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

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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-3β, nuclear factor-κB and interleukin-1β). 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 (Aoshiba 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 (βcR; also called CD131) (Brines et al., 2004). βcR 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, βcR and EpoR in spinal cord neurons and cardiomyocytes (Brines et al., 2004). Furthermore, the structural importance of an interaction between EPO and βcR 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...
**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 (βcR). 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-3β, nuclear factor-κB and interleukin-1β). Using βcR knockout mice, the authors demonstrated that the effects of EPO are dependent on the presence of βcR.

**Implications and future directions**
This study demonstrates for the first time that activation of βcR 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. pyrogulamate 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 βcR-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 TNFα (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 βcR 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 βcR 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-3β (GSK-3β) on Ser9, phosphorylation of endothelial nitric oxide synthase (eNOS) on Ser1177, activation of nuclear factor (NF)κB (measured as nuclear translocation of p65) and expression of interleukin-1beta (IL-1β). Because sepsis most frequently occurs in aging patients (Girard et al., 2005), we then investigated the effects of EPO and the role of βcR 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 mice, 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 μl incremental intraventricular volume-balloon loading up to 40 μl (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 μl (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 βcR KO mice assessed by echocardiography and the isolated Langendorff-perfused heart**
To investigate the role of the βcR subunit in the observed beneficial properties of EPO reported above, we evaluated the cardioprotective effects of EPO in βcR 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 βcR 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 βcR 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 βcR KO...
mice, endotoxemia in βcR KO mice resulted in a significant reduction in percentage EF, FS and FAC (<0.05) (Fig. 2B-D), indicating impairment in systolic contractility. Administration of EPO to endotoxemic βcR KO mice did not alter this impairment in systolic contractility. Administration of EPO to endotoxemic βcR KO mice caused no alteration in IL-1β expression (<0.05) (Fig. 3C). In contrast, administration of EPO to endotoxemic βcR KO mice caused no change in the phosphorylation of Akt on Ser473 (<0.05) (Fig. 3B) or eNOS on Ser1177 (<0.05) (Fig. 3C).

Effect of EPO on the phosphorylation of Akt, GSK-3β and eNOS in the hearts of endotoxemic WT and βcR 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 βcR KO mice subjected to LPS demonstrated no change in the phosphorylation of Akt on Ser473 (P>0.05) (Fig. 3A), GSK-3β on Ser9 (P>0.05) (Fig. 3B) and eNOS on Ser1177 (P>0.05) (Fig. 3C). In contrast, administration of EPO to endotoxemic βcR KO mice caused no change in the phosphorylation of Akt on Ser473 (P>0.05) (Fig. 3A), GSK-3β 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 NFκB subunit in the hearts of endotoxemic WT and βcR KO mice
When compared with hearts from sham mice, the hearts of endotoxemic WT and βcR KO mice exhibited significant increases in the nuclear translocation of the p65 subunit (P<0.05) (Fig. 3D), indicating activation of NFκB. Administration of EPO to endotoxemic WT mice significantly attenuated the increase in nuclear translocation of p65 and, hence, inhibition of activation of NFκB in the heart (P<0.05) (Fig. 3D). In contrast, administration of EPO to endotoxemic βcR 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-1β in the hearts of endotoxemic WT and βcR KO mice
When compared with hearts from sham mice, the hearts of endotoxemic WT and βcR KO mice demonstrated a significant increase in IL-1β expression (P<0.05) (Fig. 3E). Administration of EPO to endotoxemic WT mice significantly attenuated IL-1β expression (P<0.05) (Fig. 3E). In contrast, administration of EPO to endotoxemic βcR KO mice caused no alteration in IL-1β expression (P>0.05) (Fig. 3E).

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype</th>
<th>LVID(D) (mm)</th>
<th>LVEDV (μl)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endotoxemia in 2-month-old mice</strong></td>
<td></td>
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<tr>
<td>Sham + vehicle</td>
<td>WT</td>
<td>3.4±0.06</td>
<td>47±2.13</td>
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<tr>
<td>Sham + EPO</td>
<td>WT</td>
<td>3.1±0.14</td>
<td>39±4.34</td>
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<tr>
<td>LPS + vehicle</td>
<td>WT</td>
<td>3.6±0.07</td>
<td>54±2.60</td>
<td>15</td>
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<tr>
<td>LPS + EPO</td>
<td>WT</td>
<td>3.6±0.08</td>
<td>53±2.61</td>
<td>15</td>
</tr>
<tr>
<td>Sham + vehicle</td>
<td>KO</td>
<td>3.1±0.09*</td>
<td>39±2.91</td>
<td>9</td>
</tr>
<tr>
<td>Sham + EPO</td>
<td>KO</td>
<td>3.5±0.15</td>
<td>52±5.36</td>
<td>3</td>
</tr>
<tr>
<td>LPS + vehicle</td>
<td>KO</td>
<td>3.5±0.08</td>
<td>53±2.96</td>
<td>14</td>
</tr>
<tr>
<td>LPS + EPO</td>
<td>KO</td>
<td>3.5±0.07</td>
<td>53±2.65</td>
<td>25</td>
</tr>
<tr>
<td><strong>Polymicrobial sepsis in aged mice</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Sham + vehicle (8 months)</td>
<td>WT</td>
<td>3.5±0.30</td>
<td>51±9.44</td>
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<tr>
<td>Sham + EPO (8 months)</td>
<td>WT</td>
<td>3.5±0.12</td>
<td>51±3.98</td>
<td>3</td>
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<tr>
<td>CLP + vehicle (2 months)</td>
<td>WT</td>
<td>3.5±0.08</td>
<td>51±2.61</td>
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<tr>
<td>CLP + vehicle (5 months)</td>
<td>WT</td>
<td>4.1±0.15</td>
<td>73±6.29</td>
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<tr>
<td>CLP + vehicle (8 months)</td>
<td>WT</td>
<td>3.9±0.09</td>
<td>68±3.55</td>
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<tr>
<td>CLP + EPO (8 months)</td>
<td>WT</td>
<td>3.8±0.13</td>
<td>62±5.03</td>
<td>10</td>
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<tr>
<td>Sham + vehicle (8 months)</td>
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<tr>
<td>Sham + EPO (8 months)</td>
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<td>3.5±0.00</td>
<td>52±0.76</td>
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<tr>
<td>CLP + vehicle (8 months)</td>
<td>KO</td>
<td>3.8±0.12</td>
<td>64±4.24</td>
<td>7</td>
</tr>
<tr>
<td>CLP + EPO (8 months)</td>
<td>KO</td>
<td>4.1±0.14</td>
<td>74±6.05</td>
<td>7</td>
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</tbody>
</table>

*WT or βcR 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 ± s.e.m. for n number of observations. *P<0.05 versus LPS + vehicle (KO).
Effect of EPO on cardiac dysfunction in WT and βcR 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 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 βcR 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 mice, WT 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. Administration of EPO to WT mice significantly attenuated the impaired systolic contractility associated with CLP (P<0.05) (Fig. 5B-D).

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Fig. 1. Effect of erythropoietin (EPO) on the cardiac dysfunction in endotoxemic wild-type 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 μl incremental increases in balloon volume (E) and in response to 30 μl (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=10); sham + EPO (n=6); LPS + vehicle (n=15); LPS + EPO (n=15). (E,F) Sham + vehicle (n=11); LPS + vehicle (n=11); LPS + EPO (n=10). Data are expressed as means ± s.e.m. for n number of observations. *P<0.05 versus LPS + vehicle; ‚P<0.05 sham + vehicle versus LPS + vehicle; †P<0.05 LPS + vehicle versus LPS + EPO.

Fig. 2. Effect of erythropoietin (EPO) on the cardiac dysfunction in endotoxemic β-common receptor knockout (βcR 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 μl incremental increases in balloon volume (E) and in response to 30 μl (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 ± s.e.m. for n number of observations. *P<0.05 versus LPS + vehicle; ‚P<0.05 sham + vehicle versus LPS + vehicle.
mice, βcR 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 βcR 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 βcR 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 (Aoshiba et al., 2009). We developed a model of CLP-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; Chorinchath 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 βcR 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 βcR KO mice.

Having discovered that βcR is essential for the cardioprotective effects of EPO in sepsis, we then investigated the molecular pathways that are activated by EPO in a βcR-dependent fashion. In summary, EPO activated Akt and eNOS and inhibited the (endotoxemia-induced) activation of GSK-3β and NFκB and expression of IL-1β. Most notably, all of these beneficial effects of EPO were lost in βcR KO mice.
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 βcR 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 βcR KO mice. Similarly, a neutralizing antibody to the βcR 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 TNFα (Ueba et al., 2010). Taken together, all of the above results support the view that activation of Akt, secondary to activation of the βcR 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-3β at Ser9. GSK-3β is a serine-threonine kinase that was originally recognized as a kinase that phosphorylates glycogen synthase. In contrast to most other kinases, GSK-3β is active in a resting cell state; however, it is inactivated by phosphorylation of Ser9. Activation of Akt inactivates GSK-3β by causing Ser9 phosphorylation (Cross et al., 1995). We report here that EPO caused a significant increase in phosphorylation of GSK-3β on Ser9 (resulting in inhibition of this kinase), an effect that was lost in βcR KO mice. Our hypothesis that inhibition of GSK-3β importantly contributes to the improvement in cardiac contractility afforded by EPO is supported by the following findings: (1) inhibition of GSK-3β 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-3β (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-3β (Chong et al., 2011); and (4) prevention of the inhibition of GSK-3β 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-3β secondary to activation of βcR by EPO importantly contributes to the improvement in systolic contractility afforded by EPO in sepsis.

In addition to inhibiting the activation of GSK-3β, activation of Akt is known to phosphorylate eNOS at Ser1177 in endothelial cells (Dugo et al., 2005; Dugo 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 βcR 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 βcR subunit in the induction of NO by EPO (Sautina et al., 2010). This supports the view that the βcR 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 βcR 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 (Tyml, 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-3β, several studies have now reported an association between GSK-3β and NFκB activity in vitro (Hoeﬂich et al., 2000; Schwabe and Brenner, 2002) and in vivo (Dugo et al., 2005; Dugo et al., 2006). NFκB 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.
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(Senftleben and Karin, 2002). Treatment of TNFα-stimulated hepatocytes with a specific GSK-3β inhibitor results in decreased NFkB-dependent gene transcription (Schwade and Brenner, 2002). This study also indicated four potential phosphorylation sites for GSK-3β on the NFkB subunit p65. Most notably, pre-treatment with a number of chemically distinct inhibitors of GSK-3β 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 NFκB and NFkB-dependent proinflammatory genes, along with a reduced phosphorylation of Ser536 on the NFkB p65 subunit. In addition, GSK-3β might also inhibit the activation of NFkB by phosphorylating and degrading IκBα, which is required to prevent NFkB translocation (Takada et al., 2004). In our study, EPO attenuated the activation of NFkB caused by endotoxemia in the heart, an effect that was lost in βcR KO mice. NFkB is involved in the regulation of IL-1β transcription (Cogswell et al., 1994). EPO attenuates neuroimmune activation in neuropathic pain by inhibiting the production of TNFa, IL-1β and IL-6 (Jia et al., 2009). We report here that mice subjected to LPS show an increase in IL-1β production, which is significantly attenuated following the administration of EPO. This effect was lost in endoimmune βcR 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.’

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-3β, (c) activation of eNOS, (d) inhibition of NFκB, and (e) inhibition of the expression of IL-1β (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 βcR. Thus, targeting the tissue-protective receptor with EPO or specific agonists, which selectively activate the tissue-protective βcR-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 βcR 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 (Imipenen/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. 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 minutes 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 pellet 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-3β, dilution 1:200; goat anti-pGSK-3β 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-p-eNOS Ser1177, dilution 1:200; rabbit anti-NFκβ 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-1β by ELISA
The expression of IL-1β in mouse heart samples was determined using a mouse IL-1β/IL-1F2 immunoassay kit (R&D Systems, Minneapolis, MN) and has been normalized to the protein content.
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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 ± 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.

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COMPETING INTERESTS

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

AUTHOR CONTRIBUTIONS

C.T., N.S.A.P., A.I.K. and M.M.Y. were involved in writing the article or had substantial involvement in its revision prior to submission.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at

REFERENCES

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RESEARCH ARTICLE


