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

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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 neovascularization was studied with two distinct VEGF-neutralizing 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-alfa (10 IU/mL) was used in vitro and darbepoetin alfa (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 (STAT-3) 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-neutralizing antibodies abrogated the salutary effects of EPO on cardiac microvascularization and function. VEGF neutralization 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.

Keywords

Heart failure • Neovascularization • Cardiac function • Erythropoietin • Vascular endothelial growth factor

1. 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 neovascularization 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 neovascularization 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.9 Although these findings suggest an important role for VEGF in EPO-induced neovascularization, 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 microvasculature 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 neutralizes the effects of EPO administration, specifically improvement of cardiac microvasculature and function, using a well-established in vivo model of HF.

2. Methods

2.1 Recombinant human EPO and experimental antibodies

We used EPO-alfa (EPREX, Janssen-Cilag) in a concentration of 10 IU/mL in all in vitro experiments. The long-acting EPO analogue darbepoetin alfa (Aranesp, Amgen) was administered once every 3 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 3 weeks after the infarct when infarct healing has subsided. The VEGF neutralizing 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.15) 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 neutralizing antibodies recognize and neutralize all splice variants of rat VEGF.

2.2 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.13,14 Human cardiac fibroblasts (HCF, ScienCell Research Laboratories) were cultured in RPMI 1640 medium with l-glutamine and 10% foetal bovine serum. Before the experiment, the 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 h. 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 h. The role of well-described EPO-R 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 MAP-kinase kinase blocker p38 (SB203580, 1 μM, Calbiochem) for 30 min. After pre-incubation, 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 STAT-3 phosphorylation cells were lysated after 30 min with RIPA-buffer containing protease and phosphatase inhibitors (Invitrogen) as previously described.8 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% O2, 5% CO2. 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), quantitatively reverse transcriptions–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.

2.3 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 Ottineti with slight modifications.15 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 heparin16) with or without 10 IU/mL EPO or 1 μg/mL goat anti-VEGF AF564, R&D systems) for 7 days. After 5 and 7 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.

2.4 Animals and bone marrow labelling

Male Sprague Dawley rats (270–320 g) were purchased (Harlan). For bone marrow transplantation experiments, we used male Fischer F344 rats (200–230 g) 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.5 Briefly, whole R26-hPAP bone marrow cells were transfused to Fischer F344 recipients after total body irradiation and left to reconstitute for 6 weeks before commencing with the experimental protocol. Animals were fed and housed according to the institutional rules and regulations.

2.5 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.5 Three weeks after MI, rats were randomly assigned to treatment with darbepoetin alfa or vehicle, given once every 3 weeks i.p. as described above. Because we intended to explore the effect of EPO on established HF, the first darbepoetin dose was given 3 weeks after MI when infarct healing has subsided. The effects of VEGF in EPO-induced neovascularization were studied by administering one of the VEGF neutralizing 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 microvascularization in HF,14 we only administered neutralizing antibodies during the first week after EPO treatment to allow for a neutralization-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-neutralization, 400 µL of blood was drawn from the tail vein at weeks 3, 4, 6, and 7. After 9 weeks, at sacrifice, haemodynamic measurements were performed with a micropit pressure transducer (Millar Instr., Inc., Houston, TX, USA) as described previously.5 Heart rate, LV systolic pressure, and LV end-diastolic pressure (LVEDP) were measured. The maximal rates of increase and decrease in LV pressure (dP/dt max and dP/dt min) 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.

2.6 VEGF-neutralizing capacity of plasma

Ninety-six-well plates were coated with donkey anti-goat-IgG in PBS overnight. Next, plates were five washes 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 neutralization was determined as VEGF measured/VEGF added (1000 pg/mL) × 100%. The neutralizing characteristics of aVEGF2 have been described elsewhere.17

2.7 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.8 For determination of STAT-3 phosphorylation, we used the PhosphoPlus STAT-3 antibody kit (Cell Signalling Technology, catalogue number 9130) according to the suppliers’ guidelines.

2.8 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.8

2.9 LV histology

Infarct size, cardiomyocyte cross-sectional area, the number of cardiomyocytes/mm², the number of capillaries per tissue area (mm²), and the capillary/cardiomyocyte ratio were determined as described previously.5 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 His52 (kind gift from Dr J.L. Hillebrands).8 The envision kit (Dako-Cytomation) with Mayers haematoxin (Sigma) for nuclear staining was used for chromogenic detection. For fluorescent detection, diamidino-2-phenylindole, the biotynyl/hodamine-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 borderzone. 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 4–5 random high power fields of the non-infarcted LV-free wall remote from the infarction by blinded observers (BDW, LY).

2.10 Statistical methods

Data are expressed as 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).

2.11 Ethics

The investigation conforms with the principles outlined in the Declaration of Helsinki. In addition, the investigation conforms with 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 Center Groningen, University of Groningen, Groningen, The Netherlands. The experimental protocol was approved by the Animal Ethics Committee of the University of Groningen.

3. Results

3.1 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,6 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 STAT-3 inhibiting peptide, but not by wortmannin or PD98059 (Figure 1D), suggesting that the JAK2/STAT-3 pathway is the dominant signal transduction pathway. To confirm that STAT-3 is activated, we additionally measured STAT-3 phosphorylation, which confirmed that EPO induces STAT-3 signalling in our model (see Supplementary material online, Figure S2).

3.2 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). Neutralization of VEGF did not inhibit the effects of EPO on aortic sprouting.

3.3 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.
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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 EPO-R 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.

Figure 2  Effects of VEGF inhibition on EPO-induced angiogenesis in vitro. (A) Maximal aortic sprout length after 5 and 7 days in a Matrigel aortic implantation assay. Aortic rings were cultured in the presence or absence of EPO (10 IU/mL) or VEGF neutralizing antibody (1 µg/mL goat anti-rat VEGF). EPO significantly increased maximal sprout length compared with control cultures. The increase was however <10%. Neutralization of VEGF did not inhibit the effects of EPO on aortic sprouting. (B) Typical examples of aortic sprouting in the different experimental groups after 7 days in culture. C, Control culture; E, EPO; aV, VEGF-neutralizing antibody.
Importantly, administration of the neutralizing antibodies did not quantitatively affect VEGF-protein levels at sacrifice (Figure 3). Therefore, the results of the study were not biased by neutralization-induced changes of VEGF expression and thus represent the effects of temporary blocking VEGF signalling.

### 3.4 Effect of VEGF neutralization on EPO-induced improvement of cardiac function

General characteristics of the study groups are presented in Table 1. Temporal characteristics of the VEGF-neutralizing capacity of the plasma are presented in Supplementary material online. In brief, during the anti-VEGF antibody administration 78% of VEGF was neutralized. In the subsequent weeks without antibody administration, the neutralization capacity was normalized to control values. These data indicate that intermittent administration of neutralizing antibodies results in temporary neutralization 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 \( (dP/dt_{\text{max}} \text{ dLVP}) \) and LV-relaxation \( (dP/dt_{\text{min}}) \) and decreased LV filling pressures \( (LVEDP) \) compared with the untreated MI (all \( P < 0.01 \), Figure 3). Neutralization of VEGF with both antibodies blocked the salutary effects of EPO on cardiac function parameters \( (P = \text{NS vs. untreated MI}, P < 0.05 \) vs. MI-EPO, Figure 4), whereas intermittent neutralization itself did not further decrease cardiac function compared with the untreated MI. \( (P = \text{NS}, Figure 4) \). Correction of LV contractility and relaxation indices for instantaneous pressures did not alter the results (data not shown).

### 3.5 Effect of VEGF neutralization on EPO-induced LV neovascularization

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 neutralization completely blocked the effects of EPO on capillary density \( (P = \text{NS vs. untreated MI}, P < 0.01 \) vs. MI-EPO, Figure 5), whereas neutralization alone did not result in...
Table 1  General characteristics at sacrifice

<table>
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<th>Variable</th>
<th>SHAM + IgG</th>
<th>MI + IgG</th>
<th>MI-EPO + IgG</th>
<th>MI + EPO + aVEGF1</th>
<th>MI + EPO + aVEGF2</th>
<th>MI + aVEGF1</th>
<th>MI + aVEGF2</th>
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<td>n</td>
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<td>11</td>
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<td>Body weight (g)</td>
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<td>Haematocrit (%)</td>
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<td>58 ± 1.5</td>
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<td>49 ± 1.4</td>
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<td>Circ. EPCs (n/field)</td>
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<td>280 ± 62</td>
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<td>—</td>
<td>135 ± 21</td>
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<td>Heart/body (g/g)</td>
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<td>Cardiom-cross. (µm²)</td>
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<td>771 ± 46</td>
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<td>756 ± 24</td>
<td>806 ± 37</td>
<td>781 ± 47</td>
<td>804 ± 28</td>
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Haemodynamics

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<th>Variable</th>
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<th>MI + IgG</th>
<th>MI-EPO + IgG</th>
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<th>MI + EPO + aVEGF2</th>
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<td>Aorta-syst (mmHg)</td>
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<td>65 ± 6</td>
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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; heart/body, heart weight/body weight ratio; lung/body, lung weight/body weight ratio; b.p.m., 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.

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Figure 4  Effects of VEGF neutralization on EPO-induced improvement of LV function. (A–D) Graphic representation of LV contractility (dP/dT\text{max}), relaxation (dP/dT\text{min}), LVEDP and dLVP. MI, myocardial infarction; EPO, erythropoietin; IgG, control IgG; aVEGF, VEGF-neutralizing antibody.
further reduction of capillary density ($P = \text{NS} \text{ vs. untreated MI, Figure 5}$). Capillary/cardiomyocyte ratio was slightly but non-significantly increased in all MI groups compared with sham. EPO treatment resulted in a 42% increase in capillary/cardiomyocyte ratio compared with sham and MI, which was also blocked by VEGF inhibition (Figure 5).

### 3.6 Effect of VEGF neutralization 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 ($P < 0.001 \text{ vs. sham and MI}$). VEGF inhibition resulted in a 47% reduction in of EPO-induced endothelial cell proliferation ($P = 0.01 \text{ vs. MI-EPO}$), although the number of proliferating cells was still significantly higher than in the sham and MI-group ($P < 0.05 \text{ vs. sham and untreated MI}$).

### 3.7 Effect of VEGF neutralization on myocardial homing of EPCs

EPO significantly increased circulating EPCs after EPO treatment and VEGF neutralization 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 ($P = \text{NS} \text{ vs. untreated MI}$, $P < 0.01 \text{ vs. MI-EPO}$, Figure 6C and D), whereas VEGF neutralization alone did not further reduced the number of BMDEC ($P = \text{NS} \text{ vs. untreated MI}$, Figure 6C and D).

### 4. Discussion

The present study establishes for the first time a crucial role for VEGF in EPO-induced neovascularization and restoration of LV function. Under ischaemic conditions, EPO promoted VEGF transcription through the JAK2/STAT-3 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, neutralization of VEGF completely abrogated the salutary effects of EPO on cardiac function and microvascularization. Likewise, neutralization 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 neovascularization 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.

4.1 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. Moreover, in experimental HF without anaemia, EPO consistently results in sustained improvement of cardiac function and microvascularization even in a dose that does not increase haematocrit levels. 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 mobilization of EPCs was not affected by VEGF neutralization, myocardial neovascularization was significantly attenuated, which indicates that the stimulation of EPCs alone is not sufficient to improve cardiac function.

4.2 EPO stimulates the paracrine angiogenic response of cardiomyocytes

Disproportional (micro) vascular growth during cardiac hypertrophy causes an impaired vascular supply of cardiomyocytes. Exhaustion of VEGF release by cardiomyocytes during prolonged ischaemia has been proposed as an underlying mechanism. 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.\textsuperscript{21} Furthermore, we provide proof that EPO-induced VEGF gene transcription is mediated via the JAK2/STAT-3 signal transduction pathway. STAT-3 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.\textsuperscript{22,23} The important role for STAT-3 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 EPO-R upregulation.\textsuperscript{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.\textsuperscript{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 neutralization, as evidenced by marked attenuation of PCNA-positive endothelial cells after VEGF inhibition. Our results confirm recent observations by Asaumi et al.\textsuperscript{7} whom also demonstrated that the angiogenic effects of EPO on top of VEGF were limited. 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.\textsuperscript{16} However, to avert intrinsic negative inotropic effects of VEGF neutralization in our model, we only administered VEGF neutralizing antibodies in the first week after EPO administration. With this, we established intermittent VEGF neutralization, only during the pharmacological window of EPO (see Supplementary material online, Figure S1).

### 4.3 EPO-increased VEGF expression facilitates myocardial incorporation of EPCs

EPO mobilizes EPCs from the bone marrow and we and others have therefore postulated an important role for EPCs in EPO-induced neovascularization.\textsuperscript{26} We recently showed that EPO stimulates incorporation of EPCs into the myocardial microvasculature.\textsuperscript{8} However, the present study indicates that EPC mobilization alone does not necessarily augment neovascularization. We previously demonstrated that EPCs incorporate into healthy and diseased tissues, whereas neovascularization was specifically induced in the presence of VEGF upregulation and ischaemia.\textsuperscript{12} Moreover, transplantation of normal bone marrow to EPO-R null mice does not rescue VEGF expression nor does it restore neovascularization.\textsuperscript{27} One might therefore suggest that EPCs are dispensable for EPO-induced neovascularization. However, attenuated expression of VEGF in EPO-R null mice is also associated with impaired homing of EPCs,\textsuperscript{5} 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-mobilized EPCs.

### 4.4 Clinical implications

After several promising phase 2 studies,\textsuperscript{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,\textsuperscript{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 EPO-R should be developed, as such agents might restore cardiac function without undesirable haematopoietic or pro-thrombotic side effects.\textsuperscript{30} Possibly, (post)transcriptional cardiomyocyte-specific modification of the EPO-R occurs that might enable us to design cardiospecific EPO derivatives. Alternative drug delivery approaches might include EPO-eluting hydrogels.\textsuperscript{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.

### 4.5 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.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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### Conflict of interest

None declared.

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### References

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