diermodellen met een acuut hartinfarct suggereren dat ischemie-reperfusie (I/R) schade tot 50% van de uiteindelijke grootte van het hartinfarct kan bepalen. In I/R modellen zijn een aantal strategieën beschreven die de schade die optreedt bij reperfusie kunnen verbeteren. Helaas is de vertaling van deze strategieën naar de klinische praktijk teleurstellend. Om onze data die verzameld is in dierexperimentele modellen te kunnen bevestigen, hebben we een humaan model van I/R schade onderzocht, waarin het percentage apoptotische cellen na cardiopulmonaire bypass (CPB) en cardioplegie tijdens een coronaire bypass operatie (CABG) werd vergeleken tussen atriaal en ventriculair weefsel (**hoofdstuk 5**). Tijdens CABG operaties met CPB en cardioplegie, werden achtereenvolgende biopten genomen van het rechter hartoor en de linker ventrikel voorwand. Dit werd gedaan voor CPB en na het opheffen van de aortale klem. Wij hebben de veranderingen in het aantal apoptotische cellen en biochemische markers van ischemie van het hart en disfunctie van de nier beoordeeld. CPB was geassocieerd met een kleine, weliswaar voorbijgaande, maar significante verhoging van CK, CK-MB, troponine T and NT-proBNP levels in het bloed. Een werd een hoger aantal apoptotische cellen gemeten in de ventriculaire biopten genomen na opheffen van de aortale klem, vergeleken met de biopten genomen voor CPB. Het aantal apoptotische cellen in het rechter hartoor veranderde niet tijdens CPB. Correlatie tussen de duur van de aortale klemming en de verandering in apoptotische cellen in de linker ventriculaire voorwand was borderline significant. Wij hebben geconcludeerd dat CABG operaties met CPB en cardioplegie geassocieerd zijn met meer apoptose, wat voornamelijk in het ventriculair weefsel plaats vind. Ventriculair hartweefsel is mogelijk gevoeliger om veranderingen waar te nemen dan atriaal hartweefsel en is mogelijk beter bruikbaar om de beschermende effecten van een therapeutische interventie te beoordelen.

Downstream signaal transductie paden van erythropoietine: mogelijke targets voor interventie

In het tweede deel van dit proefschrift hebben we ons gefocust op mogelijke targets voor interventie om de effecten van ischemie schade in het hart te reduceren. In **hoofdstuk 6** hebben we onderzocht of de toediening van oestradiol aan mannelijke muizen een verhoogde mobilisatie van EPCs uit het beenmerg tot stand brengt en of deze EPCs zich nestelen in haarden van neovascularisatie na ischemie in de achterpoot, en daardoor bijdragen aan het herstel van de bloeddoorstroming. EPC culture essay, uitgevoerd twee weken na oestradiol pellet implantatie, liet een groter aantal circulerende EPCs in het bloed zien in de hoge dosis oestradiol behandelde groep vergeleken met de lage dosis oestradiol behandelde groep of de placebo groep. Drie en vier weken na het ontstaan van de ischemie in de achterpoot, was de perfusie significant groter in de hoge dosis oestradiol behandelde groep, vergeleken met de lage dosis en placebo groepen. Één en vier weken na het ontstaan van ischemie in de achterpoot, werden meer EPCs afkomstig uit het beenmerg, aangetoond als β-galactosidase positieve cellen, aangetroffen in de ischemische gebieden van de hoge dosis oestradiol behandelde groep, dan in de lage dosis of placebo groepen. Deze resultaten laten zien oestradiol dosisafhankelijk het aantal EPCs in het perifere bloed in mannelijke dieren kan verhogen, dat oestradiol het herstel van de bloeddoorstroming bevordert, dat het zorgt voor minder necrose na ischemie van de achterpoot, en dat deze verbeteringen plaatsvinden door verbeterde mobilisatie en verbeterde nesteling in haarden van neovascularisatie van EPCs afkomstig uit het beenmerg.

I/R schade, bijvoorbeeld door een acuut hartinfarct, gaat vaak samen met een serieus verhoogde productie van vrije zuurstof radicalen, gevolgd door cel dood, wat weer bijdraagt aan het verlies van vitaal hartspierweefsel na een doorgemaakt hartinfarct. In **hoofdstuk 7** hebben we de veiligheid en effectiviteit van een exogene heme oxygenase (HO)-1 stimulator (heme arginaat) in patiënten met een non-ST-elevatie hartinfarct (NSTEMI) onderzocht. Heme arginaat werd toegediend om deze vrije zuurstof radicalen weg te vangen, waarbij de afbraakproducten van HO-1 activiteit lijken te werken als anti-oxidanten. Het isoform van HO dat induceerbaar is, genaamd HO-1, kan in vrijwel alle weefsel gestimuleerd worden en is de snelheidsbepalende stap in de afbraak van het pro-oxidante heme molecuul in biliverdine, koolstofmonoxide (CO) en ijzer. Biliverdine wordt vervolgens door biliverdine reductase omgezet in bilirubine, terwijl ijzer de aanmaak stimuleert van het molecuul ferritine. In fysiologische concentraties hebben bilirubine, CO en ferritine allemaal een hoog anti-oxidante werking en beschermende eigenschappen op cellen. In onze studie werden patiënten met NSTEMI open label gerandomiseerd naar een enkele dosis heme arginaat of placebo. Heme arginaat of placebo werden binnen drie uur na opname in het ziekenhuis voor een NSTEMI toegediend. Een enkele dosis heme arginaat verhoogde de plasma concentraties van ferritine en totaal, direct en indirect bilirubine niet. Tevens verhoogde dit niet de HO-1 mRNA expressie of de HO eiwit activiteit in perifere mononucleaire bloedcellen in vergelijking met de placebo groep. Veiligheidsparameters waren vergelijkbaar tussen de twee groepen. Daarentegen activeerde NSTEMI zelf wel het HO-1 systeem. Mediane totale bilirubine waarden waren met 60% gestegen na NSTEMI. Tevens was de mediane expressie van HO-1 mRNA (17,5 keer vergeleken met de baseline waarden) en de mediane HO eiwit activiteit (780% vergeleken met

de baseline waarden) verhoogd na NSTEMI. Uit deze studie hebben we geconcludeerd dat er een endogene HO-1 respons is op het doormaken van een NSTEMI, maar dat toevoeging van een exogene HO-1 stimulator geen extra effect heeft. Wel is het veilig om heme arginaat toe te dienen in patiënten met NSTEMI. Wij bevelen aan dat voordat er begonnen wordt aan grotere studies met heme arginaat, er eerst een studie wordt verricht om de optimale dosis te bepalen voor patiënten met een hartinfarct (zowel NSTEMI als STEMI).
Een frequent voorkomende comorbiditeit die optreedt bij hartfalen is anemie. De aanwezigheid van anemie bij hartfalen is geassocieerd met een slechte uitkomst. De oorzaken van anemie bij hartfalen worden slechts ten dele begrepen, alhoewel verschillende mechanismen worden genoemd. Omdat disfunctie van het beenmerg kan bijdragen aan het ontstaan van anemie bij hartfalen, hebben wij het mechanisme van beenmerg disfunctie onderzocht in een bekend model van hartfalen (**hoofdstuk 8**), de transgene REN2 rat, welke gekarakteriseerd wordt door ernstige hypertrofie en ventriculaire dilatatie. Sprague Dawley (SD) ratten zijn gebruikt als controle ratten. Verder onderzochten we of we met stimulatie van de hematopoiese met EPO de anemie en beenmerg disfunctie kon behandelen. Het aantal burst forming units-erythroid (BFU-E) was ernstig verlaagd in het beenmerg van REN2 ratten vergeleken met het beenmerg van de SD ratten. Behandeling met EPO verhoogde het hematocriet in de SD-EPO groep, maar in de (licht) anemische REN2 ratten was er geen effect van de behandeling met EPO op het hematocriet. Dit ging samen met een 67% verlaging van het aantal BFU-E in het beenmerg van de REN2 ratten vergeleken met de SD ratten. Behandeling met EPO verhoogde het aantal BFU-E in zowel de SD als in de REN2 ratten, maar kon dit niet tot controle levels terug brengen in de REN2 ratten. Expressie van verscheidene genen betrokken bij differentiatie, mobilisatie en ijzer metabolisme in het beenmerg is gedisreguleerd in REN2 ratten en behandeling met EPO kon dit niet normaliseren. Al deze resultaten samen suggereren dat beenmerg disfunctie een belangrijke bijdrager is aan anemie bij hartfalen in de hypertensieve REN2 rat en dat EPO niet een effectief middel is om anemie bij hartfalen te behandelen, in ieder geval niet in dit model.

TRANSLATIONEEL ONDERZOEK

Voordat ik verder ga met de discussie over toekomstige experimenten die de signaal transductie paden van EPO kunnen helpen uitpluizen en de potentiële klinische toepasbaarheid van EPO of zijn derivaten, wil ik graag enkele gedachten over translationeel onderzoek delen.
Om de gezondheidszorg te verbeteren, moeten wetenschappelijke ontdekkingen vertaald worden in praktisch toepasbare toepassingen. Zulke ontdekkingen beginnen meestal in het experimentele lab met basaal onderzoek - waarin wetenschappers ziektes bestuderen op een moleculair of cellulair niveau - en vervolgens wordt het onderzoek vervolgt op een klinisch niveau, oftewel aan het bed van de patiënt. Vaak is het zo dat de preklinische onderzoekers het hoe en waarom onderzoeken, waar klinische onderzoekers zoeken naar de beste manier om hun patiënten te behandelen. Knock-out en overexpressie modellen worden gebruikt om de mechanismes achter ziektes bloot te leggen. Echter, in de humane setting is dit duidelijk niet haalbaar. Onderzoekers zijn er zich gelukkig steeds meer van bewust dat deze experimentele lab tot patiënt aanpak van translationeel onderzoek eigenlijk tweerichtingsverkeer is. Basale

onderzoekers verschaffen clinici nieuwe instrumenten om te gebruiken bij hun patiënten en om de impact van deze nieuwe instrumenten te beoordelen. En klinische onderzoekers maken nieuwe observaties over het karakter en de progressie van ziektes, die vaak nieuwe basale experimenten tot gevolg hebben.
Translationeel onderzoek heeft zich bewezen als een belangrijk proces dat klinisch onderzoek voortdrijft. Helaas, zoals maar al te vaak voorkomt in translationeel onderzoek, worden resultaten gevonden in experimentele modellen, niet altijd vertaald naar de humane situatie, of zijn niet altijd te vertalen naar de humane situatie. Preklinisch en klinisch onderzoek zijn twee aparte werelden en er is vaak een teleurstellende overeenkomst tussen deze twee werelden. Groeiende barrières tussen klinisch en basaal onderzoek, samen met de groeiende complexiteit van het uitvoeren van klinisch onderzoek, maken het moeizamer en moeizamer om nieuwe kennis te vertalen naar de kliniek en weer terug naar het experimentele lab.
Een sterke infrastructuur in het onderzoek kan dit belangrijke deel van de dagelijkse onderzoekspraktijk versterken. Van belang is de arts-onderzoeker (MD, PhD), die in staat is om het gat tussen basaal en klinisch onderzoek te overbruggen. Hun groeiende besef dat niet alleen het hoe en waarom van belang is, maar ook de toepasbaarheid van deze nieuwe bevindingen voor hun patiënten moet de gezondheidzorg voorwaarts brengen.

TOEKOMSTPERSPECTIEVEN

Nu de resultaten van de HEBE III studie gepubliceerd zijn, is het wachten op nog twee andere grote gerandomiseerde EPO studies. In het vierde kwartaal van 2010 komen waarschijnlijk de resultaten van de tweede grote studie naar de effecten van EPO bij STEMI naar buiten. De resultaten van de RED-HF studie, die de effecten van EPO in hartfalen onderzoekt, zullen naar alle waarschijnlijkheid pas in 2011 bekend worden. Beide studies moeten, samen met de resultaten van de HEBE III studie, het definitieve antwoord geven op het gebruik van EPO in cardiovasculaire aandoeningen. Echter, ongeacht de uitkomst zal het onderzoek naar cellulaire bescherming tegen ischemie en ischemie-reperfusie schade doorgaan.
De doses EPO gebruikt in voorgaande hartfalen studies veroorzaakten allemaal een significante stijging van de hematocriet waarden. Als dit in de klinische situatie zou worden toegepast, zou dit kunnen leiden tot ongewenste neveneffecten zoals hypertensie, toevallen, vasculaire trombose en overlijden, mogelijk gerelateerd aan de plotselinge verhoging van het hematocriet. Onze onderzoekgroep heeft recent het effect onderzocht van een lage dosis EPO, die geen effect had op de hematocriet. Net als een hoge dosis EPO, zorgt een lage dosis EPO voor een significante verbetering van de hartfunctie en een verbeterde myocardiale microvascularisatie, alhoewel het effect iets minder uitgesproken was. Deze resultaten suggereren niet alleen dat de positieve effecten van EPO op het hart onafhankelijk zijn van het verhoogde hematocriet, maar suggereren ook dat een lage dosis EPO een veilige en effectieve behandeloptie is voor patiënten met hartfalen.
Een andere optie om de potentieel negatieve effecten van chronische EPO gebruik op de hematocriet waarden te vermijden, is het gebruik van het recent ontdekte gecarbamyleerde EPO en andere erythropoietische derivaten van EPO, die wel hun cel beschermende werking hebben behouden, maar zonder de onwenselijke effecten op erythropoiese. Twee onafhankelijke studies

in modellen van een acuut hartinfarct hebben aangetoond dat deze niet-erythropoietische EPO's hun beschermende potentieel behouden. Het is echter onzeker of deze nieuwe EPO's in chronisch hartfalen ook de hartfunctie kunnen verbeteren. Grote gerandomiseerde studies moeten gehouden worden om deze vraag te kunnen beantwoorden.

Een volgende mogelijkheid is het verhogen van de kennis over de downstream signalering van EPO (figuur 1). Wij hebben een micro-array verricht om de te kunnen achterhalen wat de veranderingen zijn die plaatsvinden in transcriptie van genen na EPO behandeling in hartfalen dat zich ontwikkeld heeft na het doormaken van een hartinfarct. Voor deze micro-array hebben we mRNA gebruikt afkomstig uit het hypertrofische hartweefsel uit een diermodel voor hartfalen. Een micro-array is een multiplexe technologie, welke gebruikt wordt in de moleculaire biologie. Het bestaat uit een array van duizenden microscopische spots van DNA oligonucleotiden, ook wel bekend als probes. Probe-target hybridisatie wordt meestal gedetecteerd en gekwantificeerd door detectie van fluorophoro, zilver of chemiluminicent gelabelde targets om de relatieve hoeveelheid van nucleïnezuur sequenties te bepalen in het target. Omdat een array tienduizenden probes kan bevatten, is het mogelijk om met een micro-array tegelijkertijd vele genetische tests uit te voeren, en in onze studie, het gehele genoom van een rat. We hebben geprobeerd om een set fundamentele genen te identificeren die gereguleerd worden door EPO en ons nieuwe mogelijke targets voor onderzoek kunnen verschaffen.

Tabel 1. Basis karakteristieken

	Con-PL (n=5)	Con-EPO (n=4)	MI-PL (n=5)	MI-EPO (n=5)
LG (gr)	359±7	433±22	397±9	403±9
HW (mg)	1181±37	1650±172*	1959±297*	1598±89*
HW/LG (mg/gr)	3.28±0.11	3.82±0.23	4.91±0.70*	3.97±0.20*‡
MAP (mmHg)	90.8±3.8	97.0±3.1	91.0±4.5	89.6±2.9
Hf (spm)	384±36	321±13	325±13	333±9
dPdtmax (mmHg/s)	9900±764	10556±976	6225±1126*	9518±247
dPdtmin (mmHg/s)	-10775±462	-9753±743	-5737±830*	-8456±274
LVEDP (mmHg)	2.8±0.5	2.7±1.8	19.8±7.1*	12.8±2.5*‡
Ht (%)	51±1.9	63±5.2*	47±1.9	59±3.5*‡
ANP (x verhoging)	1.00±0.12	1.78±0.40	7.65±1.23*	5.17±1.06*‡

Tabel heeft een overzicht van de basis karakteristieken van de verschillende groepen geanalyseerd met de micro-array. Sprague Dawley ratten werden gebruikt als controle. Hartfalen werd geïnduceerd door permanente ligatie van de LAD. Op deze manier werd er een hartinfarct geïnduceerd. Controle ratten kregen een sham procedure. Drie weken na het hartinfarct werden de ratten gerandomiseerd naar behandeling met het langwerkende EPO darbepoetine alfa (EPO, 40 µg/kg) of placebo (PL), wat elke drie weken subcutaan toegediend werd. Con = controle raten, LG = lichaamsgewicht, HW = hart gewicht, MAP = gemiddelde bloeddruk, Hf = hartfrequentie, dPdtmax en dPdtmin zijn afgeleiden van de maximale contractie en relaxatie van de linker ventrikel, LVEDP = linker ventrikel eind diastolische druk, Ht = hematocriet. Om te bepalen of er echt sprake was van hartfalen, hebben we atriaal natriuretisch peptide gemeten in het hart (ANP, weergegeven als x verhoging ten opzichte van de SD groep). Data is gepresenteerd als gemiddelde±SEM. * $p<0.05$ vs. Con-PL, ‡ $p<0.05$ vs. MI-PL

Tabel 1 laat de basis karakteristieken zien van de groepen die met de micro-array techniek zijn geanalyseerd. Onder ander hebben we het transcriptoom van ratten met ischemisch hartfalen vergeleken met het transcriptoom van ratten met ischemisch hartfalen behandeld met EPO. Zeven genen zijn geïdentificeerd die verhoogd of verlaagd tot expressie worden gebracht na EPO behandeling.

Verhoogd tot expressie gebrachte genen

Upregulated during skeletal muscle growth 5 (Usmg5), ook wel bekend als diabetes-associated protein in insulin-sensitive tissues (Dapit), is een eiwit dat tot expressie wordt gebracht in skelet spieren. Een studie heeft aangetoond dat het verlaagd tot expressie komt in insuline sensitief weefsel in streptozotocin-geïnduceerde diabetes. Verder is het geassocieerd met ATP synthase in de mitochondria in het hart. Er is weinig bekend over de exacte functie van Usmg5. Het verhoogd tot expressie komen in response op actief uitrekken van skelet spieren en het verlaagd tot expressie komen van Usmg5 mRNA in insuline sensitief weefsel wanneer diabetes wordt geïnduceerd door streptozotocin verwijst mogelijk naar een rol in de energie huishouding van cellen in het glucose metabolisme, en/of naar een rol in oxidatieve fosforylatie.

Het tweede gen dat verhoogd tot expressie wordt gebracht, is myosin regulatory light chain 2, ventricular/cardiac muscle isoform, oftewel Myl2. Dit gen codeert voor de regulerende lichte keten welke geassocieerd is met de cardiale myosine beta zware keten. Calcium zet de fosforylatie aan van de lichte keten, dat vervolgens leidt tot contractie van de spiercellen. Mutaties in Myl2 zijn geassocieerd met linker ventriculaire hypertrofie cardiomyopathy. Het verhoogd tot expressie brengen door EPO zou kunnen betekenen dat Myl2 een rol speelt bij de preventie van linker ventrikel hypertrofie, alhoewel niet veel bekend is over de rol van Myl2 in hartfalen.

Verlaagd tot expressie gebrachte genen

Cabonic anhydrase 3 (Ca3) maakt deel uit van een familie van enzymen die de snelle conversie van CO_2 en H_2O naar bicarbonaat en protonen mogelijk maken, een reactie die zonder katalysator zeer langzaam zou verlopen. De primaire functie van Ca3 in zoogdieren is het behouden van de zuur-base balans in het bloed en weefsels en om te assisteren in het transport van CO_2 uit de weefsels. Maar één studie beschrijft een rol voor Ca3 in het ontstaan van hypertrofie van het hart. Blijkbaar is activatie van de plasma membrane NA^+-H^+ exchanger (NHE1) en de CL^--HCO_3^- exchanger (AE3) het centrale punt in de hypertrofische cascade. Zowel NHE1 als AE3 worden gebonden door Ca3, wat deze enzymen activeert. Behandeling van cardiomyocyten met phenylephirine *in vitro* om hypertrofie te induceren, verhoogde de expressie van Ca3, waar een specifieke inhibitor van Ca3 er voor zorgde dat de hypertrofie geïnduceerd door phenylephrine niet optrad. Mogelijk is de expressie van Ca3 verminderd in ons *in vivo* model als onderdeel van een beschermend mechanisme.

Het tweede gen dat verlaagd tot expressie komt, is het Eukaryotic translation elongation factor 1 alpha 1, Eef1α1, dat codeert voor een ribosomaal eiwit en een rol speelt in inflammatie. Het is bijvoorbeeld geïdentificeerd als een auto antigen in 66% van patiënten die lijden aan het Felty syndroom, een syndroom dat gekarakteriseerd wordt door een combinatie van reumatoïde artritis, splenomegalie en neutropenie. De rol van Eef1α1 in hartfalen is onbekend en vraagt om speculatie. Hartfalen wordt vaak gekarakteriseerd door een verhoging van circulerende

pro-inflammatoire cytokines (TNFα, interleukine (IL)-6, IL-1, en IL-2) en hun oplosbare receptoren of receptor antagonisten die verhoogd tot expressie komen als de hartfunctie achteruit gaat. Daarbij is het zo dat verhoogde productie van pro-inflammatoire cytokines en andere inflammatie markers patiënten kunnen identificeren die een verhoogd risico lopen tot het ontwikkelen van hartfalen in de toekomst. Verder is het mogelijk dat een oorzaakgevolg relatie bij kan dragen aan de ontwikkeling van hartfalen, omdat verhoogde bloedspiegels van TNFα en IL-6 schadelijke effecten hebben op de hartfunctie. Eef1α1 speelt mogelijk een rol in de downstream effecten van inflammatoire cytokines.

Deze benaderingswijze heeft wel een aantal limitaties. Als eerste zien we geen verhoging van bekende genen die geassocieerd zijn met de anti-apoptotische en neovasculaire eigenschappen van EPO, zoals STAT3 en VEGF. Helaas is een van de tekortkomingen van een micro-array dat de associatie met het hoeveelheid aanwezige eiwit onbekend is. Als tweede, het weefsel gebruikt voor deze micro-array is uitgenomen één week na de laatste gift EPO. We kunnen niet uitsluiten dat de effecten van deze laatste toediening van EPO niet opgepikt worden door op een transcriptioneel niveau.

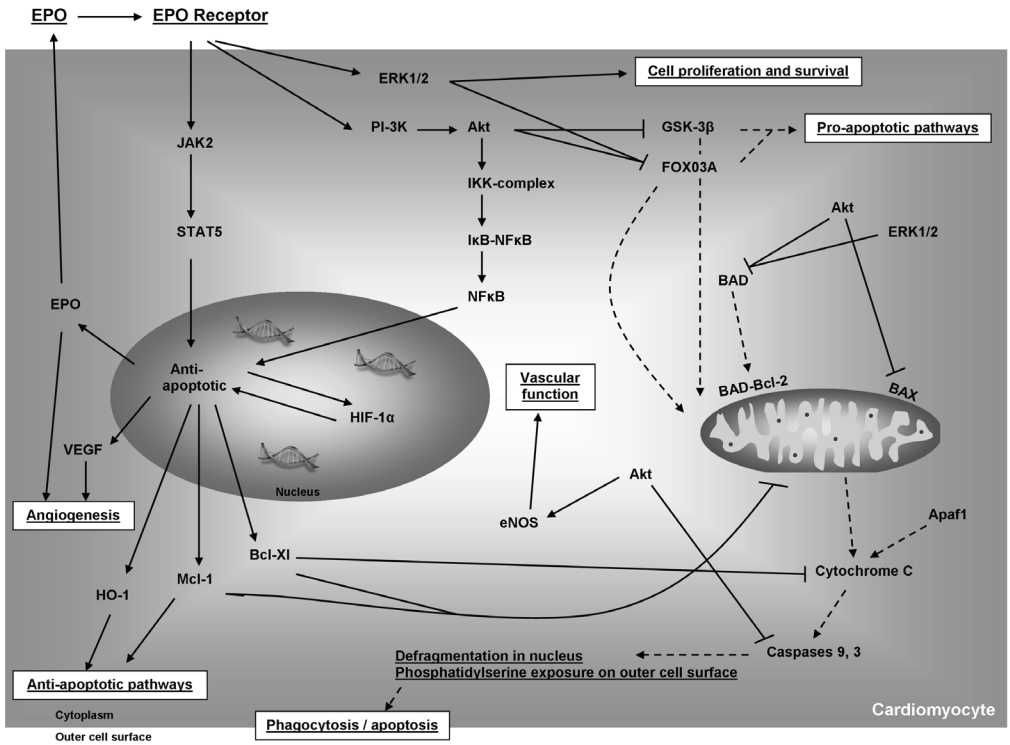
Desalniettemin kunnen deze factoren nieuwe inzichten verschaffen in de signalering en het werkingsmechanisme van EPO en zou kunnen bijdragen aan nieuwe therapeutische opties voor hartfalen.

Tabel 2. Verhoogd en verminderd tot expressie gebrachte genen in MI vergeleken met MI-EPO

Verhoogd tot expressie gebrachte genen			
Omschrijving	**x verhoging**	**p-waarde**	**Symbool**
Upregulated during skeletal muscle growth 5	2,4	0,03	Usmg5
Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	1,7	0,03	Myl2
Ribosomal protein L22 like 1	1,5	0,04	RPL22L1
Thioesterase superfamily member 2	1,5	0,04	Them2
Verminderd tot expressie gebrachte genen			
Omschrijving	**x verhoging**	**p-waarde**	**Symbool**
Carbonic anhydrase 3	8,1	0,02	Ca3
Eukaryotic translation elongation factor 1 alpha 1	1,6	0,01	Eef1a1
WW domain binding protein 5	1,5	0,003	Wbp5

Tabel heeft een overzicht van de genen die zijn gevonden met de micro-array. Hierin is de MI groep vergeleken met de MI-EPO groep, >1.5 x verhoging, $p<0.05$.

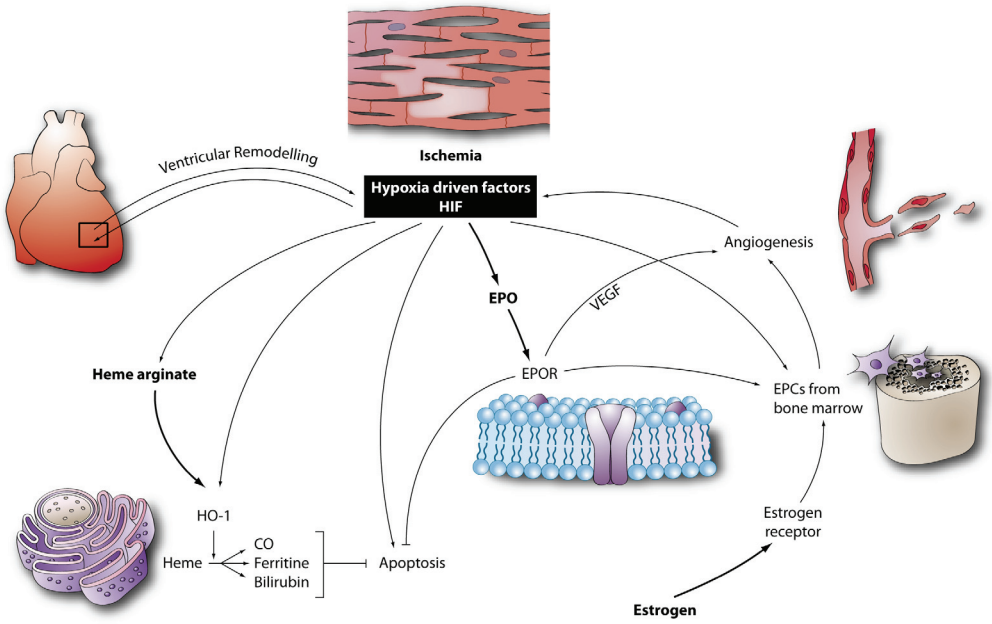
Figuur 1.



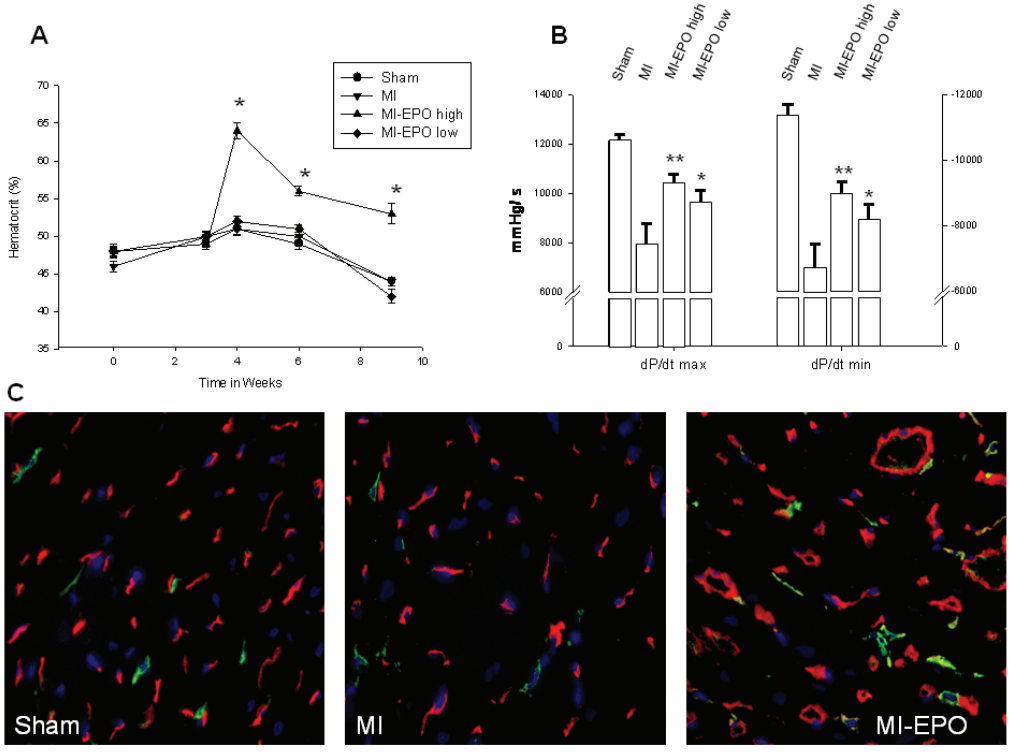
Overzicht van de potentiële beschermende mechanismes van erythropoietine (EPO) op cellen. Activatie van de EPO receptor door EPO leid tot activatie van intracellulaire signaal transductie paden, zoals PI3K/Akt, ERK1/2 en STAT. De activatie van deze signaal transductie paden starten allemaal met de binding van EPO aan de EPO receptor, waardoor JAK2 geactiveerd wordt. Vervolgens worden PI3K en Akt geactiveerd door fosforylatie. Activatie van STAT kan de cellulaire bescherming en bescherming tegen apoptose door EPO reguleren. EPO handhaaft daardoor de cellulaire integriteit en verhinderd apoptose via een aantal mechanismen, zoals de modulatie van apoptosis protease activating factor-1, de vrijzetting van cytochroom c en de verhindering van activatie van caspase 9 en 3. Gestippelde lijnen, onderdrukte signaal transductie paden; doorgetrokken lijnen, geactiveerde signaal transductie paden. Voor kleuren figuur, zie supplement 1.

SUPPLEMENT 1. COLOURED FIGURES

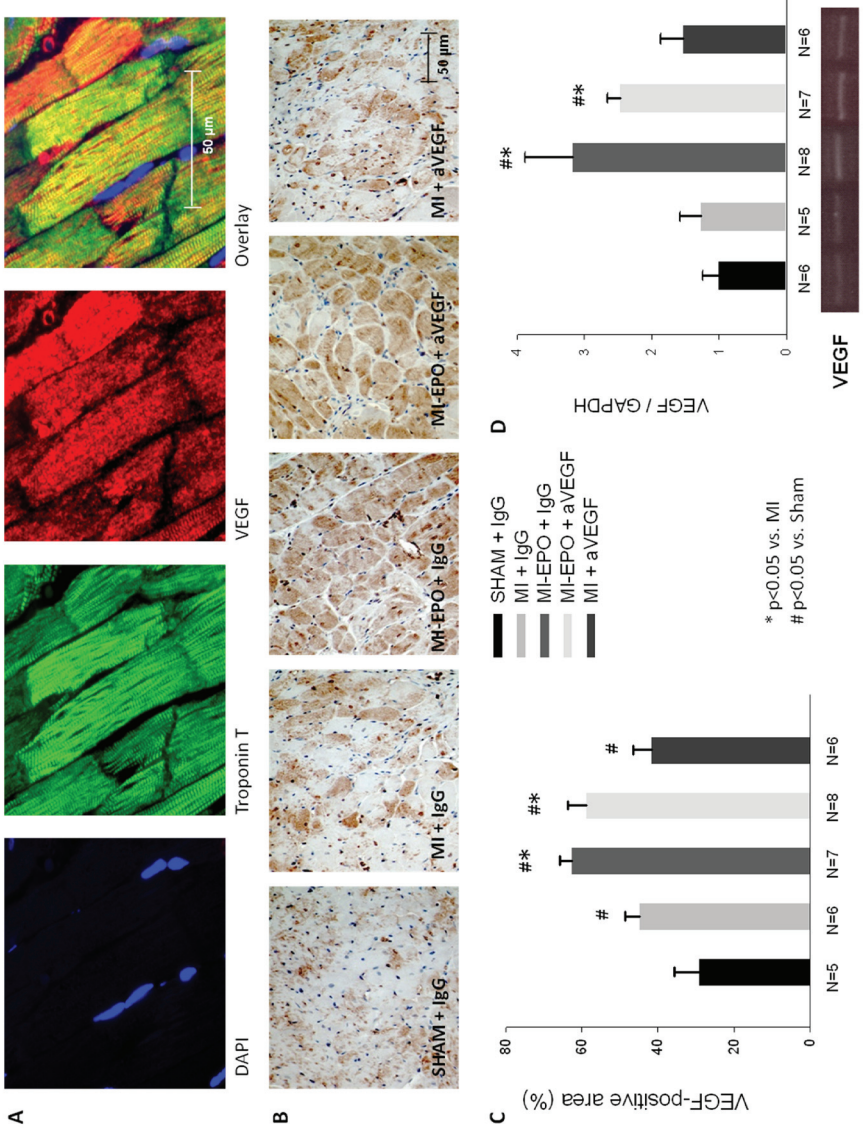
Chapter 1 - figure 1



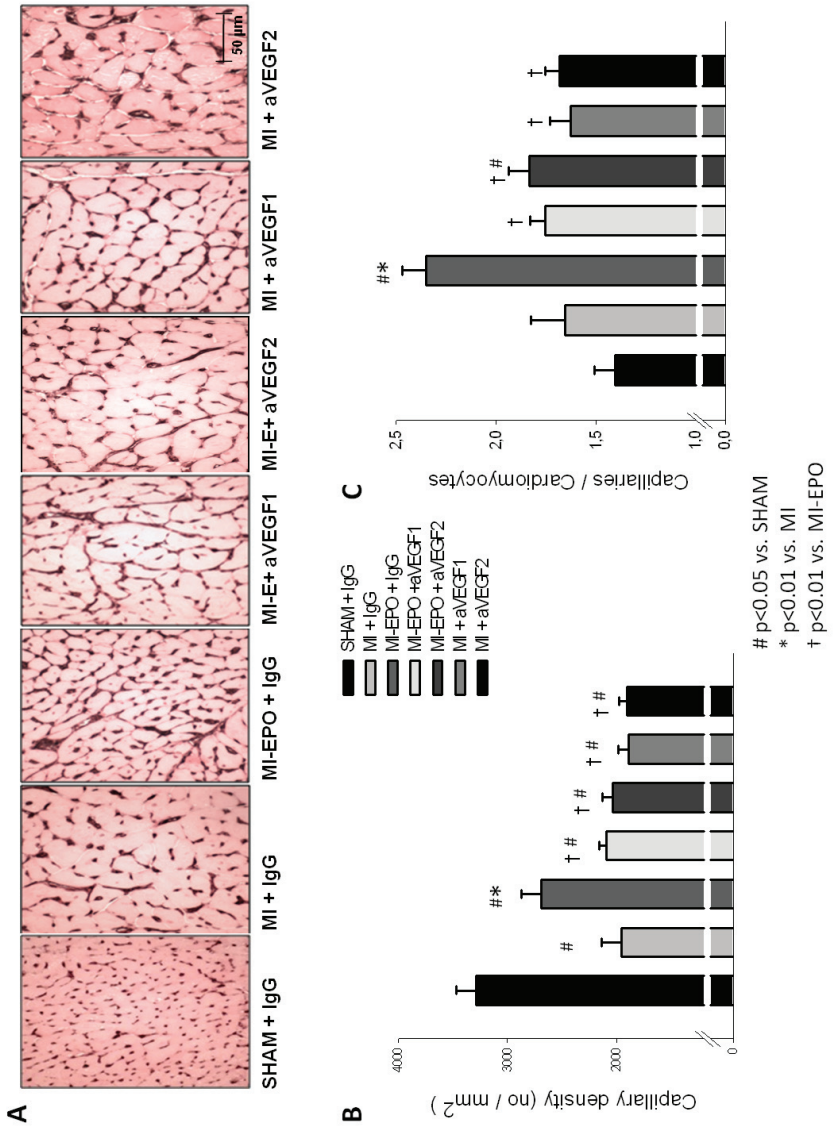
Chapter 2 - figure 3



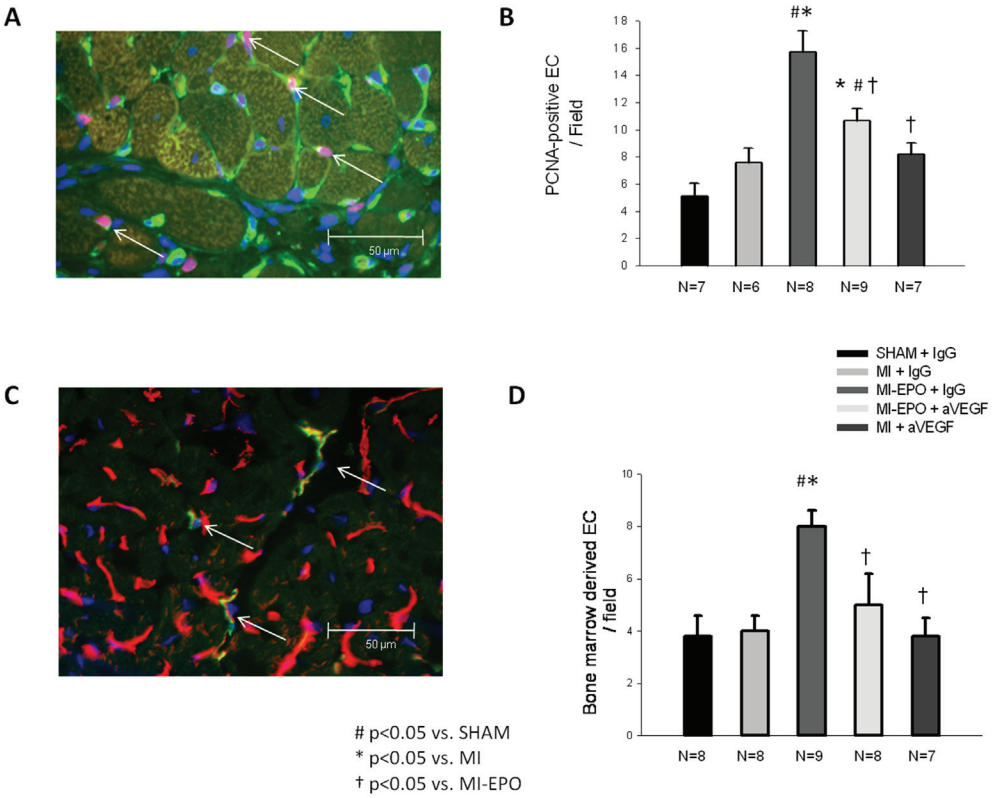
Chapter 3 - figure 3



Chapter 3 - figure 5

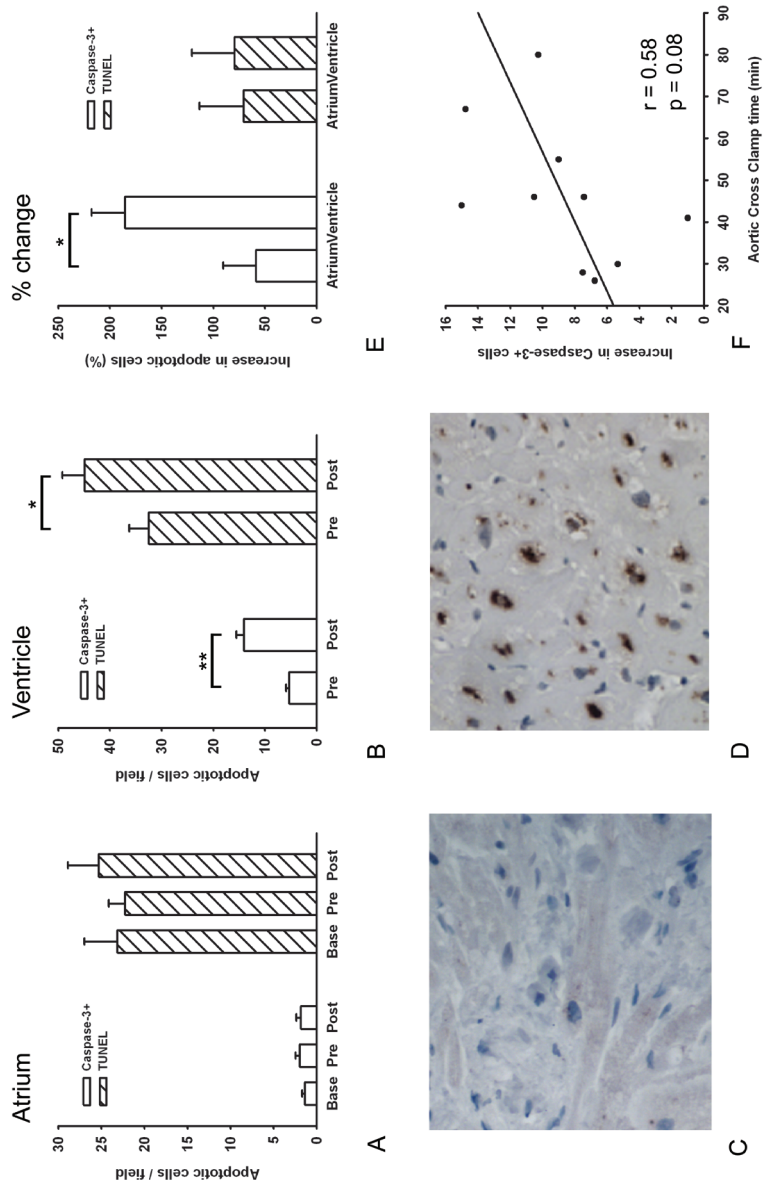


Chapter 3 - figure 6

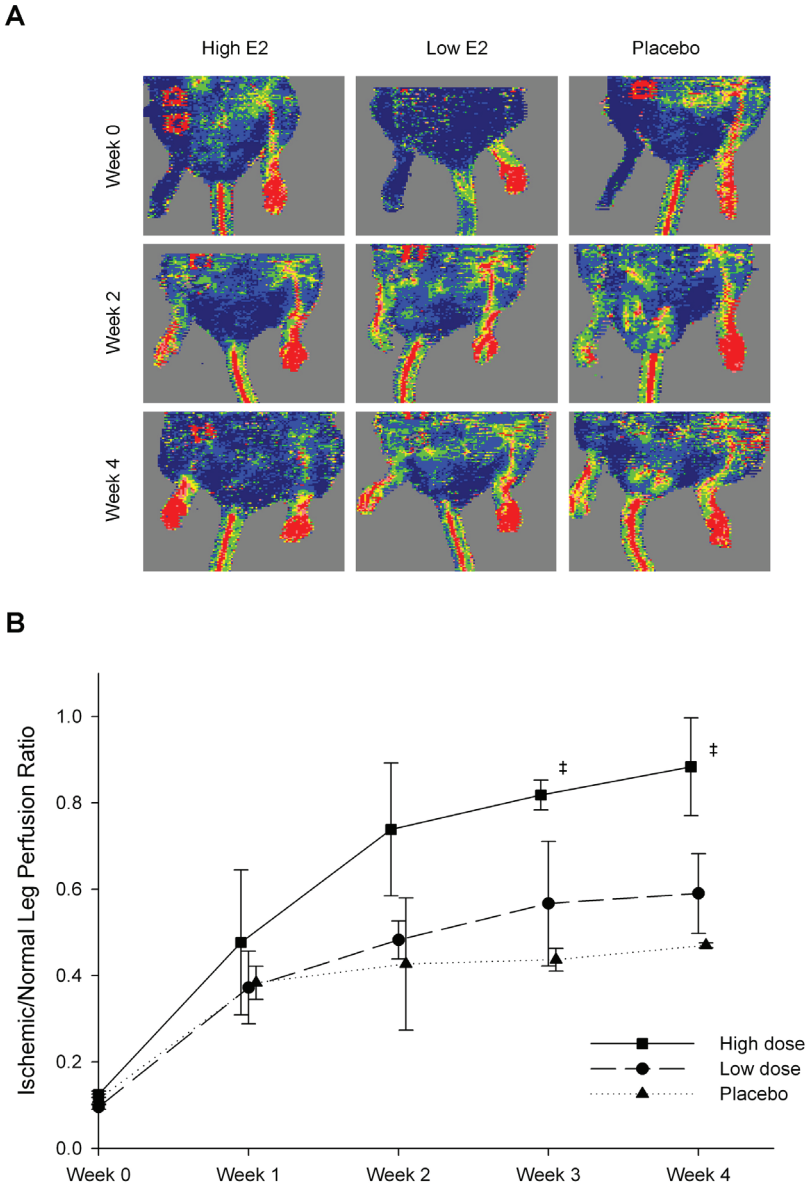


# p<0.05 vs. SHAM
* p<0.05 vs. MI
† p<0.05 vs. MI-EPO

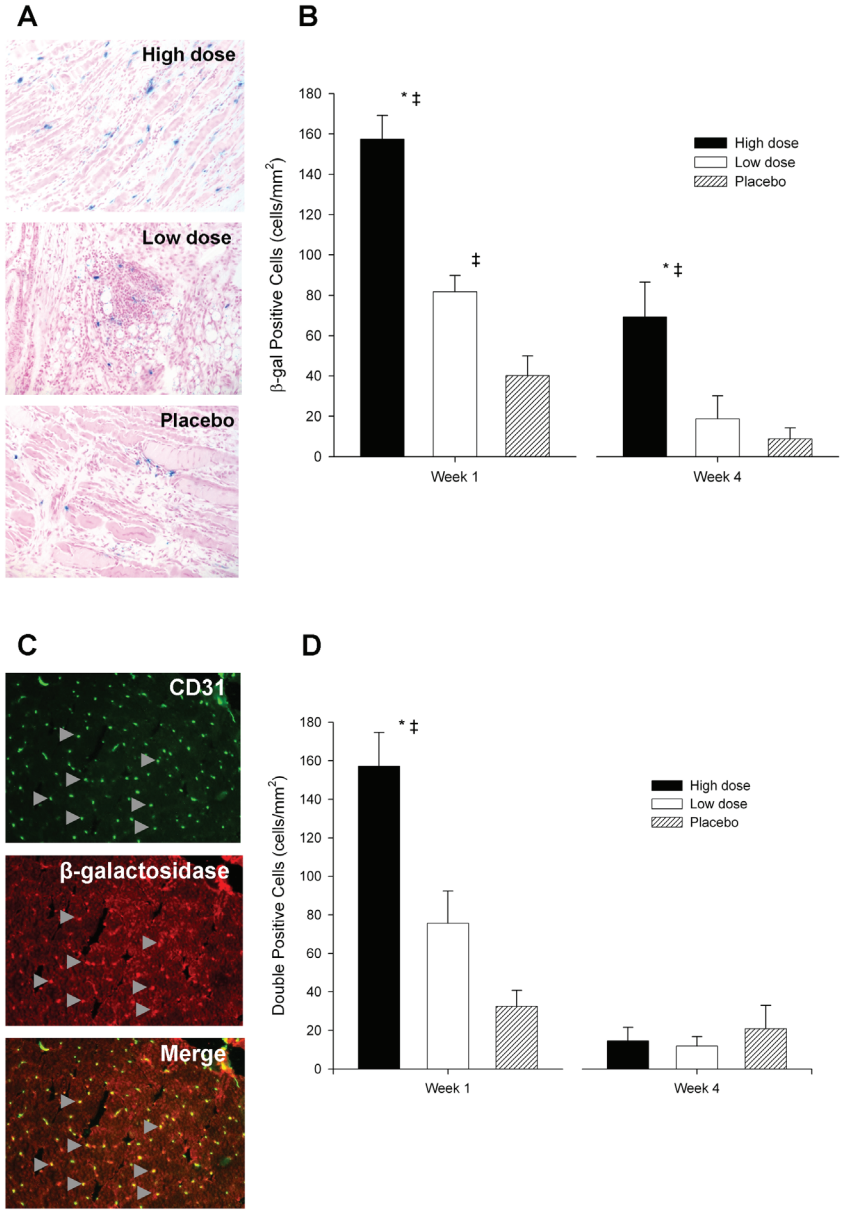
Chapter 5 - figure 2



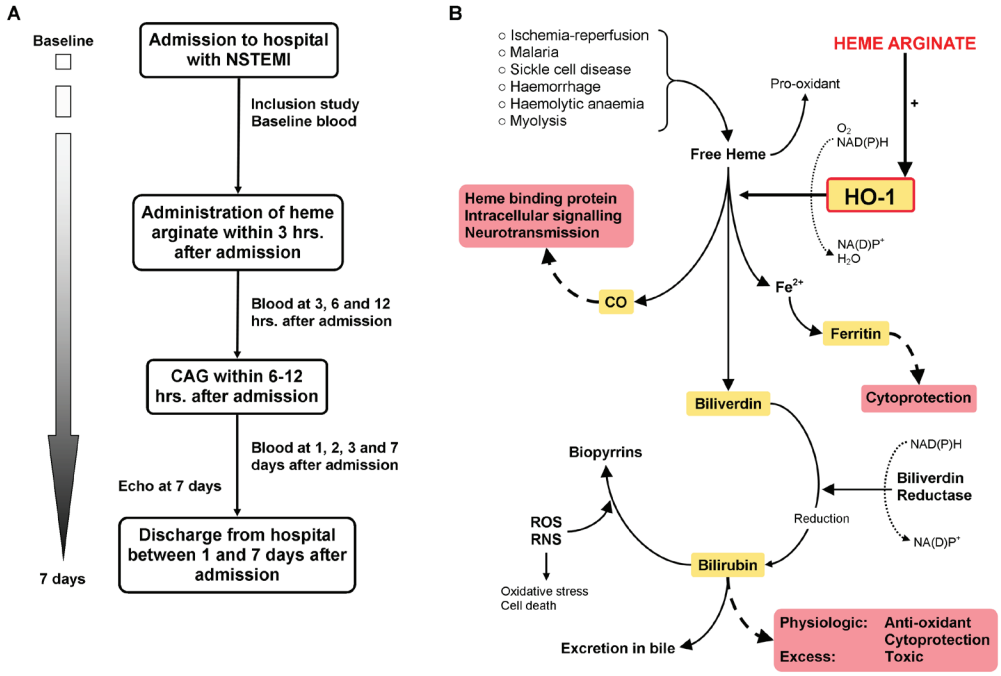
Chapter 6 - figure 2



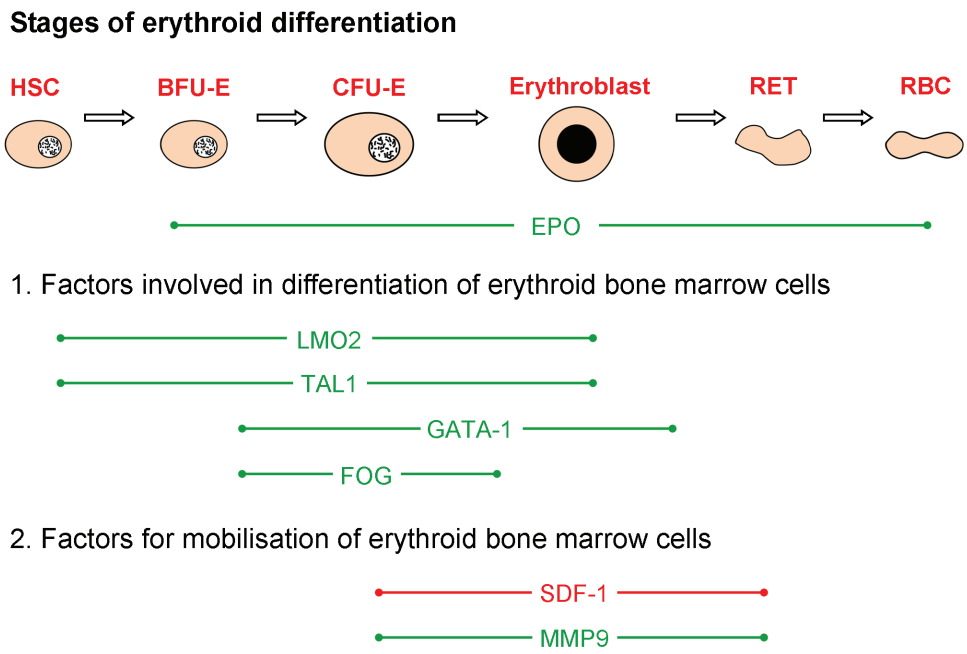
Chapter 6 - figure 4



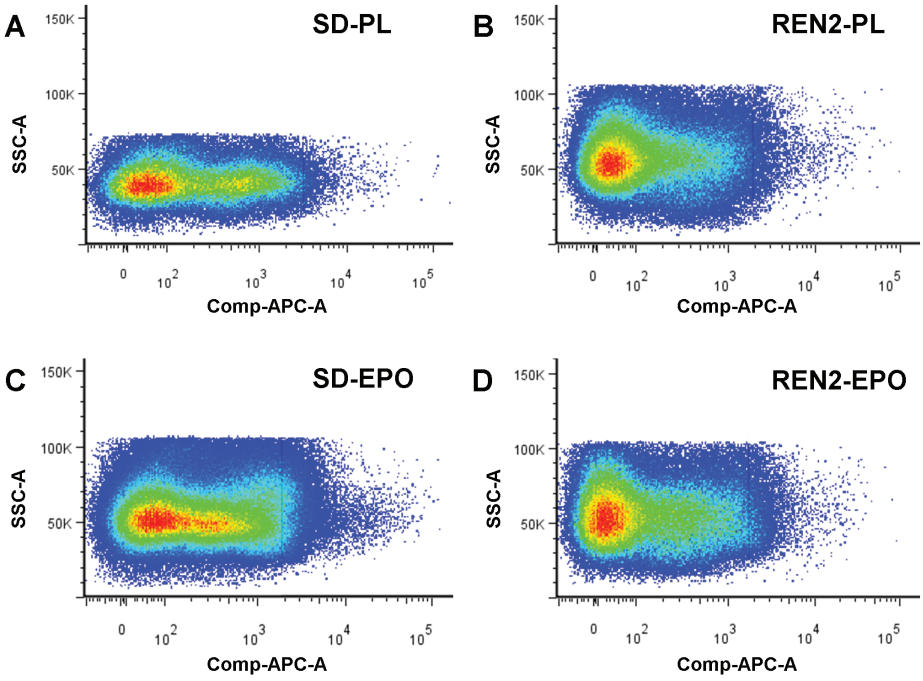
Chapter 7 - figure 1

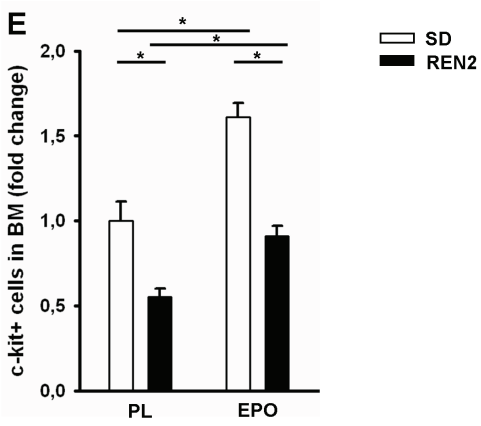


Chapter 8 - figure 1

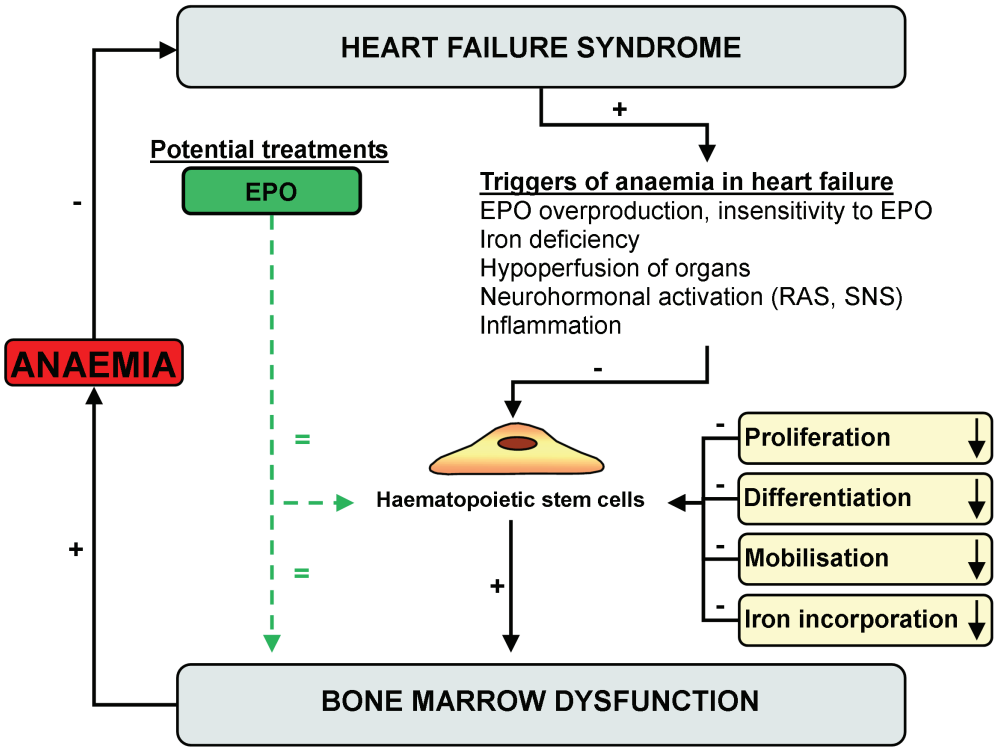


Chapter 8 - figure 4

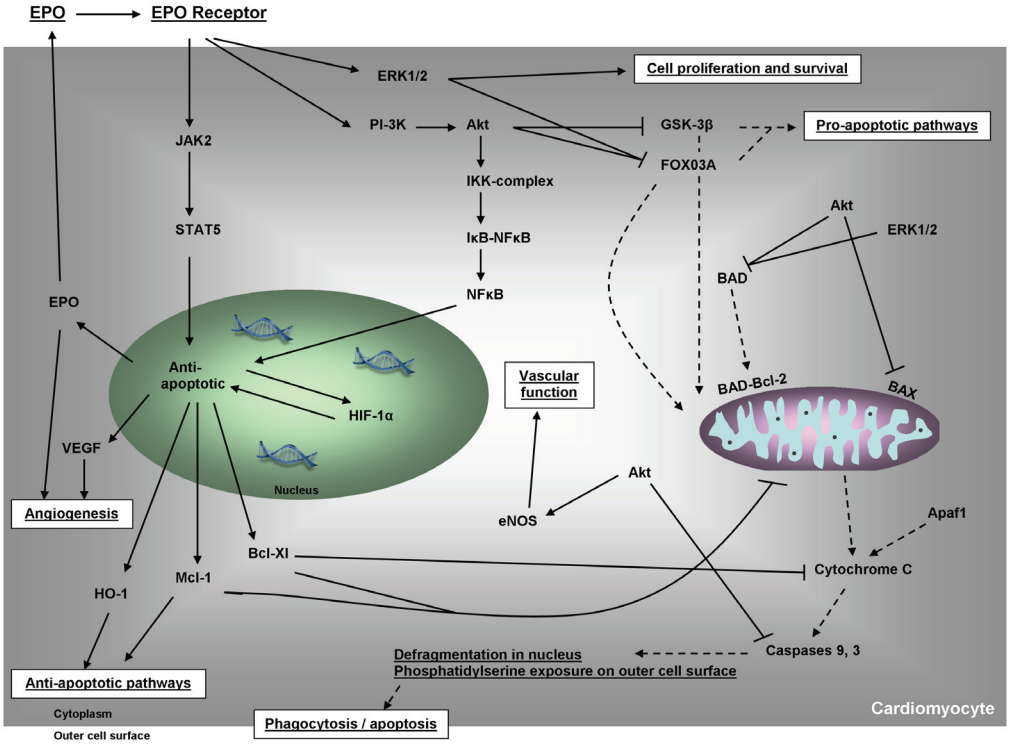




Chapter 8 - figure 6



Chapter 9 - figure 1



DANKWOORD

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