

RESEARCH PAPER

Darbepoetin-mediated cardioprotection after myocardial infarction involves multiple mechanisms independent of erythropoietin receptor–common β -chain heteroreceptor

Peter Kanellakis¹, Giovanna Pomilio¹, Alex Agrotis¹, Xiaoming Gao²,
Xiao-Jun Du², David Curtis^{3,4*} and Alexander Bobik^{1*}

¹Vascular Biology & Atherosclerosis, ²Experimental Cardiology, BakerIDI Heart and Diabetes Institute, ³Department of Medicine, University of Melbourne and ⁴Rotary Bone Marrow Research Laboratory, Royal Melbourne Hospital, Melbourne, Australia

Correspondence

Professor Alexander Bobik,
BakerIDI Heart and Diabetes
Institute, PO Box 6492 St Kilda
Road Central, Melbourne, VIC,
8008, Australia. E-mail:
alex.bobik@baker.edu.au

*Contributed equally to this study.

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BACKGROUND AND PURPOSE

Darbepoetin, a long-acting erythropoietin derivative, attenuates cardiomyocyte apoptosis and improves short-term (3 days) cardiac function, but the mechanisms responsible are unknown. We investigated potential mechanisms by which darbepoetin exerts cardioprotection following myocardial infarction in mice and the significance of the erythropoietin receptor (EPOR)–common β -chain (c- β -chain) heteroreceptor.

EXPERIMENTAL APPROACH

Mice underwent 60 min coronary occlusion followed by treatment with vehicle or a single dose of darbepoetin. Effects on gene expression, apoptosis and neutrophil accumulation in infarcted left ventricle were assessed 24 h later. Cardiac function, effects on vascularization and fibrosis were assessed 28 days later. The significance of EPOR–c- β -chain heteroreceptor was examined 28 days after infarction using mice deficient in c- β -chain.

KEY RESULTS

Twenty-four hours after darbepoetin, mRNAs encoding haeme oxygenase-1 (HO-1), iNOS and brain natriuretic peptide (BNP) were markedly elevated only in infarcted regions, and the frequency of apoptotic cells attenuated. Inflammation was also attenuated with reductions in neutrophil numbers. Darbepoetin also elevated mRNAs encoding angiogenic factors: placental growth factor, monocyte chemoattractant protein-1 and interleukin-1 β . Twenty-eight days after treatment, CD31+ vessels in the infarct zone doubled and fibrosis reduced. Cardiac haemodynamics were improved. Darbepoetin also improved cardiac haemodynamics in c- β -chain-deficient mice, increased HO-1 and iNOS expression and vessel numbers and attenuated fibrosis.

CONCLUSIONS AND IMPLICATIONS

Darbepoetin stimulates expression of haeme oxygenase, iNOS, BNP and angiogenic factors specifically in infarcted left ventricle that attenuates inflammation, apoptosis and fibrosis; elevate vessel numbers; and improve cardiac function. The EPOR–c- β -chain heteroreceptor is not essential for these effects.

Abbreviations

Akt1, serine–threonine protein kinase encoded by the *AKT1* gene; Angpt14, angiopoietin-like-4; BNP, brain natriuretic peptide; Ccl2, chemokine (C-C motif) ligand 2; CSF-3, colony-stimulating factor-3; CT, point at which fluorescence crosses a threshold value; CTGF, connective tissue growth factor; CXCL9, chemokine (C-X-C motif) ligand 9; dUTP, deoxyuridine triphosphate; Edg1, functional sphingosine-1-phosphate receptor; EFNA-1, ephrin-A1; EFNA-2, ephrin-A2; EPO, erythropoietin; EPOR, erythropoietin receptor; Flt-1, vascular endothelial growth factor receptor-1; G-CSF, granulocyte colony-stimulating factor; HO-1, haeme oxygenase-1; IL, interleukin; iNOS, inducible NOS; KATP, adenosine triphosphate-sensitive potassium channels; LVSP, left ventricular systolic pressure; LV + dp/dt(max), maximum rate of rise of left ventricular pressure; LV – dp/dt(min), maximum rate of fall in left ventricular pressure; MAPK14, mitogen-activated protein kinase-14; MDK, midkine; MMP9, matrix metalloproteinase-9; NF- κ B, nuclear factor kappa light chain enhancer of activated B cells; NPR1, natriuretic peptide receptor-A; NRP1, neurophilin-1; Nudt6, nucleoside diphosphate linked moiety X-type motif 6; PDGF-A, platelet-derived growth factor-A; PGF, placental growth factor; Pofut1, protein O-fucosyltransferase-1; PTGS1, prostaglandin endoperoxide synthase-1; PTN, pleiotrophin; P42/44 MAPK, p42/p44 mitogen-activated protein kinase; P38MAPK, p38 mitogen-activated protein kinase; Sphk1, sphingosine kinase-1; STAT3, signal transducer and activation of transcription 3; TIMP3, tissue inhibitor of metalloproteinase-3; TNFSFR, tumour necrosis factor receptor superfamily; Tek, angiopoietin-1 receptor; TGF-b3, transforming growth factor-b3; VEGF-A/B, vascular endothelial growth factor-A/B

Introduction

The mortality and morbidity of acute myocardial infarction remain a major public health challenge with a significant percentage of surviving patients being severely disabled despite advances in thrombolysis and angioplasty. This disability is the consequence of grossly inadequate cardiac repair resulting in the loss of cardiomyocytes, poor vascularization and excessive collagen accumulation. Any improvement in salvaging damaged tissue would represent a major advance in therapy for myocardial infarction. Recent studies aimed at improving healing have focused on stem cells, stem cell mobilization by agents such as granulocyte colony-stimulating factor (G-CSF) and treatment with erythropoietin (EPO) (Hanlon *et al.*, 2005; Kanellakis *et al.*, 2006; Nelson *et al.*, 2009). EPO is a glycoprotein hormone secreted by the kidney in response to hypoxia, which enhances erythropoiesis. Its effects are mediated via a specific cellular receptor erythropoietin receptor (EPOR), a member of the type 1 superfamily of single transmembrane cytokine receptors (Constantinescu *et al.*, 1999). Although still controversial (Um *et al.*, 2007), it has been suggested that EPO, in addition to signalling via a dimeric receptor for tissue protection, signals via a second receptor system consisting of EPOR and common β -chain (c- β -chain) (Brines *et al.*, 2004). Recombinant EPO protects tissues from damage and has significant therapeutic value in rodent models such as ischaemia–reperfusion injury in kidneys (Yang *et al.*, 2003), stroke (Wang *et al.*, 2004) and diabetic neuropathy (Bianchi *et al.*, 2004).

Darbepoetin is a structural analogue of EPO differing from EPO in five amino acids, resulting in addition of two extra N-linked oligosaccharides to the molecule. This results in a fourfold lower affinity

for EPOR. However, *in vivo*, a longer plasma half-life more than compensates for the reduced affinity. Darbepoetin also exerts significant tissue protective effects. It confers behavioural and histological neuroprotection after focal cerebral ischaemia (Belayev *et al.*, 2005), protects the kidney against the detrimental effects of ischaemia (Johnson *et al.*, 2006), podocytes from injury by puromycin (Eto *et al.*, 2007) and attenuates hepatocellular apoptosis induced by lipopolysaccharides (Le Minh *et al.*, 2007). Darbepoetin also has significant effects on infarcted myocardium. In rats, it reduces infarct size, attenuates cardiomyocyte apoptosis and improves short-term (3 days) cardiac function (Gao *et al.*, 2007). Its cardioprotective effects were reported to be dependent on p42/44 MAPK, p38MAPK, mitochondrial ATP-dependent potassium channels and sarcolemmal KATP channels (Baker *et al.*, 2007). In contrast, in pigs, darbepoetin did not reduce infarct size, but limited fibrosis and preserved regional myocardial wall motion (Toma *et al.*, 2007). Despite these studies, the spectrum of effects by which darbepoetin exerts protective effects on infarcted myocardium has yet to be defined. In addition, potential mechanisms and receptors utilized by darbepoetin to exert its cardioprotective effects after myocardial infarction are not known.

In the present study, we used a mouse cardiac ischaemia–reperfusion model to define the actions of darbepoetin. We confirmed its ability to attenuate apoptosis following reperfusion of ischaemic myocardium. This was associated with increased expression of inducible NOS (iNOS), haeme oxygenase-1 (HO-1) and brain natriuretic peptide (BNP). Treatment also reduced neutrophil accumulation in the infarct zone and increased the expression of angiogenic factors. Blood vessels in the infarct regions

were also increased, fibrosis attenuated and cardiac function improved. We also demonstrated that the EPOR-c- β -chain is not required for darbepoetin to exert its cardioprotective effects.

Methods

Animals

C57Bl/6 mice were from AMREP Animal Services (AMREP, Melbourne, Victoria, Australia). Mice deficient in c- β -chain were obtained from the Walter and Eliza Hall Institute of Medical Research (Melbourne, VIC, Australia) and bred at AMREP Animal Services. All experiments were performed when the mice were 10 weeks of age. The mice were housed under conventional conditions with free access to food and water. All experiments were approved by the AMREP Animal Ethics Committee.

Surgical procedures

The mice were anaesthetized with a mixture of ketamine, xylazine and atropine, and intubated and ventilated as previously described (Kanellakis *et al.*, 2006). A thoracotomy was performed in the left third intercostal space and the beating heart isolated. A 7-0 silk suture was passed under the left coronary artery at the inferior edge of the left atrium, and tied with a slipknot to produce occlusion. Air was then evacuated from the chest, the cavity closed and normal respiration restored. After 1 h of ischaemia, blood flow through the left coronary artery was re-established by pulling the slipknot (Kanellakis *et al.*, 2006). Because c- β -chain-deficient mice were much more resistant to the detrimental effects of ischaemia-reperfusion injury than wild-type C57Bl/6 mice (see Results), these mice were also subjected to permanent coronary artery ligation. In this instance, the left coronary artery was permanently ligated. Sham-operated mice underwent identical surgical procedures except that the coronary arteries were not ligated. Darbepoetin or vehicle was administered 1 h after the coronary artery had been occluded.

Experimental groups

In the first experiments, five groups of C57Bl/6 mice (15 per group) were studied. A sham-operated group was treated 1 h after chest closure with a single dose of darbepoetin ($30 \mu\text{g}\cdot\text{kg}^{-1}$ i.v.). Another group subjected to ischaemia-reperfusion was treated immediately after reperfusion with i.v. saline, and three groups subjected to ischaemia-reperfusion injury were treated immediately after reperfusion with darbepoetin (0.3, 3.0 or $30 \mu\text{g}\cdot\text{kg}^{-1}$ i.v.). Twenty-four hours after the initiation of reperfusion, five mice in

each group were culled and their hearts excised for immunohistochemistry and molecular biological studies. All mice surviving for 28 days were subjected to cardiac haemodynamic studies, and then culled and hearts collected for histological/immunohistochemical studies. We also utilized four groups of mice deficient in c- β -chain, two of which were subjected to ischaemia-reperfusion and two to permanent coronary artery ligation. The mice subjected to ischaemia-reperfusion were treated as previously indicated, while those subjected to permanent ligation were treated upon chest closure either with saline (i.v.) or darbepoetin ($30 \mu\text{g}\cdot\text{kg}^{-1}$ i.v.); sham-operated mice deficient in c- β -chain were also included. Cardiac haemodynamics was measured 28 days after infarctions, and then the mice were culled and hearts collected for histological studies.

Cardiac haemodynamics

Cardiac haemodynamics were measured using a 1.4F Millar microtipped transducer catheter (Millar Instruments, Houston, TX, USA; Kanellakis *et al.*, 2006). The mice were anaesthetized with pentobarbitone ($8 \text{ mg}\cdot 100 \text{ g}^{-1}$ i.p.) and atropine ($0.06 \text{ mg}\cdot 100 \text{ g}^{-1}$ i.p.), placed in a supine position, intubated and ventilated. The catheter was inserted via the right carotid artery into the aorta and advanced into the left ventricle. Resting aortic pressures, as well as left ventricular systolic pressure (LVSP) and $\text{LV} + \text{dp}/\text{dt}(\text{max})$ and $\text{LV} - \text{dp}/\text{dt}(\text{min})$, were recorded using a computer and ADInstruments software (ADInstruments, Bella Vista, NSW, Australia; Kanellakis *et al.*, 2006).

Cardiac fibrosis

After haemodynamic measurements, the hearts were arrested in diastole with 15% potassium chloride and fixed by immersion in 10% buffered formalin for 24–48 h. Three transverse slices from the base, mid-region and apex were embedded in paraffin. Each slice was then sectioned (6 mm thick) for histology and immunohistochemistry. The amount of collagen deposited in the left ventricle was measured as previously described after staining the sections with picrosirius red (Picrosirius Red F3BA, 0.1% solution in saturated aqueous picric acid), using an Optimus Bioscan 2 Imaging System (Thomas Optical Measurement System, Media Cybernetics, Silver Spring, MD, USA; Kanellakis *et al.*, 2006).

Immunohistochemistry

Paraffin sections were cleared in xylene, rehydrated and antigen retrieved by heating the sections in 0.01 M citrate buffer (pH 6.0) in a microwave for

8 min. After the slides had been incubated for a further 20 min, they were transferred to 0.1 M phosphate-buffered saline (PBS, pH 7.5). After peroxidase activity had been quenched, the sections were incubated in 10% horse serum for 30 min, washed in PBS and incubated for 1 h in PBS containing an anti-CD31 antibody (1–20; BD Pharmingen, Franklin Lakes, NJ, USA) (Raj *et al.*, 2006). Following multiple washings, the sections were incubated with a biotinylated anti-rat antibody [in normal goat serum (NGS)/PBS], followed by incubation with streptavidin–horseradish peroxidase complex. Antigen was visualized using DAB. Sections were counterstained with haematoxylin and CD31-positive vessels within the infarct zone counted.

Neutrophil accumulation, HO-1 and iNOS in the infarct zone 24 h after reperfusion or permanent ligation were also assessed by immunohistochemistry, using cardiac tissue embedded in OCT compound (Tissue-Tek, Sakura Finetek USA Inc, Torrance, CA, USA) and frozen (Raj *et al.*, 2006). Briefly, cryostat-cut sections (6 µm) were fixed in cold (–20°C) acetone for 20 min. The sections were then sequentially incubated in 3% hydrogen peroxide in PBS, 10% NGS/PBS and biotin/avidin blocking reagents (Vector Laboratories, Burlingame, CA, USA). Thereafter, the sections were incubated (1 h) with a rat anti-mouse neutrophil antibody (1–50, Serotec, Raleigh, NC, USA) in NGS/PBS, rabbit polyclonal anti-HO-1 (1–50, Abcam, Cambridge, MA, USA), rabbit polyclonal anti-iNOS (1–50, Abcam) or corresponding non-immune IgG. After multiple washings, the sections were processed as above after the addition of the biotinylated anti-rat antibody. Neutrophils were quantified by counting.

Apoptotic [terminal deoxynucleotidyl transferase-mediated dUTP-X nick-end labelling (TUNEL)-positive] cells in infarct zones of C57Bl/6 mice 24 h after myocardial infarction were estimated using TUNEL (*in situ* cell death detection kit, Roche, Dee Why, NSW, Australia) as previously described (Kanellakis *et al.*, 2009).

Real-time PCR

Total RNA was extracted from infarcted and non-infarcted left ventricle as previously described (Ward *et al.*, 1997). Any contaminating DNA was removed by incubating these RNA extracts with 2 U DNase (Stratagene, La Jolla, CA, USA). Then, RNA was precipitated using isopropanol and washed in 70% ethanol prior to being dissolved in sterile water and quantification at 260 nm. RNA was then reverse transcribed using TaqMan Methodology (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer. The cDNA (40 ng) was used for real-time PCR to determine the expression of each

gene using Applied Biosystems SYBR Green PCR Mix and an ABI PRISM 7500 system. Each amplification was performed in duplicate and included internal controls for ribosomal 18s. Relative amounts of each mRNA for each gene in infarcted and non-infarcted LV from control and treated mice subjected to ischaemia–reperfusion injury were calculated using comparative C_T values (Raj *et al.*, 2006). Oligonucleotide primers were: iNOS, sense: 5'-GTGGAG AGATTTTGCATGACACTCT-3' and antisense: 5'-ACCCCAAGCAAGACTTGGACTT-3'; hemoxyenase-1: sense: 5'-CAGGTGTCCAGAGAAGGCT TTAA-3' and antisense: 5'-TGCGCTCTATCTCCTC TTCCA-3'; BNP, sense: 5'-CAGTCTCCAGA GCAATTCAAGATG-3' and antisense: 5'-GAG CTGTCTCTGGGCCATTTTC-3'; survivin, sense: 5'-G GCCAGTGTTTTTCTGCTT-3' and antisense: 5'-G GGAGTGCTTTCTATGCTCCTCT-3'; Bcl-2, sense: 5'-CGGGAGAACAGGGTATGATAACC-3' and antisense: 5'-ATCTCCAGCATCCCACTCGTA-3'; Bax, sense: 5'-TGGTTGCCCTCTTCTACTTTGC-3' and antisense: 5'-TGATCAGCTCGGGCACTTTAGT-3'.

Gene arrays

RNA from infarcted and non-infarcted regions of hearts from C57Bl/6 mice was also subjected to gene expression profiling using Oligo GEArray Mouse Angiogenesis Microarrays (SABiosciences, Frederick, MD, USA). RNA was converted to biotinylated cRNA according to the manufacturer's directions, and hybridized to membrane-bound oligonucleotides representing 113 genes implicated in angiogenesis. After X-ray film development, data analyses were performed using the GEArray Expression Analysis Suite (SABiosciences).

Statistical analyses

Results are expressed as mean \pm SEM. Comparisons between groups were carried out using one-way ANOVA or Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Darbepoetin and genes regulating apoptosis

We investigated whether a single injection of low- (0.3 µg·kg⁻¹) or high- (30 µg·kg⁻¹) dose darbepoetin could attenuate apoptosis in the infarct zone 24 h after ischaemia–reperfusion injury. Both low and high doses were equally effective in attenuating apoptotic cell numbers by nearly 80% ($P < 0.05$; Figure 1A). Because expression of HO-1, iNOS and BNP has been associated with protection of the heart against ischaemia (D'Souza *et al.*, 2003; Jones

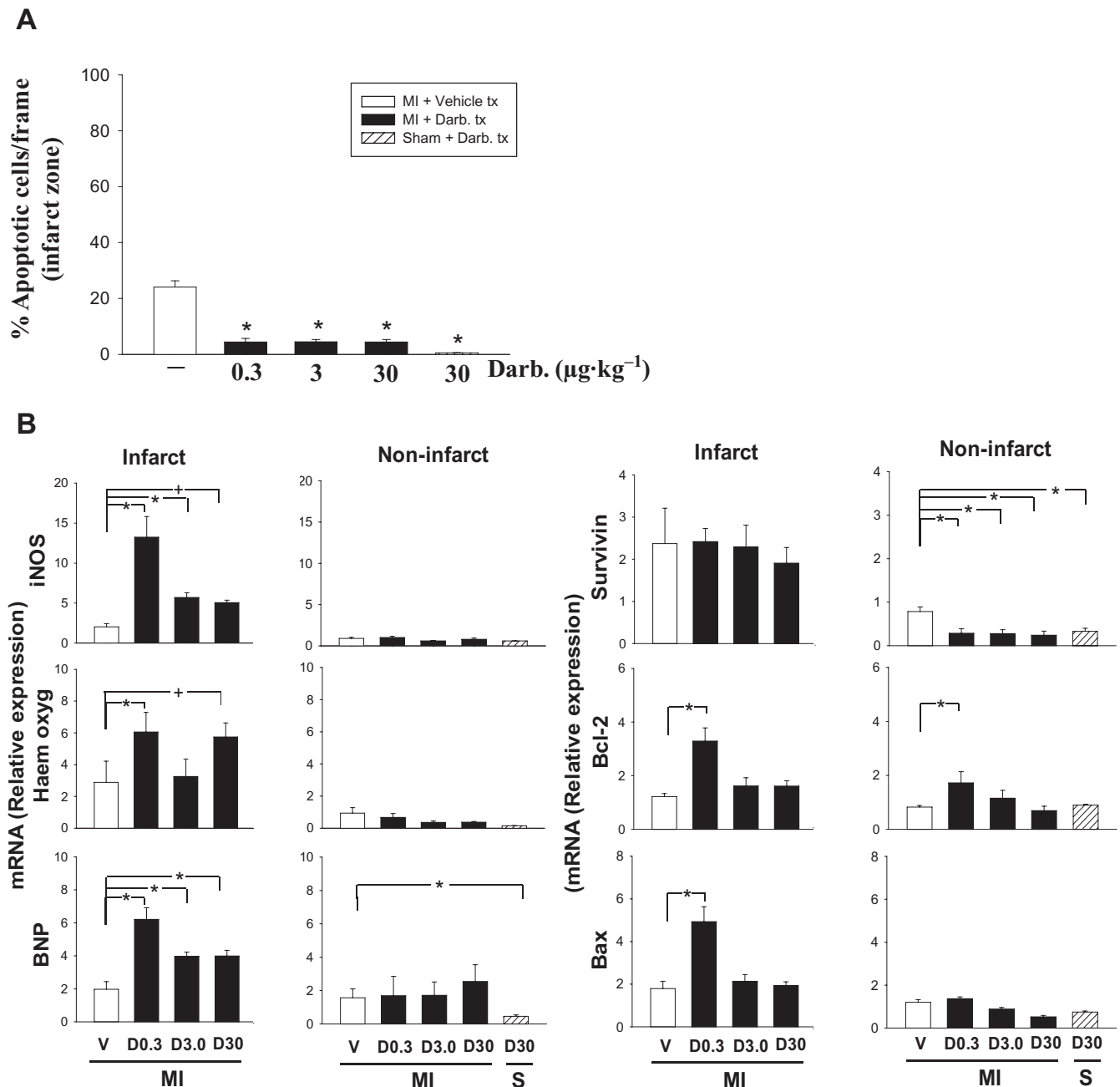


Figure 1

Darbepoetin, apoptosis and genes that regulate cell survival in infarct zone. (A) Apoptotic cells in the infarct zone 24 h after reperfusion determined using an 'in situ Cell Death Detection Kit' and direct cell counting, expressed as percentage of total cells in the infarct zone. (B) Total RNA isolated from infarct regions 24 h after reperfusion, and treatment with darbepoetin or vehicle was subjected to real-time PCR to quantify expression levels of genes that regulate responses to injury. Columns represent infarcted mice treated with vehicle (V) or darbepoetin (D; $\mu\text{g}\cdot\text{kg}^{-1}$) or sham-operated mice (S) treated with darbepoetin. All results are means \pm SEM of five mice in each group. * $P < 0.05$ from vehicle-treated mice.

and Bolli, 2006; Liu *et al.*, 2006), we investigated whether darbepoetin also affected their expression. Darbepoetin treatment doubled the expression of HO-1 specifically in the infarcted region of the heart ($P < 0.05$; Figure 1A); mRNA levels encoding HO-1 in non-infarcted regions were unaffected (Figure 1A). These effects were apparent at low and high doses of

darbepoetin. Similarly, darbepoetin also increased expression of iNOS by threefold ($P < 0.05$; Figure 1B), again only in the infarcted region of the heart with significant increases observed at all doses of darbepoetin. mRNA encoding BNP also doubled in the infarct region ($P < 0.05$; Figure 1B). In contrast, other anti-apoptotic factors, survivin and

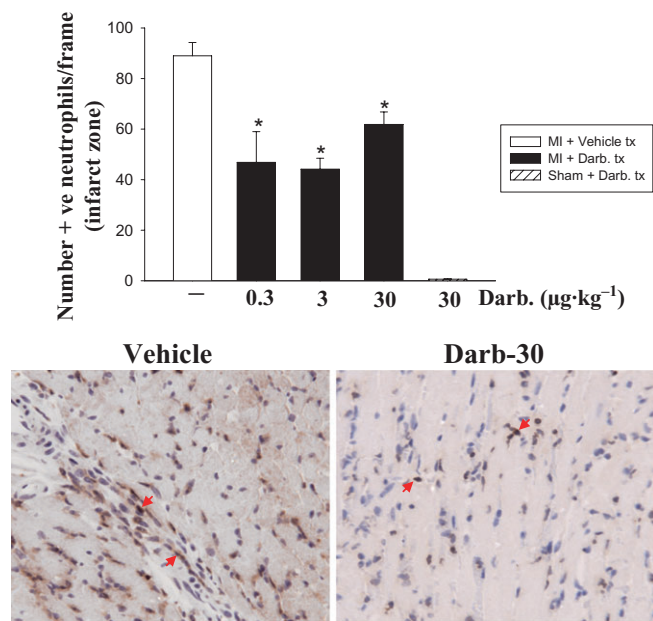


Figure 2

Darbepoetin and neutrophil accumulation in infarcted myocardium. Top: neutrophil numbers in the infarct zone were determined by counting immunopositive neutrophils in the infarct zone 24 h after reperfusion. Results are mean \pm SEM of five mice in each group. * $P < 0.05$ from infarct zone of vehicle-treated group. Bottom: photomicrographs of neutrophils accumulating in the infarct zone of mice treated with vehicle or darbepoetin (30 $\mu\text{g}\cdot\text{kg}^{-1}$). Bar represents 100 μm .

Bcl-2, were mostly unaffected, although mRNA encoding Bcl-2 and Bax was increased twofold at the lowest darbepoetin dose (Figure 1B).

Darbepoetin and neutrophil accumulation

Neutrophils are known to be involved in murine myocardial ischaemia–reperfusion injury, releasing a variety of cytotoxic mediators augmenting cardiac necrosis and apoptosis (Vinten-Johansen, 2004). Consequently, we examined the effects of darbepoetin on neutrophil accumulation in the infarct zone 24 h after ischaemia–reperfusion. Both low and high doses of darbepoetin were equally effective in attenuating neutrophil accumulation, by approximately 50% ($P < 0.05$; Figure 2).

Darbepoetin and genes regulating angiogenesis

EPO can induce angiogenesis in cultured heart tissue independently of endothelial progenitor cells (Jaquet *et al.*, 2002). To determine whether darbepoetin treatment also increases vascularization in the infarct zone after ischaemia–reperfusion injury, we assessed vessel numbers. Infarcted regions of the left ventricle of mice treated with darbepoetin con-

tained up to three times more CD31-positive vessels compared to untreated mice ($P < 0.05$; Figure 3A). This was apparent with both the low and high darbepoetin doses.

We next investigated whether this increase in vessel numbers was associated with increased expression of angiogenic factors. We assessed its ability to stimulate expression of genes encoding angiogenic factors by analyzing mRNA obtained 24 h after treatment, using angiogenesis oligonucleotide gene arrays. Twenty-four hours after infarction, a number of negative regulators of angiogenesis were down-regulated in the infarct zone, including tissue inhibitor of metalloproteinase-3 (TIMP3), midkine (MDK), ephrin-A1 (EFNA-1) and IL-10, while natriuretic peptide precursor B was up-regulated (Figure 3B).

Darbepoetin increased expression of placental growth factor (PGF) 5- to 10-fold ($P < 0.05$), specifically in infarcted myocardium. Increases in PGF were apparent after low- and high-dose darbepoetin (Figure 3B). We were unable to detect its receptor Flt-1 in the infarct zone (Figure 3B), but neuropilin-1 (NRP1) was readily detectable (Figure 3B). Chemokine (C-C motif) ligand 2 (CCL2, MCP-1) was also elevated specifically in infarcted myocardium ($P < 0.05$; Figure 3B). Although best known for inducing monocyte/macrophage recruitment to sites of inflammation, CCL2 is also capable of stimulating endothelial cell chemotaxis and inducing new vessel formation (Salcedo *et al.*, 2000). Darbepoetin also increased interleukin-1 β (IL-1 β) expression three- to fourfold ($P < 0.05$; Figure 3B), which can also exert pro-angiogenic effects (Naldini *et al.*, 2006). In contrast, vascular endothelial growth factor (VEGF)-B expression was unaffected as was VEGF-A (Figure 3B). Interestingly, increased VEGF-A expression was only apparent in non-infarcted regions of the heart and not dependent on darbepoetin, but was associated with darbepoetin-mediated elevations in Flt-1 mRNA (Figure 3B). Platelet-derived growth factor (PDGF)-A chain mRNA was also up-regulated by darbepoetin, but only in non-infarcted regions of the heart as was TEK receptor tyrosine kinase ($P < 0.05$; Figure 3B).

Darbepoetin and fibrosis

Because the frequency of cardiomyocyte apoptosis was attenuated and BNP elevated by darbepoetin, we reasoned that this may result in less fibrosis. Accordingly, we investigated whether treatment with darbepoetin also attenuated fibrosis following cardiac ischaemia–reperfusion. Treatment with darbepoetin attenuated fibrosis by more than 50% ($P < 0.05$; Figure 4) and was apparent with low and high doses of darbepoetin.

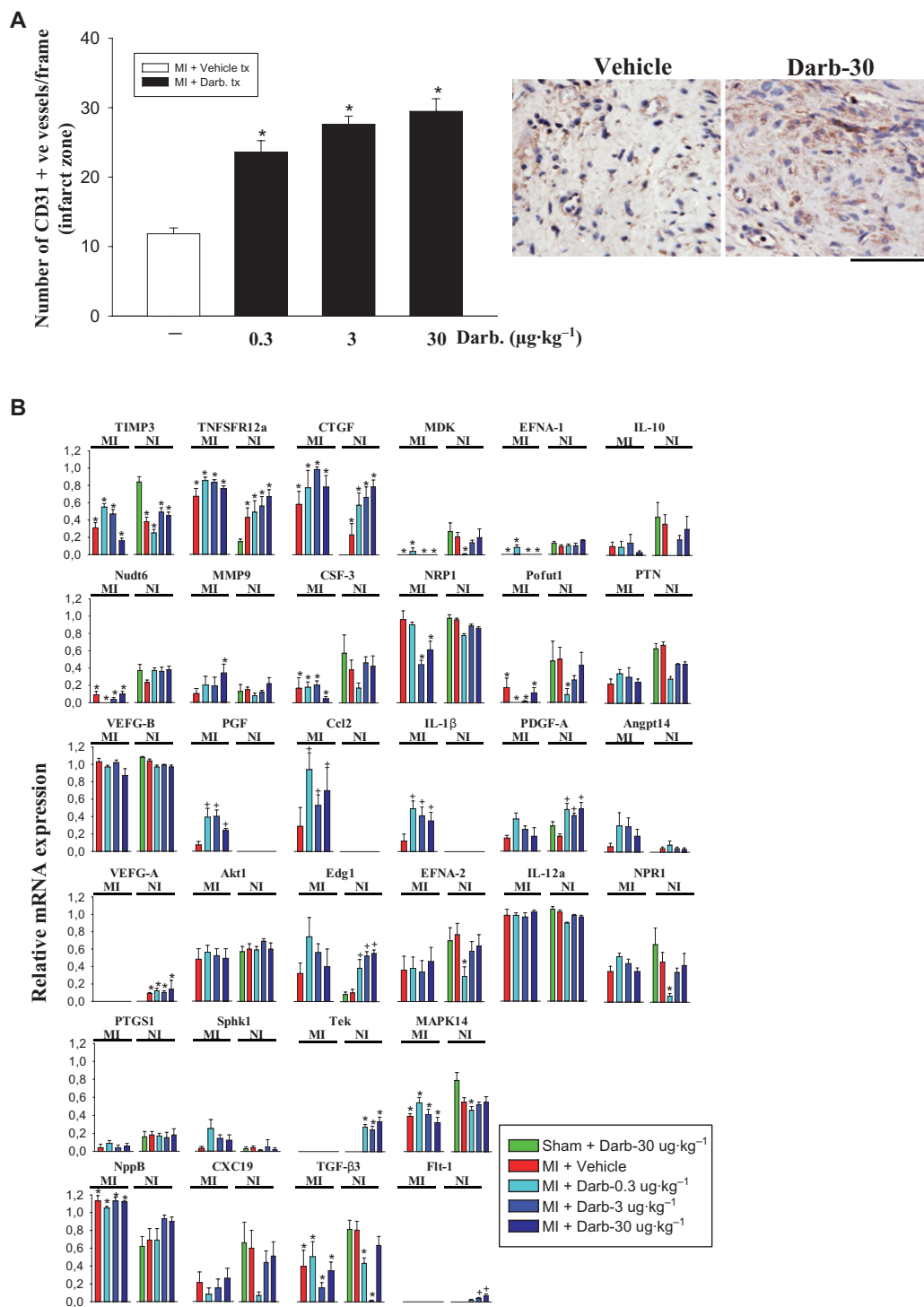


Figure 3

Darbepoetin affects vessels in infarct zone and angiogenic genes. (A) Left: CD31-positive vessels in infarcted region of myocardium 28 days after treatment with vehicle or darbepoetin. Right: Cd31-positive vessels in the infarct zone of mice treated with vehicle or darbepoetin ($30 \mu\text{g}\cdot\text{kg}^{-1}$). The bar represents $100 \mu\text{m}$. (B) Expression of 28 angiogenic genes 24 h after myocardial infarction/sham operation and treatment with vehicle or darbepoetin. TNFSFR12a, tumour necrosis super family receptor 12a; CTGF, connective tissue growth factor; IL-10, interleukin-10; Nudt6, nudix (nucleoside diphosphate linked moiety X)-type motif 6; MMP9, matrix metalloproteinase-9; CSF-3, G-CSF; NRP1, neuropilin-1; Pofut 1, protein O-fucosyltransferase-1; PTN, pleiotrophin; CCL2, monocyte chemoattractant protein-1; Angpt14, angiopoietin-like-4; PTGS1, prostaglandin endoperoxide synthase-1; Sphk1, sphingosine kinase-1. All other genes follow standard accepted abbreviations. In (A), results are means \pm SEM of five mice in each group. $*P < 0.05$ from sham-operated, darbepoetin-treated mice. In (B), results are means \pm SEM of 10 mice in each group. $*P < 0.05$ from darbepoetin-treated, sham-operated mice; $+P < 0.05$ from infarcted, vehicle-treated mice.

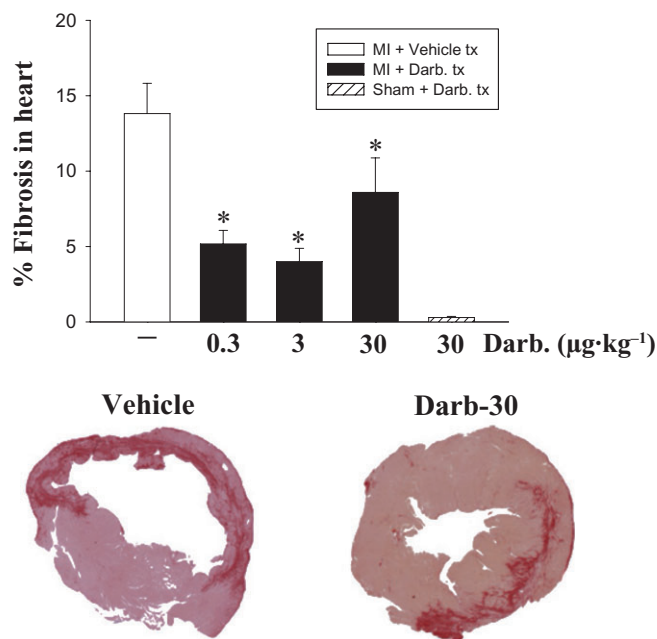


Figure 4

Darbepoetin and cardiac fibrosis. Fibrosis was assessed 28 days after ischaemia/sham operation and treatment with vehicle or darbepoetin. Top: cross-sections were stained with picosirius red, and fibrosis expressed as % area stained with picosirius red in each cross section. Results are means \pm SEM from 10 mice in each group. * $P < 0.05$ from infarcted, vehicle-treated mice. Bottom: photomicrographs of cross sections of infarcted left ventricle stained with picosirius red, demonstrating the extent of fibrosis in mice treated with vehicle or darbepoetin (30 µg·kg⁻¹).

Darbepoetin improved cardiac haemodynamics

Because darbepoetin reduced inflammation and improved healing of infarcted hearts, we reasoned that this may also result in improved cardiac haemodynamics. Darbepoetin improved cardiac haemodynamics, increasing LVSP, LV + dp/dt and LV – dp/dt ($P < 0.05$; Figure 5), effects that were most apparent at intermediate and high doses of darbepoetin.

Darbepoetin and c-β-chain

It has been suggested that the tissue-protective effects of EPO might be dependent on its interaction with an EPO–c-β-chain heteroreceptor complex (Brines *et al.*, 2004). To determine whether the cardioprotective effects of darbepoetin were dependent on an EPO–c-β-chain heteroreceptor complex, we evaluated its effects in c-β-chain-deficient mice. c-β-Chain-deficient mice were resistant to the detrimental effects of 1 h ischaemia followed by reperfusion. In these mice, LVSP, LV + dp/dt and LV – dp/dt were unaffected 28 days after ischaemia–reperfusion and similar to sham-operated c-β-chain-deficient mice (P

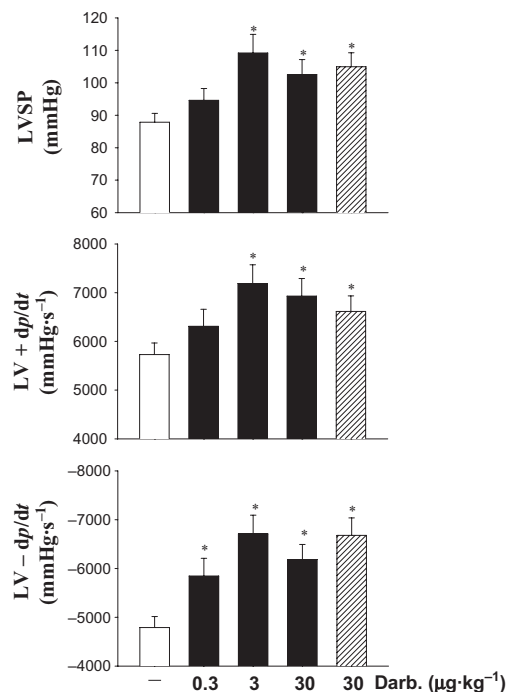


Figure 5

Cardiac haemodynamics following ischaemic injury. LVSP, and LV + dp/dt and LV – dp/dt were measured in anaesthetized C57Bl6 mice using an intraventricular Miller catheter. Results are means \pm SEM of 10 mice in each group. * $P < 0.05$ from infarcted, vehicle-treated mice.

for differences >0.05 ; Figure 6). Administration of darbepoetin to c-β-chain-deficient mice subjected to ischaemia–reperfusion did not further improve already normal cardiac haemodynamics (Figure 6). Consequently, we assessed its effects on c-β-chain-deficient mice after more severe cardiac damage, induced by permanent ligating the coronary artery. Following permanent coronary ligation, LVSP, LV + dp/dt and LV – dp/dt in the c-β-chain-deficient mice were reduced by 25–35% (all $P < 0.05$; Figure 6). In these mice, darbepoetin administered 1 h after the onset of cardiac ischaemia significantly ($P < 0.05$) increased LVSP 28 days later; LV + dp/dt and LV – dp/dt also tended to be increased (Figure 6). To determine whether other tissue responses were affected, we also assessed its effects on the expression of HO-1 and iNOS protein, as well as fibrosis and vessel numbers within the infarct border zone. Treatment of the c-β-chain-deficient mice with darbepoetin markedly increased expression of HO-1 and iNOS protein in cardiomyocytes within the infarct zone 24 h later (Figure 7). Treatment also resulted in 50% less cardiac fibrosis ($P < 0.05$; Figure 7) and significantly increased the number of CD31+ vessels within the infarct border zone 28 days later ($P < 0.05$; Figure 7).

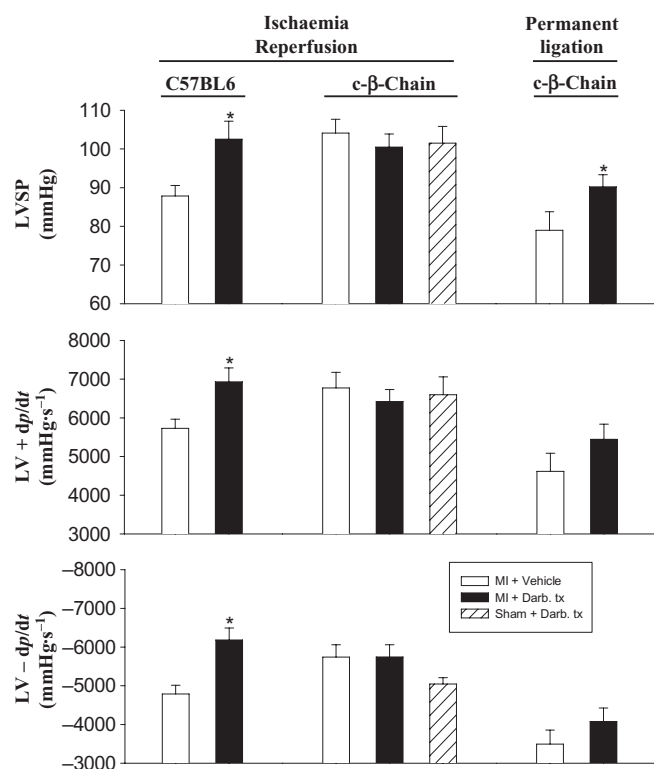


Figure 6

Effects of darbepoetin on cardiac haemodynamics (LVSP, LV + dp/dt and LV - dp/dt) 28 days after subjecting C57BL6 and c-β-chain-deficient mice to 1 h of ischaemia followed by reperfusion and also c-β-chain-deficient mice to permanent coronary ligation. Results are means ± SEM of six to nine mice in each group. **P* < 0.05 from vehicle-treated, infarcted mice.

Discussion

Our results indicate that darbepoetin administration at the time of reperfusion initiates multiple cellular responses in the ischaemic heart, which contributes to its cardioprotective effects. This includes activation of anti-inflammatory and angiogenic genes: iNOS, BNP, HO-1, PGF, CCL2 and IL-1β. Their elevations were associated with reduced inflammation, reduced apoptosis and reduced fibrosis, as well as improved vascularization and cardiac function, effects that are independent of signalling via an EPOR-c-β-chain heteroreceptor complex.

Treatment with darbepoetin markedly elevated the expression of three cardioprotective genes specifically within the infarcted region of the heart: iNOS, HO-1 and BNP. Over-expression of iNOS protects against cardiac ischaemia-reperfusion injury by preventing mitochondrial permeability transition and attenuating free radical generation (West *et al.*, 2008). NO generated by iNOS can also attenuate apoptosis by inhibiting caspase-3 via S-nitrosation (Rossig *et al.*, 1999). While not speci-

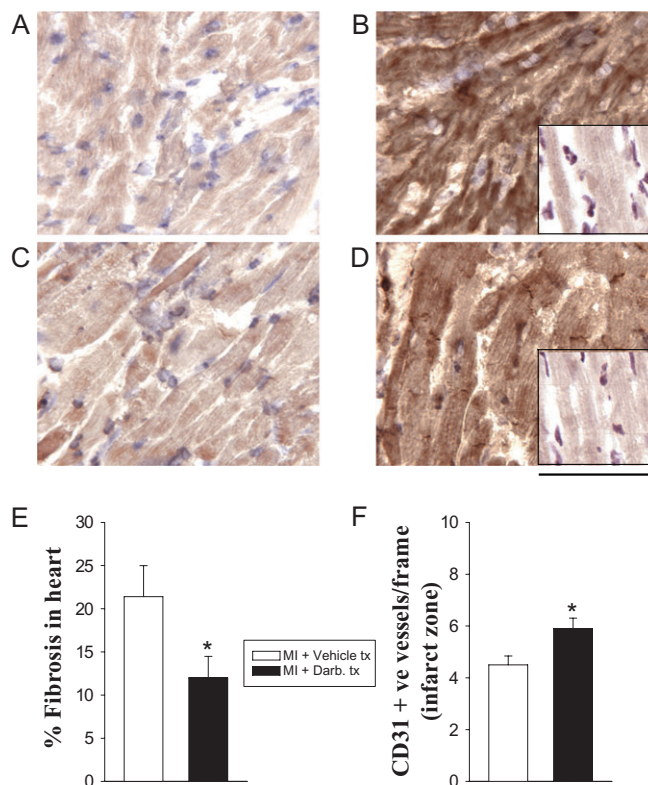


Figure 7

Effects of darbepoetin on the expression of HO-1 and iNOS in infarcted left ventricle of c-β-chain-deficient mice 24 h after permanent coronary ligation, and fibrosis and number of vessels 28 days later. (A) iNOS expression in infarcted left ventricle of vehicle-treated mice. (B) iNOS expression in infarcted left ventricle of darbepoetin-treated mice; insert: non-immune IgG control. (C) HO-1 expression in infarcted left ventricle of vehicle-treated mice. (D) HO-1 expression in infarcted left ventricle of darbepoetin-treated mice; insert: non-immune IgG control. (E) Left ventricular fibrosis (F) vessels in border infarct zone of left ventricle. Results are means ± SEM of six to nine mice in each group. **P* < 0.05 from vehicle-treated, infarcted mice.

cally studied, it is likely that darbepoetin stimulates iNOS over-expression in the infarct zone via a STAT3 pathway. Darbepoetin activates STAT3 pathways in autoimmune cardiomyopathies (Mao *et al.*, 2008), and STAT3 is crucial for up-regulation of iNOS (Ziesche *et al.*, 2007). Our study suggests that B-type natriuretic peptide (BNP) may also contribute to darbepoetin's cardioprotective effects; darbepoetin up-regulated BNP expression specifically in the infarct zone. BNP is a cardiac anti-fibrotic factor *in vivo* and a local regulator of ventricular remodelling (Tamura *et al.*, 2000).

Our finding that darbepoetin elevated HO-1 expression is consistent with its effects on iNOS expression; NO produced by iNOS stabilizes mRNAs and via this mechanism increases HO-1 (Kuвано *et al.*, 2009). HO-1 is a phase II enzyme, a member of

the family of heat shock protein that is associated with defence responses against oxidative and cellular stress, and also promotes angiogenesis (Dulak *et al.*, 2008). It interacts with haeme to yield equimolar quantities of iron, biliverdin and carbon monoxide (Dulak *et al.*, 2008). Increased carbon monoxide production is most likely the mechanism by which HO-1 exerts its cardioprotective, anti-apoptotic and anti-inflammatory effects. Carbon monoxide protects against cardiac ischaemia-reperfusion injury (Fujimoto *et al.*, 2004), and the HO-1/carbon monoxide-biliverdin pathway down-regulates neutrophil rolling, adhesion and migration in acute inflammation (Freitas *et al.*, 2006). This latter mechanism explains why darbepoetin attenuates neutrophil accumulation following cardiac ischaemia-reperfusion. HO-1-derived carbon monoxide also protects endothelial cells against apoptosis, by elevating NF- κ B-inducible anti-apoptotic genes A20, c-IAP2 and manganese superoxide dismutase (Brouard *et al.*, 2002). Whether such a mechanism also protects cardiomyocytes against apoptosis remains to be determined. Over-expression of cardiac HO-1 also attenuates fibrosis after myocardial infarction (Liu *et al.*, 2006); hence, it is likely that the darbepoetin-induced over-expression of HO-1 also contributes to its anti-fibrotic effect after infarction.

Our study confirms earlier studies in pigs that darbepoetin can increase vessel number in the infarct zone (Toma *et al.*, 2007). This increase could be due to a number of mechanisms including anti-apoptotic effects of darbepoetin on endothelial cells and/or increased angiogenesis stimulated by growth factors. Our gene array studies indicate that darbepoetin elevates the angiogenic factors, PGF, CCL2 and IL-1 β , specifically within the infarct zone, while VEGF-A was elevated only within non-infarcted regions of the heart. Because Flt-1 was only elevated in non-infarcted regions of the heart, it is unlikely that PGF directly contributes to the increase in vessel number. It is possible that CCL2 and IL-1 β contribute to vessel numbers within the infarct zone. CCL2 recruits macrophages to the infarct zone, as well as stimulating angiogenesis (Schwarz *et al.*, 2004), while IL-1 β induces angiogenesis independently of VEGF or FGF-2 (Naldini *et al.*, 2006). Although adeno-associated adenoviral over-expression of HO-1 promotes vascularization in ischaemia heart, which is associated with increased expression of VEGF (Lin *et al.*, 2008), the elevation in endogenous HO-1 induced by darbepoetin is unlikely to contribute to angiogenesis, as we did not observe any associated increase in VEGF.

Recently, non-erythropoietic derivatives of EPO have also been shown to be cardioprotective

(Fiordaliso *et al.*, 2005). Because these compounds do not bind to 'classical' homodimeric EPORs (Leist *et al.*, 2004), it has been suggested that cardioprotection may be mediated via an EPOR-c- β -chain heteroreceptor; EPO failed to protect cultured cardiomyocytes deficient in the common β -subunit against staurosporin-induced apoptosis (Brines *et al.*, 2004). Our studies with darbepoetin in mice deficient in the c- β -chain indicate that expression of the c- β -chain is not essential for its cardioprotective effects *in vivo*, because it still improved cardiac haemodynamics, stimulated the expression of HO-1 and iNOS, reduced fibrosis and increased vessel numbers in c- β -chain-deficient mice. Rather, our results are consistent with darbepoetin mediating its cardioprotective effects via the classical dimeric EPOR. In these studies, we unexpectedly found that c- β -chain-deficient mice are much more resistant to ischaemia-reperfusion injury than C57Bl6 mice, necessitating us to permanently ligate the coronary artery to significantly impair cardiac haemodynamics, so that we could assess the ability of darbepoetin to improve cardiac function. The reason for this resistance of c- β -chain-deficient mice requires further studies and may involve reductions in M1 macrophages and a change in the balance between inflammatory M1- and healing M2-macrophages, which accumulate in infarcted regions of the heart (Nahrendorf *et al.*, 2007); c- β -chain is essential for GM-CSF-mediated induction of M1 macrophages (Leidi *et al.*, 2009).

In conclusion, our results demonstrate that darbepoetin stimulates multiple cardioprotective mechanisms in infarcted myocardium to improve cardiac function. Its cardioprotective effects appear to be mediated via molecular mechanisms that attenuate apoptosis, inflammation and fibrosis, as well as increasing vessel numbers within the infarcted regions. These effects are not dependent on signalling via an EPO-c- β -chain heteroreceptor complex.

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Conflict of interest

All authors have no conflict of interest.

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