

Erythropoietin attenuates cardiac dysfunction in experimental sepsis in mice via activation of the β -common receptor

Areeg I. Khan^{1,*‡}, Sina M. Coldewey^{1,2,*}, Nimesh S. A. Patel¹, Mara Rogazzo³, Massimo Collino³, Muhammed M. Yaqoob⁴, Peter Radermacher⁵, Amar Kapoor¹ and Christoph Thiemermann^{1,‡}

SUMMARY

There is limited evidence that the tissue-protective effects of erythropoietin are mediated by a heterocomplex of the erythropoietin receptor and the β -common receptor ('tissue-protective receptor'), which is pharmacologically distinct from the 'classical' erythropoietin receptor homodimer that is responsible for erythropoiesis. However, the role of the β -common receptor and/or erythropoietin in sepsis-induced cardiac dysfunction (a well known, serious complication of sepsis) is unknown. Here we report for the first time that the β -common receptor is essential for the improvements in the impaired systolic contractility afforded by erythropoietin in experimental sepsis. Cardiac function was assessed *in vivo* (echocardiography) and *ex vivo* (Langendorff-perfused heart) in wild-type and β -common receptor knockout mice, that were subjected to lipopolysaccharide (9 mg/kg body weight; young mice) for 16-18 hours or cecal ligation and puncture (aged mice) for 24 hours. Mice received erythropoietin (1000 IU/kg body weight) 1 hour after lipopolysaccharide or cecal ligation and puncture. Erythropoietin reduced the impaired systolic contractility (*in vivo* and *ex vivo*) caused by endotoxemia or sepsis in young as well as old wild-type mice in a β -common-receptor-dependent fashion. Activation by erythropoietin of the β -common receptor also resulted in the activation of well-known survival pathways (Akt and endothelial nitric oxide synthase) and inhibition of pro-inflammatory pathways (glycogen synthase kinase-3 β , nuclear factor- κ B and interleukin-1 β). All the above pleiotropic effects of erythropoietin were lost in β -common receptor knockout mice. Erythropoietin attenuates the impaired systolic contractility associated with sepsis by activation of the β -common receptor, which, in turn, results in activation of survival pathways and inhibition of inflammation.

INTRODUCTION

Erythropoietin (EPO) is widely used for the treatment of anemia in patients (Drüeke et al., 2006). However, during the past two decades, our understanding of the actions of EPO has shifted from a belief that the hormone acts exclusively on erythroid progenitor cells to the knowledge that this agent exerts significant protection in conditions such as myocardial ischemia-reperfusion (Calvillo et al., 2003) and haemorrhagic shock (Abdelrahman et al., 2004), and improves survival in sepsis (Aoshiha et al., 2009). The beneficial effects of EPO are allegedly mediated by a putative 'tissue-protective receptor' that is distinct from the 'classical' EPO receptor (EpoR)

known to mediate erythropoiesis (Leist et al., 2004). This receptor has been proposed to be a heteromer between the EPO receptor and the β -common receptor (β cR; also called CD131) (Brines et al., 2004). β cR is a common subunit of other heteroreceptors, including those of interleukin (IL)-3, IL-5 and granulocyte-macrophage colony stimulating factor (Murphy and Young, 2006). The 'tissue-protective receptor' specifically binds to EPO with a lower affinity than does the classical EpoR (Masuda et al., 1993). Indeed, Brines et al. were able to demonstrate cellular colocalization of EPO, β cR and EpoR in spinal cord neurons and cardiomyocytes (Brines et al., 2004). Furthermore, the structural importance of an interaction between EPO and β cR was first highlighted by Sautina et al. (Sautina et al., 2010); however, the clinical implication of this in sepsis is unknown.

Sepsis, the systemic inflammatory response syndrome to infection, has high incidence and mortality rates around the world (Fernandes and Cesar de Assuncao, 2012; Rudiger and Singer, 2007). Myocardial dysfunction is a recognized manifestation of sepsis and septic shock, with myocardial depression occurring in almost 40-50% of patients (Rudiger and Singer, 2007). Contractile dysfunction is characterized by biventricular dilatation, blunted blood pressure response to intravenous fluids, a reversible reduction in ejection fraction (EF) and a diminished ability to augment cardiac output despite increased levels of circulating catecholamines (Flynn et al., 2010; Hoesel et al., 2007). Various mechanisms have been proposed for this myocardial dysfunction, including excessive cardiac inflammation (Merx and Weber, 2007), mitochondrial dysfunction (Suliman et al., 2004; Watts et al., 2004), cardiac cell death by apoptosis or necrosis (Sharma, 2007), impaired contractility

¹Centre for Translational Medicine and Therapeutics, Queen Mary University of London, Barts and The London School of Medicine and Dentistry, The William Harvey Research Institute, EC1M 6BQ, London, UK

²Department of Anesthesia and Intensive Care Medicine, Hannover Medical School, Hannover 30625, Germany

³Department of Drug Science and Technology, University of Turin, Turin 10125, Italy

⁴Department of Nephrology, Barts Health NHS Trust, The Royal London Hospital, London, E1 1BB, UK

⁵Division of Pathophysiology and Process Development, Department of Anesthesiology, University Hospital, Ulm 89081, Germany

*These authors contributed equally to this work

‡Authors for correspondence (a.i.a.khan@qmul.ac.uk; c.thiemermann@qmul.ac.uk)

Received 21 January 2013; Accepted 7 March 2013

© 2013. Published by The Company of Biologists Ltd
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Share Alike License (<http://creativecommons.org/licenses/by-nc-sa/3.0/>), which permits unrestricted non-commercial use, distribution and reproduction in any medium provided that the original work is properly cited and all further distributions of the work or adaptation are subject to the same Creative Commons License terms.

TRANSLATIONAL IMPACT

Clinical issue

Myocardial dysfunction occurs as a complication in almost 40-50% of sepsis cases and is a major contributor to morbidity and mortality in these patients. There are currently no specific therapeutic interventions available to treat this condition. In the last decade, erythropoietin (EPO), a key player in erythropoiesis, has emerged as a pleiotropic cytokine that protects against tissue injury and dysfunction. Clinical trials have demonstrated the tissue-protective role of EPO in various conditions, but the positive effects were accompanied by significant adverse side effects. The beneficial effects of EPO are thought to be mediated by a 'tissue-protective receptor' that is distinct from the 'classical' erythropoiesis-associated receptor, EpoR. It has been proposed that the tissue-protective receptor is a heteromer of EpoR together with the β -common receptor (β cR). There is an urgent need to fully understand the mechanisms underlying the tissue-protective role of EPO, to facilitate the development of an effective and safe treatment for sepsis-associated myocardial dysfunction.

Results

In this study, the effects of EPO on cardiac function were assessed *in vivo* and *ex vivo* (using Langendorff-perfused hearts) in mouse models of endotoxemia and polymicrobial sepsis. EPO treatment improved systolic contractility in young as well as aged mice with either condition. The observed beneficial effects of EPO were shown to be associated with activation of well-known survival pathways (Akt and endothelial nitric oxide synthase) and inhibition of pro-inflammatory pathways (glycogen synthase kinase-3 β , nuclear factor- κ B and interleukin-1 β). Using β cR knockout mice, the authors demonstrated that the effects of EPO are dependent on the presence of β cR.

Implications and future directions

This study demonstrates for the first time that activation of β cR by EPO is essential for the observed improvement in systolic contractility afforded by EPO treatment in endotoxemia or sepsis. Recently, it has been demonstrated that chemically modified versions of EPO (e.g. carbamylated EPO) and peptides that mimic the structure of EPO [e.g. pyroglutamate surface B helix peptide (pHBSP)] show tissue-protective effects, but do not mediate erythropoiesis, suggesting that these agonists are specific for the tissue-protective receptor. These agonists potentially do not give rise to the complications associated with EPO treatment, such as thrombosis or hypertension. In light of the data presented here, targeting the β cR-EpoR heterocomplex with agonists such as pHBSP might represent a novel, specific approach for the treatment of sepsis-associated cardiac dysfunction. The clinical evaluation of the effects of EPO-like agonists in conditions associated with tissue injury and inflammation is eagerly awaited.

secondary to the generation of TNF α (Khadour et al., 2002) or induction of inducible nitric oxide synthase (iNOS) (Khadour et al., 2002; Barth et al., 2006). The effects of EPO in sepsis-induced cardiac dysfunction are unknown.

This study investigates the effects of EPO and, specifically, the role of β cR in the cardiac dysfunction associated with endotoxemia [caused by lipopolysaccharide (LPS)] and polymicrobial sepsis [caused by cecal ligation and puncture (CLP)]. Specifically, we have investigated: (1) the effect that EPO has on the impairment of systolic contractility (measured *in vivo* by echocardiography or *ex vivo* in the isolated Langendorff-perfused heart) associated with endotoxemia and polymicrobial sepsis using wild-type (WT) C57BL/6 mice; (2) whether the effect of EPO is mediated through the proposed tissue protective receptor, with the use of β cR knockout (KO) mice; (3) the mechanisms underlying the observed beneficial effects of EPO, including the phosphorylation of Akt on Ser473, phosphorylation of glycogen synthase kinase-3 β (GSK-3 β)

on Ser9, phosphorylation of endothelial nitric oxide synthase (eNOS) on Ser1177, activation of nuclear factor (NF) κ B (measured as nuclear translocation of p65) and expression of interleukin-1 β (IL-1 β). Because sepsis most frequently occurs in aging patients (Girard et al., 2005), we then investigated the effects of EPO and the role of β cR in aging animals with sepsis.

RESULTS

Effect of EPO on cardiac dysfunction in endotoxemic WT mice assessed by echocardiography and the isolated Langendorff-perfused heart

To investigate the effect that EPO has on the cardiac dysfunction caused by LPS, left ventricular (LV) function was assessed using echocardiography in WT mice, 18 hours after administration of vehicle or LPS. We saw no differences in LV dimensions {left ventricular internal-diastolic dimension [LVID(D)] and left ventricular end-diastolic volume (LVEDV)} between the WT groups (Table 1). Fig. 1A shows representative M-mode echocardiograms of sham + vehicle, sham + EPO, LPS + vehicle, and LPS + EPO in WT mice. When compared with sham + vehicle mice, sham mice treated with EPO demonstrated no significant alterations in percentage EF, fractional shortening (FS) or fractional area of change (FAC) ($P>0.05$) (Fig. 1B-D). When compared with sham mice, mice subjected to 18 hours of endotoxemia demonstrated a significant reduction in percentage EF, FS and FAC ($P<0.05$) (Fig. 1B-D), indicating impairment in systolic contractility *in vivo*. Administration of EPO significantly attenuated the impairment in systolic contractility associated with endotoxemia ($P<0.05$) (Fig. 1B-D).

To investigate whether the impairment in systolic contractility observed *in vivo* can be confirmed in an isolated Langendorff-perfused heart *ex vivo* (under conditions of constant cardiac preload or afterload), the alterations in isovolumic left ventricular developed pressure (LVDP) were assessed in WT mice 16-18 hours after administration of vehicle or LPS. Pressure-volume curves were generated to assess alterations in LVDP in response to 5 μ l incremental intraventricular volume-balloon loading up to 40 μ l (Fig. 1E). When compared with sham mice, WT mice subjected to LPS exhibited impairment in systolic contractility, measured as a significant reduction in LVDP in response to 30 μ l (maximum response) volume loading ($P<0.05$) (Fig. 1E,F). Administration of EPO to endotoxemic WT mice significantly attenuated the impairment in systolic contractility at this volume load ($P<0.05$) (Fig. 1E,F).

Effect of EPO on cardiac dysfunction in endotoxemic β cR KO mice assessed by echocardiography and the isolated Langendorff-perfused heart

To investigate the role of the β cR subunit in the observed beneficial properties of EPO reported above, we evaluated the cardioprotective effects of EPO in β cR KO mice. We saw no differences in LV dimensions between the KO groups with the exception of a significant increase in LVID(D) between sham + vehicle and endotoxemic β cR KO mice, which was not altered with the administration of EPO (Table 1). Fig. 2A shows representative M-mode echocardiograms of sham + vehicle, sham + EPO, LPS + vehicle, and LPS + EPO in β cR KO mice. When compared with sham + vehicle mice, sham mice treated with EPO demonstrated no significant alterations in EF, FS and FAC ($P>0.05$) (Fig. 2B-D). When compared with sham β cR KO

Table 1. Effect of EPO on echocardiographic parameters (diastolic function) in WT or β cR KO mice subjected to endotoxemia for 16-18 hours or CLP for 24 hours

Group	Genotype	LVID(D) (mm)	LVEDV (μ l)	n
Endotoxemia in 2-month-old mice				
Sham + vehicle	WT	3.4 \pm 0.06	47 \pm 2.13	10
Sham + EPO	WT	3.1 \pm 0.14	39 \pm 4.34	6
LPS + vehicle	WT	3.6 \pm 0.07	54 \pm 2.60	15
LPS + EPO	WT	3.6 \pm 0.08	53 \pm 2.61	15
Sham + vehicle	KO	3.1 \pm 0.09*	39 \pm 2.91	9
Sham + EPO	KO	3.5 \pm 0.15	52 \pm 5.36	3
LPS + vehicle	KO	3.5 \pm 0.08	53 \pm 2.96	14
LPS + EPO	KO	3.5 \pm 0.07	53 \pm 2.65	25
Polymicrobial sepsis in aged mice				
Sham + vehicle (8 months)	WT	3.5 \pm 0.30	51 \pm 9.44	3
Sham + EPO (8 months)	WT	3.5 \pm 0.12	51 \pm 3.98	3
CLP + vehicle (2 months)	WT	3.5 \pm 0.08	51 \pm 2.61	5
CLP + vehicle (5 months)	WT	4.1 \pm 0.15	73 \pm 6.29	5
CLP + vehicle (8 months)	WT	3.9 \pm 0.09	68 \pm 3.55	9
CLP + EPO (8 months)	WT	3.8 \pm 0.13	62 \pm 5.03	10
Sham + vehicle (8 months)	KO	4.2 \pm 0.11	80 \pm 4.83	5
Sham + EPO (8 months)	KO	3.5 \pm 0.00	52 \pm 0.76	2
CLP + vehicle (8 months)	KO	3.8 \pm 0.12	64 \pm 4.24	7
CLP + EPO (8 months)	KO	4.1 \pm 0.14	74 \pm 6.05	7

WT or β cR KO mice received either LPS (9 mg/kg i.p.) or vehicle (5 ml/kg 0.9% saline i.p.) or underwent CLP surgery. At 1 hour after induction of endotoxemia or CLP surgery, mice were treated either with EPO (1000 IU/kg s.c.) or vehicle (10 ml/kg 0.9% saline s.c.). All echocardiographic images were assessed for changes in LVID(D) and LVEDV. Data are expressed as means \pm s.e.m. for n number of observations. * P <0.05 versus LPS + vehicle (KO).

mice, endotoxemia in β cR KO mice resulted in a significant reduction in percentage EE, FS and FAC (P <0.05) (Fig. 2B-D), indicating impairment in systolic contractility. Administration of EPO to endotoxemic β cR KO mice did not alter this impairment in systolic contractility (P >0.05) (Fig. 2B-D).

We sought to confirm these effects in the isolated Langendorff-perfused heart. When compared with sham mice, β cR KO mice subjected to LPS exhibited impaired systolic contractility, measured as a significant reduction in LVDP in response to 30 μ l (maximum response) volume loading (P <0.05) (Fig. 2E,F). Administration of EPO to endotoxemic β cR KO mice caused no significant change in the impairment in systolic contractility associated with endotoxemia (P >0.05) (Fig. 2E,F).

Effect of EPO on the phosphorylation of Akt, GSK-3 β and eNOS in the hearts of endotoxemic WT and β cR KO mice

In order to gain a better insight into the potential mechanism(s) underlying the observed cardioprotective effects of EPO, we investigated the effects of EPO on cell signalling pathways known to confer tissue protection or to inhibit inflammation, by semi-quantitative western blot analysis of the heart. When compared with sham mice, WT and β cR KO mice subjected to LPS demonstrated no change in the phosphorylation of Akt on Ser473 (P >0.05) (Fig. 3A), GSK-3 β on Ser9 (P >0.05) (Fig. 3B) or eNOS on Ser1177 (P >0.05) (Fig. 3C). Administration of EPO to endotoxemic WT mice, however, resulted in a significant increase in the phosphorylation of Akt on Ser473 (P <0.05) (Fig. 3A), GSK-3 β on

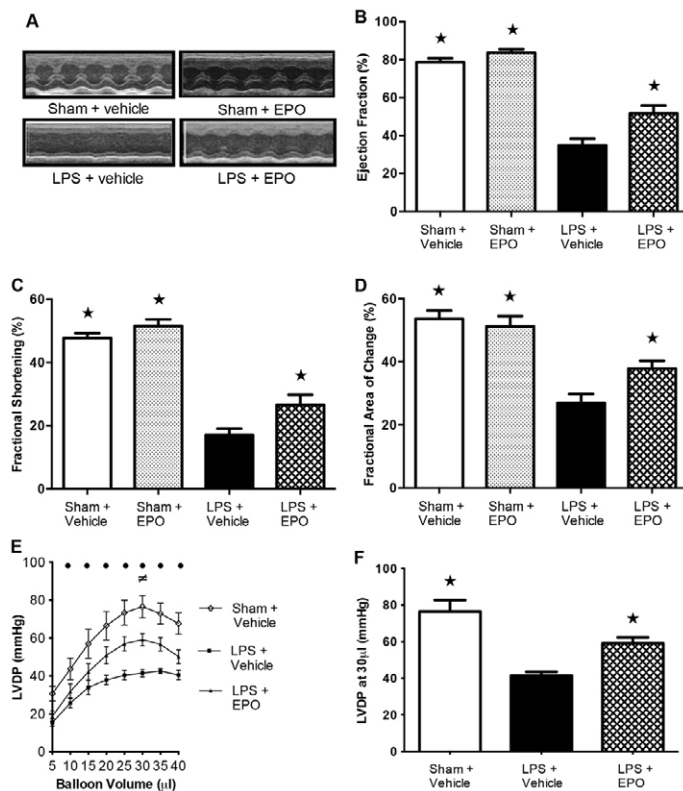
Ser9 (P <0.05) (Fig. 3B) and eNOS on Ser1177 (P <0.05) (Fig. 3C). In contrast, administration of EPO to endotoxemic β cR KO mice caused no change in the phosphorylation of Akt on Ser473 (P >0.05) (Fig. 3A), GSK-3 β on Ser9 (P >0.05) (Fig. 3B) or eNOS on Ser1177 (P >0.05) (Fig. 3C).

Effect of EPO on the nuclear translocation of the p65 NF κ B subunit in the hearts of endotoxemic WT and β cR KO mice

When compared with hearts from sham mice, the hearts of endotoxemic WT and β cR KO mice exhibited significant increases in the nuclear translocation of the p65 subunit (P <0.05) (Fig. 3D), indicating activation of NF κ B. Administration of EPO to endotoxemic WT mice significantly attenuated the increase in nuclear translocation of p65 and, hence, inhibition of activation of NF κ B in the heart (P <0.05) (Fig. 3D). In contrast, administration of EPO to endotoxemic β cR KO mice did not attenuate the nuclear translocation of p65 caused by LPS (P >0.05) (Fig. 3D).

Effect of EPO on the expression of IL-1 β in the hearts of endotoxemic WT and β cR KO mice

When compared with hearts from sham mice, the hearts of endotoxemic WT and β cR KO mice demonstrated a significant increase in IL-1 β expression (P <0.05) (Fig. 3E). Administration of EPO to endotoxemic WT mice significantly attenuated IL-1 β expression (P <0.05) (Fig. 3E). In contrast, administration of EPO to endotoxemic β cR KO mice caused no alteration in IL-1 β expression (P >0.05) (Fig. 3E).



Effect of EPO on cardiac dysfunction in WT and β cR KO mice that underwent CLP

The murine model of CLP with fluid resuscitation and antibiotics used in this study is regarded as a clinically relevant model of abdominal polymicrobial human sepsis. We established an aged model of polymicrobial sepsis-induced cardiac dysfunction, using 2-, 5- and 8-month-old mice, which demonstrated an age-dependent decrease in systolic contractility (see supplementary material Fig. S1). Therefore, 8-month-old mice were used in all the subsequent experiments.

We saw no differences in LV dimensions (Table 1) between the WT groups. Fig. 4A shows representative M-mode echocardiograms of sham + vehicle, sham + EPO, CLP + vehicle, and CLP + EPO in WT mice. When compared with sham + vehicle

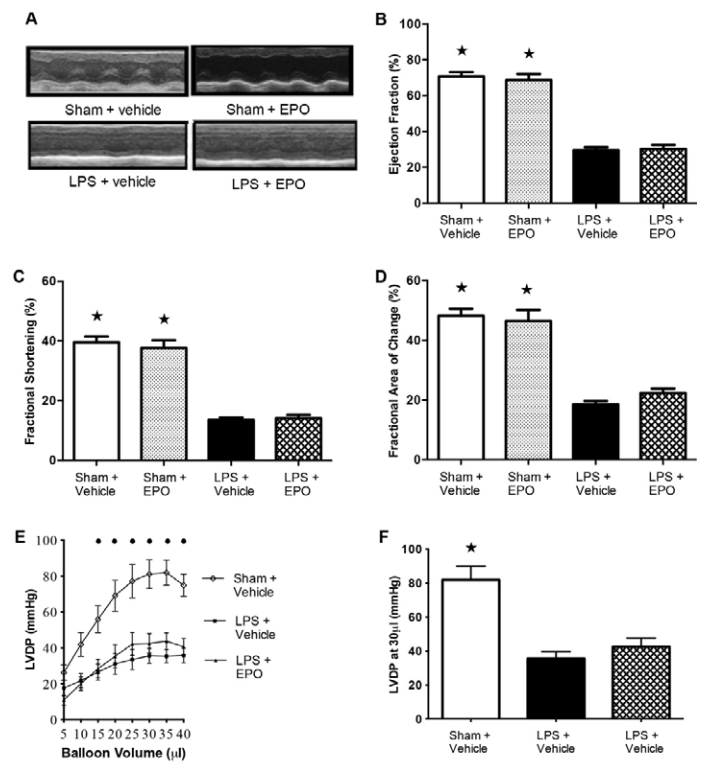


Fig. 2. Effect of erythropoietin (EPO) on the cardiac dysfunction in endotoxemic β -common receptor knockout (β cR KO) mice.

(A-D) Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) 18 hours subsequent to sham-operation or lipopolysaccharide (LPS) administration. (E,F) Volume-pressure curves were generated from isolated Langendorff-perfused hearts to assess alterations in isovolumic left ventricular developed pressure (LVDP) in response to 5 μ l incremental increases in balloon volume (E) and in response to 30 μ l (maximum response) volume loading (F), 16-18 hours subsequent to sham operation or LPS administration. Mice received either LPS (9 mg/kg i.p.) or vehicle (5 ml/kg 0.9% saline i.p.). At 1 hour after induction of endotoxemia, mice were treated either with EPO (1000 IU/kg s.c.) or vehicle (10 ml/kg 0.9% saline s.c.). (B-D) Sham + vehicle ($n=9$); sham + EPO ($n=3$); LPS + vehicle ($n=14$); LPS + EPO ($n=25$). (E,F) Sham + vehicle ($n=11$); LPS + vehicle ($n=9$); LPS + EPO ($n=9$). Data are expressed as means \pm s.e.m. for n number of observations. * $P<0.05$ versus LPS + vehicle; * $P<0.05$ sham + vehicle versus LPS + vehicle.

mice, sham mice treated with EPO demonstrated no significant alterations in EF, FS or FAC ($P>0.05$) (Fig. 4B-D). When compared with sham mice, WT mice subjected to CLP demonstrated a significant reduction in percentage EF, FS and FAC ($P<0.05$) (Fig. 4B-D), indicating the development of impaired systolic contractility *in vivo*. Administration of EPO to WT mice significantly attenuated the impaired systolic contractility associated with CLP ($P<0.05$) (Fig. 4B-D).

We saw no differences in LV dimensions between the KO groups (Table 1). Fig. 5A shows representative M-mode echocardiograms of sham + vehicle, sham + EPO, CLP + vehicle, and CLP + EPO in β cR KO mice. When compared with sham + vehicle mice, sham mice treated with EPO demonstrated no significant alterations in EF, FS or FAC ($P>0.05$) (Fig. 5B-D). When compared with sham

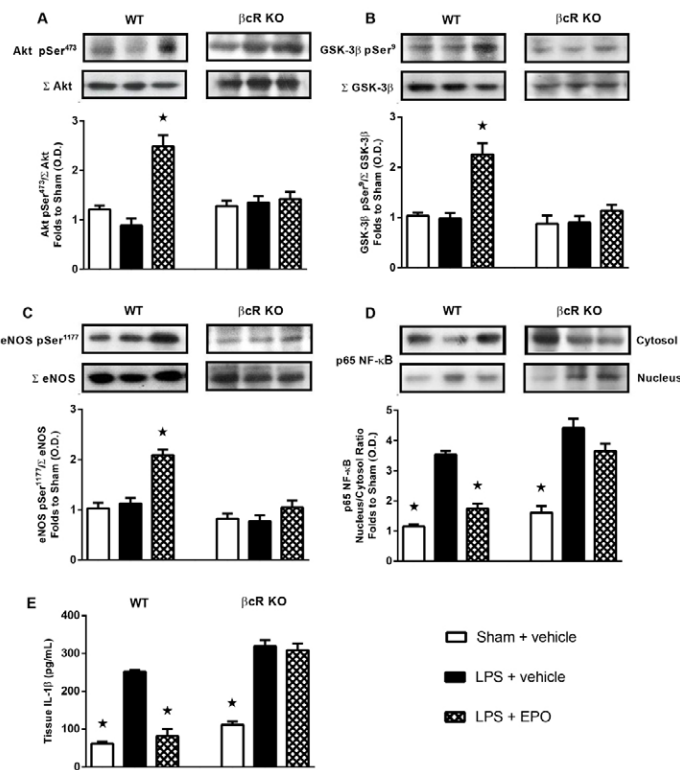


Fig. 3. Effect of erythropoietin (EPO) on signalling pathways and IL-1 β expression in the hearts of wild-type and β -common receptor knockout (β cR KO) mice with endotoxemia. Mice received either lipopolysaccharide (LPS; 9 mg/kg i.p.) or vehicle (5 ml/kg 0.9% saline i.p.). At 1 hour after induction of endotoxemia, mice were treated either with EPO (1000 IU/kg s.c.) or vehicle (10 ml/kg 0.9% saline s.c.). Densitometric analysis of the bands is expressed as relative optical density (O.D.) of (A) Akt phosphorylation at Ser473 (pSer473), corrected for the corresponding total Akt (Σ Akt) content and normalized using the related sham band, (B) glycogen synthase kinase (GSK)-3 β phosphorylation at Ser9 (pSer9), corrected for the corresponding total GSK-3 β (Σ GSK-3 β) content and normalized using the related sham band, (C) endothelial nitric oxide synthase (eNOS) phosphorylation at Ser1177 (pSer1177), corrected for the corresponding total eNOS (Σ eNOS) content and normalized using the related sham band, (D) nuclear factor (NF)- κ B p65 subunit levels in both cytosolic and nuclear fractions and expressed as a nucleus:cytosol ratio, and (E) IL-1 β expression in heart tissue of endotoxemic mice. Each immunoblot is from a single experiment and is representative of four separate experiments. Data are expressed as means \pm s.e.m. for n number of observations. * P <0.05 versus LPS + vehicle.

mice, β cR KO mice subjected to CLP demonstrated a significant reduction in percentage EF, FS and FAC (P <0.05) (Fig. 5B-D), indicating the development of impaired systolic contractility *in vivo*. Treatment of β cR KO mice with EPO did not alter this cardiac dysfunction (P >0.05) (Fig. 5B-D).

DISCUSSION

We report here, for the first time, that activation of β cR by EPO attenuates the impaired systolic contractility caused by endotoxemia in young mice or by polymicrobial sepsis in aged mice. Sepsis most frequently occurs in older patients (Girard et al., 2005), yet most of the animal studies carried out to date use young and healthy animals (Aoshiha et al., 2009). We developed a model of CLP-

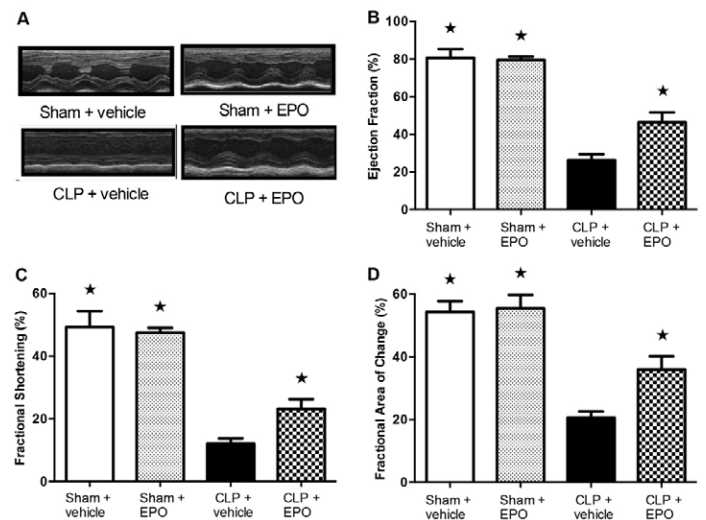


Fig. 4. Effect of erythropoietin (EPO) on the cardiac dysfunction in wild-type mice that underwent cecal ligation and puncture (CLP).

Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) in wild-type mice 24 hours subsequent to CLP surgery. At 1 hour after induction of CLP, mice were treated either with EPO (1000 IU/kg s.c.) or vehicle (10 ml/kg 0.9% saline s.c.). (B-D) Sham + vehicle (n =3); sham + EPO (n =3); CLP + vehicle (n =9); CLP + EPO (n =10). Data are expressed as means \pm s.e.m. for n number of observations. * P <0.05 versus CLP + vehicle.

induced cardiac dysfunction and discovered that the degree of impaired systolic contractility increased with age. We were unable to document a significant degree of impaired systolic contractility (at 24 hours) in young (2- and 5-month-old) mice, whereas 8-month-old mice exhibited severe impairment in systolic contractility. Although we have not investigated the effect of age on the cardiac dysfunction caused by LPS, there is very good evidence from a multitude of studies documenting that the mortality caused by endotoxins in rodents increases significantly with age (Chang et al., 1996; Chorinath et al., 1996; Tateda et al., 1996). It should be noted that an investigation into the effects of age on the pathophysiology of sepsis was not the main focus of our study, but rather a by-product of the development of a reproducible model of CLP-induced cardiac dysfunction. Most notably, EPO attenuated the severe impairment in systolic contractility caused by polymicrobial sepsis in aged mice. This effect of EPO was lost in age-matched β cR KO mice. Our finding that the beneficial effects of EPO were of a similar magnitude when cardiac dysfunction was measured either *in vivo* or *ex vivo* (in an isolated heart) also indicates that the effects of EPO are secondary to a specific improvement in cardiac contractility, independent of preload or afterload. Most notably, whether determined *in vivo* or *ex vivo*, the observed beneficial effect of EPO was lost in β cR KO mice.

Having discovered that β cR is essential for the cardioprotective effects of EPO in sepsis, we then investigated the molecular pathways that are activated by EPO in a β cR-dependent fashion. In summary, EPO activated Akt and eNOS and inhibited the (endotoxemia-induced) activation of GSK-3 β and NF κ B and expression of IL-1 β . Most notably, all of these beneficial effects of EPO were lost in β cR KO mice.

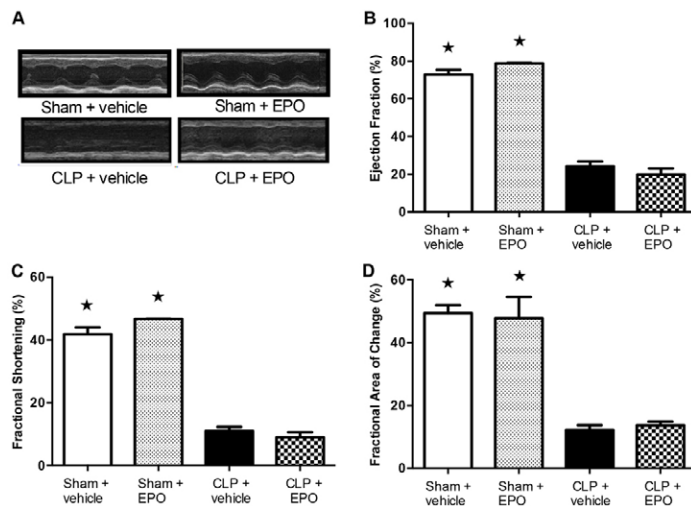


Fig. 5. Effect of erythropoietin (EPO) on the cardiac dysfunction in β -common receptor knockout (β cR KO) mice that underwent cecal ligation and puncture (CLP). Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) 24 hours subsequent to CLP surgery. At 1 hour after induction of CLP, mice were treated either with EPO (1000 IU/kg s.c.) or vehicle (10 ml/kg 0.9% saline s.c.). (B-D) Sham + vehicle ($n=5$); sham + EPO ($n=2$); CLP + vehicle ($n=7$); CLP + EPO ($n=7$). Data are expressed as means \pm s.e.m. for n number of observations. * $P < 0.05$ versus CLP + vehicle.

Akt is a member of the phosphoinositide 3-kinase (PI3K) signal transduction enzyme family and regulates cellular activation, inflammatory responses, chemotaxis and apoptosis (Cantley, 2002). When phosphorylated by its upstream regulator, phosphoinositide-dependent kinase, Akt modulates cell survival and growth (Cantley, 2002). In our study, EPO caused a significant increase in phosphorylation of Akt on Ser473 (resulting in activation of this kinase), an effect that was lost in β cR KO mice. Our hypothesis that activation of Akt importantly contributes to the improvement in cardiac contractility afforded by EPO is supported by the following findings: (1) transgenic mice with cardiac-specific expression of Akt exhibit a significant increase in cardiac contractility compared with WT mice (Condorelli et al., 2002); (2) protection by EPO against doxorubicin-induced cardiotoxicity is mediated by PI3K activation (Kim et al., 2008); (3) the reduction of infarct size afforded by EPO in a murine model of myocardial infarction is associated with activation of Akt (Calvillo et al., 2003); and (4) both the activation of Akt and the cardioprotective effects of EPO are lost when animals are pre-treated with an inhibitor of the PI3K-Akt pathway (Cai and Semenza, 2004). Interestingly, Recknagel et al. demonstrated that PI3K signalling also plays a crucial role in the development of liver dysfunction in septic rats (Recknagel et al., 2012). We report here that the increase in Ser473 phosphorylation on Akt afforded by EPO was lost in β cR KO mice. Similarly, a neutralizing antibody to the β cR subunit in endothelial cells also abolished the phosphorylation of Akt caused by EPO in these cells (Su et al., 2011). It is likely that Akt is a key molecule for the prevention of apoptosis in the heart and that activation of Akt by EPO elicits cytoprotection through an Akt-dependent pathway, in part by antagonizing the effects of TNF α (Ueba et al., 2010). Taken together, all of the above results support the view that activation of Akt, secondary to activation of the β cR

subunit, by EPO importantly contributes to the improvement in cardiac function afforded by EPO in sepsis.

Activation of Akt is associated with a pronounced increase in the phosphorylation of GSK-3 β at Ser9. GSK-3 β is a serine-threonine kinase that was originally recognized as a kinase that phosphorylates glycogen synthase. In contrast to most other kinases, GSK-3 β is active in a resting cell state; however, it is inactivated by phosphorylation of Ser9. Activation of Akt inactivates GSK-3 β by causing Ser9 phosphorylation (Cross et al., 1995). We report here that EPO caused a significant increase in phosphorylation of GSK-3 β on Ser9 (resulting in inhibition of this kinase), an effect that was lost in β cR KO mice. Our hypothesis that inhibition of GSK-3 β importantly contributes to the improvement in cardiac contractility afforded by EPO is supported by the following findings: (1) inhibition of GSK-3 β attenuates the multiple organ dysfunction caused by co-administration of LPS and peptidoglycan in the rat (Dugo et al., 2005) and improves survival in murine endotoxemia (Martin et al., 2005); (2) the cardioprotective effects of EPO are secondary to inhibition of GSK-3 β (measured as Ser9 phosphorylation) (Nishihara et al., 2006); (3) prevention by EPO of vascular integrity in animals with diabetes is dependent on activation of Akt and inhibition of GSK-3 β (Chong et al., 2011); and (4) prevention of the inhibition of GSK-3 β in the heart of diabetic mice results in the loss of the cardioprotective effects of EPO (Ghaboura et al., 2011). Taken together, all of the above results support the view that inhibition of GSK-3 β secondary to activation of β cR by EPO importantly contributes to the improvement in systolic contractility afforded by EPO in sepsis.

In addition to inhibiting the activation of GSK-3 β , activation of Akt is known to phosphorylate eNOS at Ser1177 in endothelial cells (Dimmeler et al., 1999; Fulton et al., 1999) and cardiomyocytes (Burger et al., 2006). There is evidence that EPO induces nitric oxide (NO) production and eNOS phosphorylation (Su et al., 2011; Souza et al., 2012; Kao et al., 2011), and that its cardioprotective effects are, in part, mediated by the upregulation of eNOS, demonstrated *in vitro* (Burger et al., 2006; Rui et al., 2005) and *in vivo* (Rui et al., 2005). Inhibition of the β cR subunit also abolishes the EPO-induced increase and phosphorylation of eNOS, Akt, Src and Janus kinase 2 in endothelial cells (Su et al., 2011). Indeed Sautina et al. demonstrated the requirement of the β cR subunit in the induction of NO by EPO (Sautina et al., 2010). This supports the view that the β cR subunit plays a key role in the activation of eNOS by EPO in endothelial cells. We report here that the increase in Ser1177 phosphorylation of eNOS by EPO in the heart is lost in β cR KO mice. In conditions associated with sepsis, activation of eNOS is beneficial because enhanced formation of NO causes local vasodilation, inhibition of platelets and neutrophils, and regulates angiogenesis (Tymil, 2011; Khan et al., 2010). Thus, it can be said that the activation of eNOS contributes to the beneficial effects of EPO reported here.

Downstream of GSK-3 β , several studies have now reported an association between GSK-3 β and NF κ B activity *in vitro* (Hoeflich et al., 2000; Schwabe and Brenner, 2002) and *in vivo* (Dugo et al., 2005; Dugo et al., 2006). NF κ B is a transcriptional factor that plays an important role in regulating the transcription of a number of genes, especially those involved in producing mediators involved in local and systemic inflammation, such as cytokines, chemokines, cell adhesion molecules, apoptotic factors and other mediators

(Senftleben and Karin, 2002). Treatment of TNF α -stimulated hepatocytes with a specific GSK-3 β inhibitor results in decreased NF κ B-dependent gene transcription (Schwabe and Brenner, 2002). This study also indicated four potential phosphorylation sites for GSK-3 β on the NF κ B subunit p65. Most notably, pre-treatment with a number of chemically distinct inhibitors of GSK-3 β attenuates organ injury and dysfunction caused by endotoxemia (Dugo et al., 2005; Martin et al., 2005). This protective effect was associated with inhibition of the activation of NF κ B and NF κ B-dependent proinflammatory genes, along with a reduced phosphorylation of Ser536 on the NF κ B p65 subunit. In addition, GSK-3 β might also inhibit the activation of NF κ B by phosphorylating and degrading I κ B α , which is required to prevent NF κ B translocation (Takada et al., 2004). In our study, EPO attenuated the activation of NF κ B caused by endotoxemia in the heart, an effect that was lost in β cR KO mice. NF κ B is involved in the regulation of IL-1 β transcription (Cogswell et al., 1994). EPO attenuates neuroimmune activation in neuropathic pain by inhibiting the production of TNF α , IL-1 β and IL-6 (Jia et al., 2009). We report here that mice subjected to LPS show an increase in IL-1 β production, which is significantly attenuated following the administration of EPO. This effect was lost in endotoxemic β cR KO mice.

Clinical relevance

Myocardial function is depressed in sepsis and has a significant impact on patient outcome (Rudiger et al., 2013). Using a long-term rat model of fecal peritonitis, Rudiger et al. demonstrated most recently that significant differences in stroke volume and heart rate assessed 6 hours after insult could predict a 3-day mortality with positive and negative predictive values of 93% and 80%, respectively. Based on these findings the authors suggest a crucial role for early cardiovascular performance as a prognosticator with clear therapeutic implications (Rudiger et al., 2013).

It could be argued that the clinical relevance of the EPO-related improvement in systolic contractility is limited. It is well established that survivors of septic shock present with a reversible ventricular dilatation, which is referred to as an adaptation to impaired systolic contraction (Parker et al., 1984; Parrillo et al., 1990). These authors showed that the initial ejection fraction determined by radionuclide cineangiography is significantly lower in the survivors. Improved outcome of 'dilators' versus 'non-dilators' is also shown in resuscitated murine CLP-induced septic shock (Zanotti Cavazzoni et al., 2010). However, other authors demonstrated that mortality is higher (47% versus 16%) in patients with a subnormal fractional area contraction upon initial echocardiography (Charpentier et al., 2004). Moreover, Kumar et al. did not find a significant difference in baseline LVEF as determined by radionuclide cineangiography, but a lacking increase of LVEF during dobutamine infusion allowed distinguishing between survivors and non-survivors (Kumar et al., 2008). Finally, it is noteworthy that, in the present experiment, any EPO-induced increase in LVEF coincided with unchanged end-diastolic diameter and volume. Hence, diastolic relaxation was not affected by the treatment. This observation is in contrast to our previous investigation in murine CLP-induced septic shock (Barth et al., 2006); genetic deletion and pharmacological blockade of inducible nitric oxide synthase increased systolic contractility and improved myocardial catecholamine responsiveness at the expense of impaired diastolic relaxation, i.e. 'stiffening of the ventricle'.

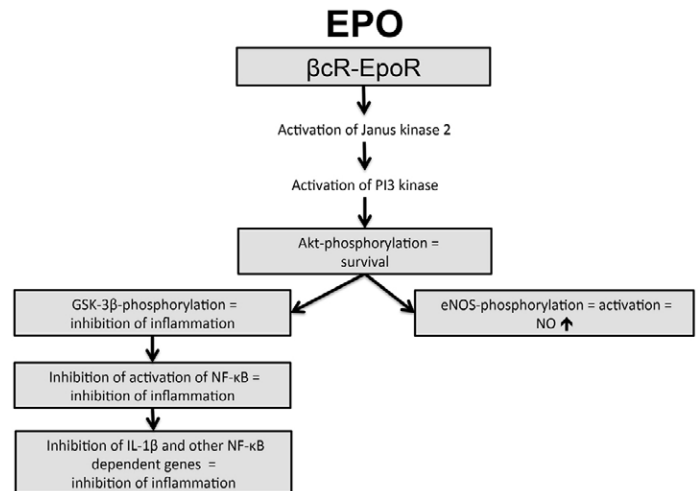


Fig. 6. Schematic overview of the signalling events modulated by erythropoietin (EPO) in the hearts of endotoxemic wild-type mice.

Activation of β cR-EpoR by EPO is associated with (a) activation of the phosphoinositide 3-kinase (PI3K)-Akt signalling pathway, which results in the inhibition of the activation of glycogen synthase kinase-3 β (GSK-3 β), which is known to suppress the activation of nuclear factor- κ B (NF κ B), resulting in a blunted expression of the NF κ B-driven gene transcription of pro-inflammatory mediators such as interleukin-1 β (IL-1 β), and (b) activation of the PI3K-Akt signalling pathway, which results in the increased activation of endothelial nitric oxide synthase (eNOS) and thus the enhanced formation of nitric oxide (NO). The signalling steps that were analyzed in our study are highlighted in light grey.

Conclusions

Our results show for the first time that the administration of EPO reduces the impaired systolic contractility associated with sepsis. In endotoxemia the observed beneficial effects of EPO are associated with: (a) activation of Akt, (b) inhibition of GSK-3 β , (c) activation of eNOS, (d) inhibition of NF κ B, and (e) inhibition of the expression of IL-1 β (Fig. 6). Most notably, attenuation of the impairment of systolic contractility as well as all of the above signalling events afforded by EPO were dependent on the presence of a functional β cR. Thus, targeting the tissue-protective receptor with EPO or specific agonists, which selectively activate the tissue-protective β cR-EpoR heterocomplex (Brines et al., 2008), could represent a therapeutic approach for the treatment of sepsis-induced cardiac dysfunction.

MATERIALS AND METHODS

The animal protocols followed in this study were approved by the local Animal Use and Care Committee in accordance with the derivatives of both the Home Office guidance on the Operation of Animals (Scientific Procedures Act 1986) published by Her Majesty's Stationery Office and the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Animals and quantification of organ dysfunction

This study was performed on 113 wild-type (WT) C57BL/6 mice (Harlan Laboratories, Wyton, UK) and 101 β cR KO mice (B6.129S1-Csf2rbtm1Cgb/J) on a C57BL/6J genetic background (bred and maintained at Queen Mary University of London, Biological Services Unit) weighing 20-30 g (2 months old) or 30-

50 g (8 months old), receiving a standard diet and water *ad libitum*. Mice were anesthetized with a ketamine (100 mg/ml) and xylazine (20 mg/ml) mixture (2:1; 1.5 ml/kg body weight i.p.) before being sacrificed.

Experimental design

Cardiac function was assessed in mice subjected to endotoxemia for 16–18 hours (2 months old) or CLP for 24 hours (8 months old). The following, specific groups were studied for endotoxemic and CLP experiments: (1) sham (5 ml/kg body weight 0.9% saline i.p.) + vehicle (10 ml/kg 0.9% saline s.c.); (2) sham (5 ml/kg 0.9% saline i.p.) + EPO (1000 IU/kg s.c.); (3) LPS (9 mg/kg i.p.) + vehicle (10 ml/kg 0.9% saline s.c.); (4) LPS (9 mg/kg i.p.) + EPO (1000 IU/kg s.c.); (5) sham (no CLP) + vehicle (10 ml/kg 0.9% saline s.c.); (6) sham (no CLP) + EPO (1000 IU/kg s.c.); (7) CLP + vehicle (10 ml/kg 0.9% saline s.c.); and (8) CLP + EPO (1000 IU/kg s.c.).

Cecal ligation and puncture

We followed the original CLP protocol introduced by Wichterman et al. (Wichterman et al., 1980) with slight modifications, including analgesia (buprenorphine; 0.05 mg/kg) and antibiotic therapy (Imipenem/Cilastin; 20 mg/kg). Based on previous evidence and preliminary data, an 18-G needle was used with the double puncture technique in order to generate cardiac dysfunction during the early phase of sepsis (24 hours). Briefly, mice were anesthetized i.p. with 1.5 ml/kg of a ketamine (100 mg/ml)/xylazine (20 mg/ml) solution in a 2:1 ratio. Buprenorphine was injected additionally to provide adequate analgesia. The rectal temperature of the animals was maintained at 37°C with a homeothermic blanket. The abdomen was opened via a 1.5 cm midline incision, and the cecum exposed. The cecum was ligated just below the ileocecal valve and punctured at both opposite ends. After a small amount of fecal matter was extruded from both ends, the cecum was placed back in its anatomical position. The abdomen was sutured; 1 ml of Ringer's solution was given for resuscitation s.c. and the mice were placed back in their cages. Antibiotic therapy and analgesia was administered 6 hours after surgery and every 12 hours after that. We evaluated the susceptibility of mice of increasing ages (2, 5 and 8 months old) to develop cardiac dysfunction 24 hours after CLP. Finally, we established a model of severe polymicrobial sepsis in 8-month-old male WT mice, which developed reliable cardiac dysfunction 24 hours after CLP.

Assessment of cardiac function *in vivo* (echocardiography)

Cardiac function was assessed in mice by echocardiography *in vivo* as reported previously (Kapoor et al., 2010). At 18 hours after administration of LPS or 24 hours after CLP surgery, anesthesia was induced with 3% isoflurane and maintained at 1% for the duration of the procedure. Two-dimensional and M-mode echocardiography images were recorded using a Vevo-770 imaging system (VisualSonics, Toronto, Ontario, Canada) by two blinded operator. Percent FAC was assessed with a two-dimensional trace at papillary muscle level. We measured LVID(D) in M-mode in the parasternal short axis view at the level of the papillary muscles. FS, EF and LVEDV were calculated from the M-mode. During echocardiography the heart rate was obtained from ECG tracing and the temperature was monitored with a rectal thermometer. Additionally, heart samples were taken and stored at -80°C for further analysis.

Assessment of cardiac function *ex vivo* (isolated Langendorff-perfused heart)

Cardiac function was assessed in mice by the isolated Langendorff-perfused heart *ex vivo* as reported previously (Kapoor et al., 2010). At 16–18 hours after the administration of LPS, mice were anesthetized and heparinized (heparin sodium, 1000 IU/100 g, i.p.). Following thoracotomy, the heart was excised and rapidly transferred to ice-cold Krebs-Henseleit buffer (KHB), containing (in mmol/l) NaCl 118, KCl 3.8, MgSO₄ 1.19, NaHCO₃ 25, CaCl₂ 1.25, KH₂PO₄ 1.18, sodium pyruvate 5, and glucose 10; equilibrated with 95% O₂/5% CO₂ (pH 7.4). Aortic cannulation was performed and hearts were perfused immediately with filtered KHB, gassed continuously with 95% O₂/5% CO₂ and maintained at 37°C. Hearts were retrogradely perfused in a non-recirculating Langendorff mode. The flow was measured and could be adjusted using a flow meter to achieve a coronary perfusion pressure of 75±5 mmHg. A small water-filled polyethylene balloon was carefully inserted into the LV via a small incision in the left atrium made near the opening for the pulmonary vein. The hearts were electrically paced at ~590 beats per minute via a silver electrode attached to the wall of the right atrium and then allowed to stabilise for at least 10 minute before any experimental protocols were carried out. Alterations in isovolumic LVDP in response to 5 µl incremental intraventricular loading of the balloon up to 40 µl were assessed. Pressure volume curves were generated to assess LVDP.

Western blot analysis

Briefly, mouse heart samples were homogenized in 10% homogenization buffer and centrifuged at 1500 g for 5 minutes at 4°C. Supernatants were removed and centrifuged at 18,600 g at 4°C for 40 minutes to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer and centrifuged at 18,600 g for 20 minutes at 4°C. The resulting supernatants containing nuclear proteins were carefully removed, and protein content was determined on both nuclear and cytosolic extracts using a bicinchoninic acid (BCA) protein assay following the manufacturer's directions (Thermo Fisher Scientific, Rockford, IL). Proteins were separated by 8% sodium dodecyl sulphate-PAGE (SDS-PAGE) and transferred to a polyvinylidenedifluoride (PVDF) membrane, which was then incubated with a primary antibody (rabbit anti-total GSK-3 β , dilution 1:200; goat anti-pGSK-3 β Ser9, dilution 1:200; rabbit anti-total-Akt, dilution 1:1000; mouse anti-pAkt Ser473, dilution 1:1000; rabbit anti-total-eNOS, dilution 1:200; goat anti-peNOS Ser1177, dilution 1:200; rabbit anti-NF κ B p65, dilution 1:1000). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1:10,000) for 30 minutes at room temperature and developed with the ECL detection system. The immunoreactive bands were visualized by autoradiography. Densitometric analysis of the bands was performed using the Gel Pro Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD). Each group was then adjusted against corresponding sham data to establish relative protein expression when compared with sham animals.

Quantitative determination of tissue IL-1 β by ELISA

The expression of IL-1 β in mouse heart samples was determined using a mouse IL-1 β /IL-1F2 immunoassay kit (R&D Systems, Minneapolis, MN) and has been normalized to the protein content.

Materials

Unless otherwise stated, all compounds in this study were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). All solutions were prepared using non-pyrogenic saline [0.9% (w/v) NaCl; Baxter Healthcare Ltd, Thetford, Norfolk, UK]. Recombinant human EPO (epoetin beta) was manufactured by Roche Diagnostics (Sussex, UK).

Statistical analyses

All values described in the text and figures are presented as mean \pm standard error of the mean (s.e.m.) of n observations, where n represents the number of animals studied. Statistical analysis was performed using GraphPad Prism 5.0d (GraphPad Software, San Diego, CA). Data without repeated measurements were assessed by a one-way ANOVA followed by Bonferroni post-hoc test. Data with repeated measurements were assessed by a two-way ANOVA followed by a Bonferroni post-hoc test. A P -value of less than 0.05 was considered to be significant.

ACKNOWLEDGEMENTS

We thank Dr Yasunori Shintani for his expert technical assistance during the echocardiography experiments.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

C.T., N.S.A.P., S.M.C., A.I.K., A.K. and M.M.Y. were involved in the conception, hypotheses delineation and design of the study; A.I.K., S.M.C., C.T., N.S.A.P., A.K., M.R. and M.C. were involved in the acquisition of the data or the analysis and interpretation of such information; and A.I.K., S.M.C., N.S.A.P., P.R. and C.T. were involved in writing the article or had substantial involvement in its revision prior to submission.

FUNDING

A.I.K. is supported by a PhD-studentship of the Medical Research Council. S.M.C. is supported by a Research Fellowship of the German Research Foundation (Deutsche Forschungsgemeinschaft; DFG CO 912/1-1 and DFG CO 912/1-2). A.K. and N.S.A.P. are supported in part by the William Harvey Research Foundation and by the British Heart foundation (PG/11/30/28849). N.S.A.P. is supported by a Kidney Research UK Post Doctoral Fellowship (PDF4/2009). This work is supported, in part, by the William Harvey Research Foundation. This work forms part of the research themes contributing to the translational research portfolio of Barts and the London Cardiovascular Biomedical Research Unit, which is supported and funded by the National Institute of Health Research. This work also contributes to the Organ Protection research theme of the Barts Centre for Trauma Sciences, supported by the Barts and The London Charity (Award 753/1722).

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at <http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.011908/-/DC1>

REFERENCES

- Abdelrahman, M., Sharples, E. J., McDonald, M. C., Collin, M., Patel, N. S., Yaqoob, M. M. and Thiemermann, C. (2004). Erythropoietin attenuates the tissue injury associated with hemorrhagic shock and myocardial ischemia. *Shock* **22**, 63-69.
- Aoshiba, K., Onizawa, S., Tsuji, T. and Nagai, A. (2009). Therapeutic effects of erythropoietin in murine models of endotoxin shock. *Crit. Care Med.* **37**, 889-898.
- Barth, E., Radermacher, P., Thiemermann, C., Weber, S., Georgieff, M. and Albuszies, G. (2006). Role of inducible nitric oxide synthase in the reduced responsiveness of the myocardium to catecholamines in a hyperdynamic, murine model of septic shock. *Crit. Care Med.* **34**, 307-313.
- Brines, M., Grasso, G., Fiordaliso, F., Sfacteria, A., Ghezzi, P., Fratelli, M., Latini, R., Xie, Q. W., Smart, J., Su-Rick, C. J. et al. (2004). Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. *Proc. Natl. Acad. Sci. USA* **101**, 14907-14912.
- Brines, M., Patel, N. S., Villa, P., Brines, C., Mennini, T., De Paola, M., Erbayraktar, Z., Erbayraktar, S., Sepodes, B., Thiemermann, C. et al. (2008). Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *Proc. Natl. Acad. Sci. USA* **105**, 10925-10930.
- Burger, D., Lei, M., Geoghegan-Morphet, N., Lu, X., Xenocostas, A. and Feng, Q. (2006). Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase. *Cardiovasc. Res.* **72**, 51-59.
- Cai, Z. and Semenza, G. L. (2004). Phosphatidylinositol-3-kinase signaling is required for erythropoietin-mediated acute protection against myocardial ischemia/reperfusion injury. *Circulation* **109**, 2050-2053.
- Calvillo, L., Latini, R., Kajstura, J., Leri, A., Anversa, P., Ghezzi, P., Salio, M., Cerami, A. and Brines, M. (2003). Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. *Proc. Natl. Acad. Sci. USA* **100**, 4802-4806.
- Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657.
- Chang, H. N., Wang, S. R., Chiang, S. C., Teng, W. J., Chen, M. L., Tsai, J. J., Huang, D. F., Lin, H. Y. and Tsai, Y. Y. (1996). The relationship of aging to endotoxin shock and to production of TNF-alpha. *J. Gerontol. A Biol. Sci. Med. Sci.* **51**, M220-M222.
- Charpentier, J., Luyt, C. E., Fulla, Y., Vinsonneau, C., Cariou, A., Grabar, S., Dhainaut, J. F., Mira, J. P. and Chiche, J. D. (2004). Brain natriuretic peptide: A marker of myocardial dysfunction and prognosis during severe sepsis. *Crit. Care Med.* **32**, 660-665.
- Chong, Z. Z., Hou, J., Shang, Y. C., Wang, S. and Maiese, K. (2011). EPO relies upon novel signaling of Wnt1 that requires Akt1, FoxO3a, GSK-3 β , and β -catenin to foster vascular integrity during experimental diabetes. *Curr. Neurovasc. Res.* **8**, 103-120.
- Chorinchath, B. B., Kong, L. Y., Mao, L. and McCallum, R. E. (1996). Age-associated differences in TNF-alpha and nitric oxide production in endotoxic mice. *J. Immunol.* **156**, 1525-1530.
- Cogswell, J. P., Godlevski, M. M., Wisely, G. B., Clay, W. C., Leesnitzer, L. M., Ways, J. P. and Gray, J. G. (1994). NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. *J. Immunol.* **153**, 712-723.
- Condorelli, G., Drusco, A., Stassi, G., Bellacosa, A., Roncarati, R., Iaccarino, G., Russo, M. A., Gu, Y., Dalton, N., Chung, C. et al. (2002). Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. *Proc. Natl. Acad. Sci. USA* **99**, 12333-12338.
- Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789.
- Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. and Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**, 601-605.
- Drüeke, T. B., Locatelli, F., Clyne, N., Eckardt, K. U., Macdougall, I. C., Tsakiris, D., Burger, H. U. and Scherhag, A. (2006). Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *N. Engl. J. Med.* **355**, 2071-2084.
- Dugo, L., Collin, M., Allen, D. A., Patel, N. S., Bauer, I., Mervaala, E. M., Louhelainen, M., Foster, S. J., Yaqoob, M. M. and Thiemermann, C. (2005). GSK-3beta inhibitors attenuate the organ injury/dysfunction caused by endotoxemia in the rat. *Crit. Care Med.* **33**, 1903-1912.
- Dugo, L., Collin, M., Allen, D. A., Murch, O., Foster, S. J., Yaqoob, M. M. and Thiemermann, C. (2006). Insulin reduces the multiple organ injury and dysfunction caused by coadministration of lipopolysaccharide and peptidoglycan independently of blood glucose: role of glycogen synthase kinase-3beta inhibition. *Crit. Care Med.* **34**, 1489-1496.
- Fernandes, C. J., Jr and Cesar de Assuncao, M. S. (2012). Myocardial dysfunction in sepsis: a large, unsolved puzzle. *Crit. Care Res. Pract.* **2012**, 896430.
- Flynn, A., Chokkalingam Mani, B. and Mather, P. J. (2010). Sepsis-induced cardiomyopathy: a review of pathophysiologic mechanisms. *Heart Fail. Rev.* **15**, 605-611.
- Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A. and Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597-601.
- Ghaboura, N., Tamareille, S., Ducluzeau, P. H., Grimaud, L., Loufrani, L., Croué, A., Tourmen, Y., Henrion, D., Furber, A. and Prunier, F. (2011). Diabetes mellitus abrogates erythropoietin-induced cardioprotection against ischemic-reperfusion injury by alteration of the RISK/GSK-3 β signaling. *Basic Res. Cardiol.* **106**, 147-162.
- Girard, T. D., Opal, S. M. and Ely, E. W. (2005). Insights into severe sepsis in older patients: from epidemiology to evidence-based management. *Clin. Infect. Dis.* **40**, 719-727.
- Hoeflich, K. P., Luo, J., Rubie, E. A., Tsao, M. S., Jin, O. and Woodgett, J. R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* **406**, 86-90.
- Hoessel, L. M., Niederbichler, A. D. and Ward, P. A. (2007). Complement-related molecular events in sepsis leading to heart failure. *Mol. Immunol.* **44**, 95-102.
- Jia, H., Feng, X., Li, W., Hu, Y., Zeng, Q., Liu, J. and Xu, J. (2009). Recombinant human erythropoietin attenuates spinal neuroimmune activation of neuropathic pain in rats. *Ann. Clin. Lab. Sci.* **39**, 84-91.

- Kao, R. L., Martin, C. M., Xenocostas, A., Huang, W. and Rui, T. (2011). Erythropoietin improves skeletal muscle microcirculation through the activation of eNOS in a mouse sepsis model. *J. Trauma* **71** Suppl. 1, S462-S467.
- Kapoor, A., Shintani, Y., Collino, M., Osuchowski, M. F., Busch, D., Patel, N. S., Sepodes, B., Castiglia, S., Fantozzi, R., Bishop-Bailey, D. et al. (2010). Protective role of peroxisome proliferator-activated receptor- β/δ in septic shock. *Am. J. Respir. Crit. Care Med.* **182**, 1506-1515.
- Khadour, F. H., Panas, D., Ferdinandy, P., Schulze, C., Csont, T., Lalu, M. M., Wildhirt, S. M. and Schulz, R. (2002). Enhanced NO and superoxide generation in dysfunctional hearts from endotoxemic rats. *Am. J. Physiol. Heart Circ. Physiol.* **283**, H1108-H1115.
- Khan, R., Kirschenbaum, L. A., LaRow, C., Berna, G., Griffin, K. and Astiz, M. E. (2010). Augmentation of platelet and endothelial cell eNOS activity decreases sepsis-related neutrophil-endothelial cell interactions. *Shock* **33**, 242-246.
- Kim, K. H., Oudit, G. Y. and Backx, P. H. (2008). Erythropoietin protects against doxorubicin-induced cardiomyopathy via a phosphatidylinositol 3-kinase-dependent pathway. *J. Pharmacol. Exp. Ther.* **324**, 160-169.
- Kumar, A., Schupp, E., Bunnell, E., Ali, A., Milcarek, B. and Parrillo, J. E. (2008). Cardiovascular response to dobutamine stress predicts outcome in severe sepsis and septic shock. *Crit. Care* **12**, R35.
- Leist, M., Ghezzi, P., Grasso, G., Bianchi, R., Villa, P., Fratelli, M., Savino, C., Bianchi, M., Nielsen, J., Gerwien, J. et al. (2004). Derivatives of erythropoietin that are tissue protective but not erythropoietic. *Science* **305**, 239-242.
- Martin, M., Rehani, K., Joje, P. S. and Michalek, S. M. (2005). Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* **6**, 777-784.
- Masuda, S., Nagao, M., Takahata, K., Konishi, Y., Gallyas, F., Jr, Tabira, T. and Sasaki, R. (1993). Functional erythropoietin receptor of the cells with neural characteristics. Comparison with receptor properties of erythroid cells. *J. Biol. Chem.* **268**, 11208-11216.
- Merx, M. W. and Weber, C. (2007). Sepsis and the heart. *Circulation* **116**, 793-802.
- Murphy, J. M. and Young, I. G. (2006). IL-3, IL-5, and GM-CSF signaling: crystal structure of the human beta-common receptor. *Vitam. Horm.* **74**, 1-30.
- Nishihara, M., Miura, T., Miki, T., Sakamoto, J., Tanno, M., Kobayashi, H., Ikeda, Y., Ohori, K., Takahashi, A. and Shimamoto, K. (2006). Erythropoietin affords additional cardioprotection to preconditioned hearts by enhanced phosphorylation of glycogen synthase kinase-3 beta. *Am. J. Physiol. Heart Circ. Physiol.* **291**, H748-H755.
- Parker, M. M., Shelhamer, J. H., Bacharach, S. L., Green, M. V., Natanson, C., Frederick, T. M., Damske, B. A. and Parrillo, J. E. (1984). Profound but reversible myocardial depression in patients with septic shock. *Ann. Intern. Med.* **100**, 483-490.
- Parrillo, J. E., Parker, M. M., Natanson, C., Suffredini, A. F., Danner, R. L., Cunnion, R. E. and Ognibene, F. P. (1990). Septic shock in humans. Advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. *Ann. Intern. Med.* **113**, 227-242.
- Recknagel, P., Gonnert, F. A., Westermann, M., Lambeck, S., Lupp, A., Rudiger, A., Dyson, A., Carré, J. E., Kortgen, A., Krafft, C. et al. (2012). Liver dysfunction and phosphatidylinositol-3-kinasesignalling in early sepsis: experimental studies in rodent models of peritonitis. *PLoS Med.* **9**, e1001338
- Rudiger, A. and Singer, M. (2007). Mechanisms of sepsis-induced cardiac dysfunction. *Crit. Care Med.* **35**, 1599-1608.
- Rudiger, A., Dyson, A., Felsmann, K., Carré, J. E., Taylor, V., Hughes, S., Clatworthy, I., Protti, A., Pellerin, D., Lemm, J. et al. (2013). Early functional and transcriptomic changes in the myocardium predict outcome in a long-term rat model of sepsis. *Clin. Sci.* **124**, 391-401.
- Rui, T., Feng, Q., Lei, M., Peng, T., Zhang, J., Xu, M., Abel, E. D., Xenocostas, A. and Kvietys, P. R. (2005). Erythropoietin prevents the acute myocardial inflammatory response induced by ischemia/reperfusion via induction of AP-1. *Cardiovasc. Res.* **65**, 719-727.
- Sautina, L., Sautin, Y., Beem, E., Zhou, Z., Schuler, A., Brennan, J., Zharikov, S. I., Diao, Y., Bungert, J. and Segal, M. S. (2010). Induction of nitric oxide by erythropoietin is mediated by the beta common receptor and requires interaction with VEGF receptor 2. *Blood* **115**, 896-905.
- Schwabe, R. F. and Brenner, D. A. (2002). Role of glycogen synthase kinase-3 in TNF-alpha-induced NF-kappaB activation and apoptosis in hepatocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* **283**, G204-G211.
- Senftleben, U. and Karin, M. (2002). The IKK/NF-kappaB pathway. *Crit. Care Med.* **30** Suppl., S18-S26.
- Sharma, A. C. (2007). Sepsis-induced myocardial dysfunction. *Shock* **28**, 265-269.
- Souza, A. C., Volpini, R. A., Shimizu, M. H., Sanches, T. R., Camara, N. O., Smedo, P., Rodrigues, C. E., Seguro, A. C. and Andrade, L. (2012). Erythropoietin prevents sepsis-related acute kidney injury in rats by inhibiting NF-kB and upregulating endothelial nitric oxide synthase. *Am. J. Physiol. Renal Physiol.* **302**, F1045-F1054.
- Su, K. H., Shyue, S. K., Kou, Y. R., Ching, L. C., Chiang, A. N., Yu, Y. B., Chen, C. Y., Pan, C. C. and Lee, T. S. (2011). β Common receptor integrates the erythropoietin signaling in activation of endothelial nitric oxide synthase. *J. Cell. Physiol.* **226**, 3330-3339.
- Suliman, H. B., Welty-Wolf, K. E., Carraway, M., Tatro, L. and Piantadosi, C. A. (2004). Lipopolysaccharide induces oxidative cardiac mitochondrial damage and biogenesis. *Cardiovasc. Res.* **64**, 279-288.
- Takada, Y., Fang, X., Jamaluddin, M. S., Boyd, D. D. and Aggarwal, B. B. (2004). Genetic deletion of glycogen synthase kinase-3beta abrogates activation of IkkappaBalpha kinase, JNK, Akt, and p44/p42 MAPK but potentiates apoptosis induced by tumor necrosis factor. *J. Biol. Chem.* **279**, 39541-39554.
- Tateda, K., Matsumoto, T., Miyazaki, S. and Yamaguchi, K. (1996). Lipopolysaccharide-induced lethality and cytokine production in aged mice. *Infect. Immun.* **64**, 769-774.
- Tymil, K. (2011). Critical role for oxidative stress, platelets, and coagulation in capillary blood flow impairment in sepsis. *Microcirculation* **18**, 152-162.
- Ueba, H., Brines, M., Yamin, M., Umemoto, T., Ako, J., Momomura, S., Cerami, A. and Kawakami, M. (2010). Cardioprotection by a nonerythropoietic, tissue-protective peptide mimicking the 3D structure of erythropoietin. *Proc. Natl. Acad. Sci. USA* **107**, 14357-14362.
- Watts, J. A., Kline, J. A., Thornton, L. R., Grattan, R. M. and Brar, S. S. (2004). Metabolic dysfunction and depletion of mitochondria in hearts of septic rats. *J. Mol. Cell. Cardiol.* **36**, 141-150.
- Wichterman, K. A., Baue, A. E. and Chaudry, I. H. (1980). Sepsis and septic shock – a review of laboratory models and a proposal. *J. Surg. Res.* **29**, 189-201.
- Zanotti Cavazzoni, S. L., Guglielmi, M., Parrillo, J. E., Walker, T., Dellinger, R. P. and Hollenberg, S. M. (2010). Ventricular dilation is associated with improved cardiovascular performance and survival in sepsis. *Chest* **138**, 848-855.