Pharmacological preconditioning with erythropoietin attenuates the organ injury and dysfunction induced in a rat model of hemorrhagic shock

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SUMMARY

Pre-treatment with erythropoietin (EPO) has been demonstrated to exert tissue-protective effects against 'ischemia-reperfusion'-type injuries. This protection might be mediated by mobilization of bone marrow endothelial progenitor cells (EPCs), which are thought to secrete paracrine factors. These effects could be exploited to protect against tissue injury induced in cases where hemorrhage is foreseeable, for example, prior to major surgery. Here, we investigate the effects of EPO pre-treatment on the organ injury and dysfunction induced by hemorrhagic shock (HS). Recombinant human EPO (1000 IU/kg/day i.p.) was administered to rats for 3 days. Rats were subjected to HS on day 4 (pre-treatment protocol). Mean arterial pressure was reduced to 35±5 mmHg for 90 minutes, followed by resuscitation with 20 ml/kg Ringer's lactate for 10 minutes and 50% of the shed blood for 50 minutes. Rats were sacrificed 4 hours after the onset of resuscitation. EPC (CD34$^+$/flk-1$^+$ cell) mobilization was measured following the 3-day pre-treatment with EPO and was significantly increased compared with rats pre-treated with phosphate-buffered saline. EPO pre-treatment significantly attenuated organ injury and dysfunction (renal, hepatic and neuromuscular) caused by HS. In livers from rats subjected to HS, EPO enhanced the phosphorylation of Akt (activation), glycogen synthase kinase-3β (GSK-3β; inhibition) and endothelial nitric oxide synthase (eNOS; activation). In the liver, HS also caused an increase in nuclear translocation of p65 (activation of NF-κB), which was attenuated by EPO. This data suggests that repetitive dosing with EPO prior to injury might protect against the organ injury and dysfunction induced by HS, by a mechanism that might involve mobilization of CD34$^+$/flk-1$^+$ cells, resulting in the activation of the Akt-eNOS survival pathway and inhibition of activation of GSK-3β and NF-κB.

INTRODUCTION

Hemorrhagic shock (HS) occurs when there is severe blood loss associated with trauma resulting in a state of global ischemia. Trauma is the leading cause of death in under-45 year olds in the USA (Heron et al., 2009), with severe hemorrhage being a leading cause of preventable death (Stewart et al., 2003). Hemorrhage is managed by fluid resuscitation with crystalloid fluids and blood products, including fresh frozen plasma, packed red blood cells and whole blood transfusions (Finfer et al., 2010), which restore the circulating volume and cardiac output. However, the return of oxygen to ischemic tissues promotes the production of reactive oxygen species and activation of immune cells. This induces a systemic inflammatory response syndrome that contributes to apoptosis and tissue necrosis, leading to further organ injury (Rushing and Britt, 2008). In patients with trauma, failure of more than four organs is linked to certain mortality, highlighting the need for interventions that may reduce or prevent the deterioration in organ injury and function (Fry et al., 1980).

Erythropoietin (EPO) is a 34-KDa glycoprotein secreted by the kidneys that controls erythropoiesis by regulating the proliferation of erythroid progenitor cells in the bone marrow. It acts via an anti-apoptotic mechanism to prevent death of erythroid progenitors, allowing them to differentiate into circulating mature erythrocytes. More recently, EPO has been shown to possess many pleiotropic actions that are beneficial in various ischemia-reperfusion-type injuries, including acute kidney injury (Sharples et al., 2004), myocardial infarction (Brunner et al., 2009), stroke (Sirén et al., 2001) and HS (Abdelrahman et al., 2004). In the majority of studies, EPO is administered either during or prior to reperfusion; however, there are also several studies that have investigated the protective effects exerted by EPO pre-treatment in similar contexts. For example, daily pre-treatment with EPO for a period of 3 days has been demonstrated to protect against the injury induced by ischemia-reperfusion in the hind-limb (Heeschen et al., 2003) and kidney (Patel et al., 2004).

Repetitive dosing with EPO could potentially activate numerous different targets. In particular, EPO is a known stimulus of endothelial progenitor cell (EPC) mobilization; EPCs are mobilized in response to hypoxia in order to promote angiogenesis but are also able to secrete paracrine proteins, which include anti-inflammatory cytokines such as IL-10 and growth factors including vascular endothelial growth factor (VEGF) (Heeschen et al., 2003). EPCs express numerous cell surface markers, which are used to characterize and identify these cells; examples include CD34, CD45, CD133, stem cell antigen-1 (sca-1) and vascular endothelial growth factor receptor-2 (VEGFR2). VEGFR2 is a type III tyrosine kinase receptor and its activation results in increased proliferation,

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TRANSLATIONAL IMPACT

Clinical issue
Trauma causes ~6 million deaths every year worldwide. Blood loss or hemorrhage, which leads to a state of global ischemia, is responsible for 40% of trauma-related deaths. Gunshot and stab wounds, injuries sustained in road traffic accidents and invasive surgery can all result in hemorrhage, which can be managed with fluid resuscitation and blood transfusion. However, because the combination of hemorrhage and resuscitation can result in global ischemia-reperfusion-type injuries and hemorrhagic shock, it is common that organ damage and dysfunction follow the treatment of hemorrhage. Because the failure of four organs is associated with certain death, there is an urgent need for pharmaceutical interventions that limit the damage associated with resuscitation. The hormone erythropoietin (EPO), which controls the production of red blood cells by regulating the proliferation of erythroid progenitor cells in the bone marrow, exerts anti-apoptotic and anti-inflammatory effects in many models of ischemia-reperfusion-type injury, including hemorrhagic shock. Interestingly, EPO can exert these effects when given up to 24 hours before the ischemic insult.

Results
In this study, the authors investigate how EPO pre-treatment exerts this tissue-priming effect. They show that daily treatment of rats with EPO for 3 days prior to the induction of hemorrhagic shock significantly attenuates renal (glomerular) dysfunction and liver and neuromuscular injury compared with pre-treatment with vehicle. Daily treatment of rats for 3 days with EPO results in mobilization of endothelial progenitor cells. Finally, they show that there is significant activation of the Akt-eNOS pro-survival pathway in rats pre-treated with EPO and then subjected to hemorrhagic shock.

Implications and future directions
These findings indicate that the mobilization of endothelial progenitor cells might contribute to the tissue-protective effects observed with EPO pre-treatment, either by secretion of anti-inflammatory cytokines, growth factors and other paracrine proteins and/or by direct interaction with the Akt-eNOS pro-survival pathway. Importantly, they suggest that pre-treatment of patients with EPO prior to foreseeable hemorrhage (for example, before surgery) could be one way to limit the tissue injury associated with hemorrhage and resuscitation. Finally, the mechanism of action reported here suggests that dosing with EPO is a possible alternative to using stem cell-based therapies in other clinical settings because it mobilizes erythroid progenitor cells that, in turn, could potentiate the tissue-protective effects of EPO.

migration, survival and permeability of vascular endothelial cells and EPCs. These actions are beneficial in hypoxia as they promote improved perfusion of ischemic tissues (reviewed by Holmes et al., 2007). This suggests that EPO-induced mobilization of EPCs could result in tissue-protective effects either indirectly through the secretion of paracrine proteins and/or by direct interaction of EPCs with host cells.

EPO can activate anti-inflammatory and anti-apoptotic pathways either by interaction with its classical receptor, the EPO receptor (EPO-R) (reviewed by Chateauvieux et al., 2011), or with the proposed molecular target responsible for EPO's tissue-protective effects, the β common receptor (βcR) (Brines et al., 2004). Therefore, EPO could have the potential to be used as a therapy that is administered prior to the onset of an ischemia-reperfusion-type injury. However, many ischemic insults occur spontaneously and cannot be predicted (e.g. trauma due to road traffic accident) and the preferred treatment timepoint is at the location of the accident or upon hospital admission, i.e. when reperfusion is performed. Alternatively, in situations where the ischemic insult is

foreseeable, such as prior to major surgery in which blood loss is likely, EPO pre-treatment could be a viable therapeutic option. EPO is already clinically used as a treatment prior to surgery in order to reduce the risk of post-operative anemia and to reduce the requirement of peri-operative blood transfusions (Naran et al., 2012; Yoo et al., 2011). It is possible that, in addition to the erythropoietic effects that EPO exerts in these situations, EPO could protect tissues against the ischemic damage associated with blood loss in surgery.

This study investigates whether daily pre-treatment with EPO for a period of 3 days attenuates the organ injury and dysfunction induced by HS. We have used a well-characterized rat model of HS to investigate the early development of the renal and glomerular dysfunction, and liver and neuromuscular injury associated with severe hemorrhage and resuscitation (Nandra et al., 2012; Patel et al., 2011). Having discovered that EPO pre-treatment does indeed attenuate the organ injury and dysfunction induced by HS, we investigated the potential mechanism(s) behind this protective effect by evaluating the ability of EPO to mobilize EPCs and its effects on the activation of various cellular signaling pathways [in particular phosphorylation of Akt on Ser473, phosphorylation of glycogen synthase kinase-3β (GSK-3β) on Ser9, phosphorylation of eNOS on Ser1177 and activation of nuclear factor-κB (NF-κB, measured as nuclear translocation of the p65 subunit)].
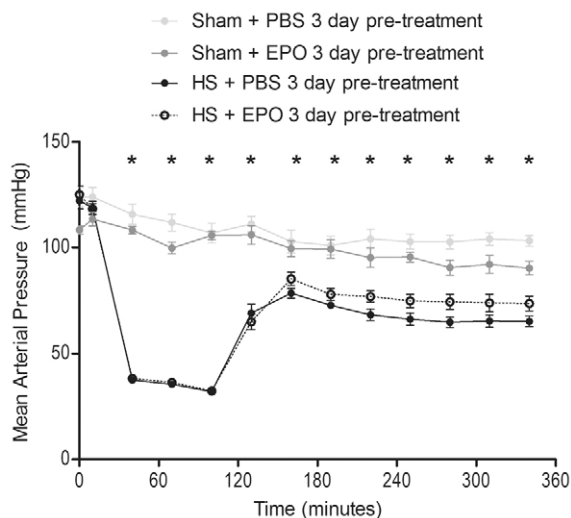
RESULTS
Effect of EPO pre-treatment on the circulatory failure caused by hemorrhagic shock
Compared with sham-operated rats, rats subjected to hemorrhage exhibited a significant decline in mean arterial pressure (MAP; $P<0.05$, Fig. 1). Consequent resuscitation increased MAP but this was still significantly lower than in sham-operated rats ($P<0.05$, Fig. 1). In rats subjected to HS, pre-treatment with EPO had no significant effect on the decline in MAP; although there was a slight increase in MAP during the resuscitation phase compared with phosphate-buffered saline (PBS) pre-treated rats ($P>0.05$, Fig. 1). EPO pre-treatment also had no significant effect on the MAP of sham-operated rats pre-treated with PBS ($P>0.05$, Fig. 1).

Effect of EPO pre-treatment on the organ injury and dysfunction induced by hemorrhagic shock
Compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed significant increases in serum creatinine ($P<0.05$, Fig. 2A), AST ($P<0.05$, Fig. 2C), ALT ($P<0.05$, Fig. 2D) and creatine kinase ($P<0.05$, Fig. 2E), and a significant decrease in creatinine clearance ($P<0.05$, Fig. 2B), indicating the development of renal dysfunction, liver injury, neuromuscular injury and glomerular dysfunction, respectively. Pre-treatment of HS-rats with EPO significantly attenuated the rises in serum creatinine ($P<0.05$, Fig. 2A), AST ($P<0.05$, Fig. 2C), ALT ($P<0.05$, Fig. 2D) and creatine kinase ($P<0.05$, Fig. 2E), and attenuated the decrease in creatinine clearance ($P<0.05$, Fig. 2B). There was no significant difference in levels of serum creatinine ($P>0.05$, Fig. 2A), AST ($P>0.05$, Fig. 2C), ALT ($P>0.05$, Fig. 2D) and creatine kinase ($P>0.05$, Fig. 2E), nor in the creatinine clearance ($P>0.05$, Fig. 2B) in sham-operated rats treated with PBS or EPO.

Fig. 1. Effect of EPO pre-treatment on the circulatory failure caused by hemorrhagic shock. Effect of daily EPO pre-treatment over 3 days on MAP in rats subjected to sham-operation on the fourth day (Sham + PBS 3-day pre-treatment, n=4; Sham + EPO 3-day pre-treatment, n=4) or hemorrhagic shock on the fourth day (HS + PBS 3-day pre-treatment, n=12; HS + EPO 3-day pre-treatment, n=12). Data represent mean ± s.e.m. for n observations; *P<0.05, Sham + PBS 3-day pre-treatment versus HS + PBS 3-day pre-treatment.

Effect of EPO pre-treatment on the hematocrit and serum EPO concentration

Compared with PBS pre-treated rats, rats pre-treated with EPO had a slightly elevated hematocrit (P>0.05, Table 1) and a significantly higher serum concentration of recombinant human EPO (rhEPO) (P<0.0001, Table 1); however, this concentration is less than 0.1 IU/ml.

Effect of EPO concentration on the percentage of circulating CD34$^+$/flk-1$^+$ cells

Compared with PBS pre-treated rats, pre-treatment with EPO significantly increased the percentage of CD34$^+$/flk-1$^+$ cells (cells positive for CD34 and fetal liver kinase-1, the murine homolog of VEGFR2) present within the mononuclear cell component of the blood (P<0.05, Fig. 3B); the data are also shown in the form of a representative histogram (Fig. 3A).

Effect of EPO pre-treatment on the phosphorylation of Akt, GSK-3β and eNOS, and the nuclear translocation of p65 in the livers of rats subjected to hemorrhagic shock

EPO is known to activate several cell signaling pathways as a result of its interaction with the EPO receptor (EPO-R). In particular, EPO can act on the Akt survival pathway, which could confer tissue-protective and anti-inflammatory effects; therefore, we investigated the effect of EPO pre-treatment on the activation of Akt and proteins that interact with Akt such as GSK-3β (Cross et al., 1995) and eNOS (Dimmeler et al., 1999). Compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed a reduction in the phosphorylation of Akt on Ser473, GSK-3β on Ser9 and eNOS on Ser1177 in the liver (P<0.05, Fig. 4A-C). Pre-treatment with EPO attenuated the decline in phosphorylation induced by HS, therefore restoring the

activity of these proteins to that of sham-operated rats (P<0.05, Fig. 4A-C).

A downstream effect of the activation of Akt and inhibition of GSK-3β is the inhibition of the transcription factor NF-κB. The p65 subunit of NF-κB is known to influence the gene expression of many proteins that are involved in apoptosis and inflammation. Compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed a significant increase in the nuclear translocation of the p65 NF-κB subunit in the liver, indicating the activation of NF-κB (P<0.05, Fig. 4D). Pre-treatment with EPO significantly attenuated the increase in NF-κB activation induced by HS (P<0.05, Fig. 4D).

There was no significant effect on the phosphorylation of Akt on Ser473, GSK-3β on Ser9 and eNOS on Ser1177 or on the nuclear translocation of the p65 NF-κB subunit in the livers of sham-operated rats pre-treated with EPO compared with those pre-treated with PBS (P>0.05, Fig. 4A-D).

DISCUSSION

Over the past 20 years, EPO has been reported to reduce tissue injury in many animal models of disease associated with ischemia-reperfusion injury (Brunner et al., 2009; Sharples et al., 2004; Sirén et al., 2001). Here, we demonstrate the tissue-protective action of EPO when administered as a daily treatment prior to the induction of hemorrhage. EPO pre-treatment (1000 IU/kg/day i.p. for 3 days) significantly attenuated the renal (glomerular) dysfunction and the liver and neuromuscular injury induced by hemorrhage and resuscitation in the anesthetized rat. This is the first time this particular pharmacological 'pre-conditioning' approach has been used as a therapeutic strategy in a pre-clinical model of HS.

We have previously reported that administration of EPO prior to resuscitation (after severe hemorrhage) reduces organ injury and dysfunction. Specifically, administration of EPO (300 IU/kg i.v. 5 minutes prior to resuscitation) reduced the renal dysfunction and liver injury caused by hemorrhage and resuscitation in the rat. The beneficial effect of EPO was associated with prevention of the HS-induced increase in caspase activity in the kidney (Abdelrahman et al., 2004); in particular, caspases 3, 8 and 9, which are known to play a pivotal role in the process of apoptosis (Du et al., 2000). The degree of protection exerted by EPO in the previous study is comparable to the degree of protection reported here, indicating that EPO is still able to induce a protective effect when administered over a long period prior to hemorrhage. This suggests that during the 3-day pre-treatment period EPO is able to increase the resistance of tissues and organs to withstand a later insult.

How does EPO exert this tissue-protective (priming) effect? It could be argued that continuous treatment with EPO could result in an increase in basal blood pressure, which could improve the perfusion of organs. It is reported that a third of patients receiving long-term EPO treatment do develop hypertension (Rossi et al., 2011). However, in this study there was no difference in the baseline MAP values of PBS and EPO pre-treated rats, suggesting that EPO pre-treatment did not have a hypertensive effect. Additionally, EPO might improve the perfusion of tissues by promoting erythropoiesis and increasing the oxygen-carrying capability of the blood. Long-term EPO treatment is reported to increase hematocrit in both experimental animals (Brunner et al.,
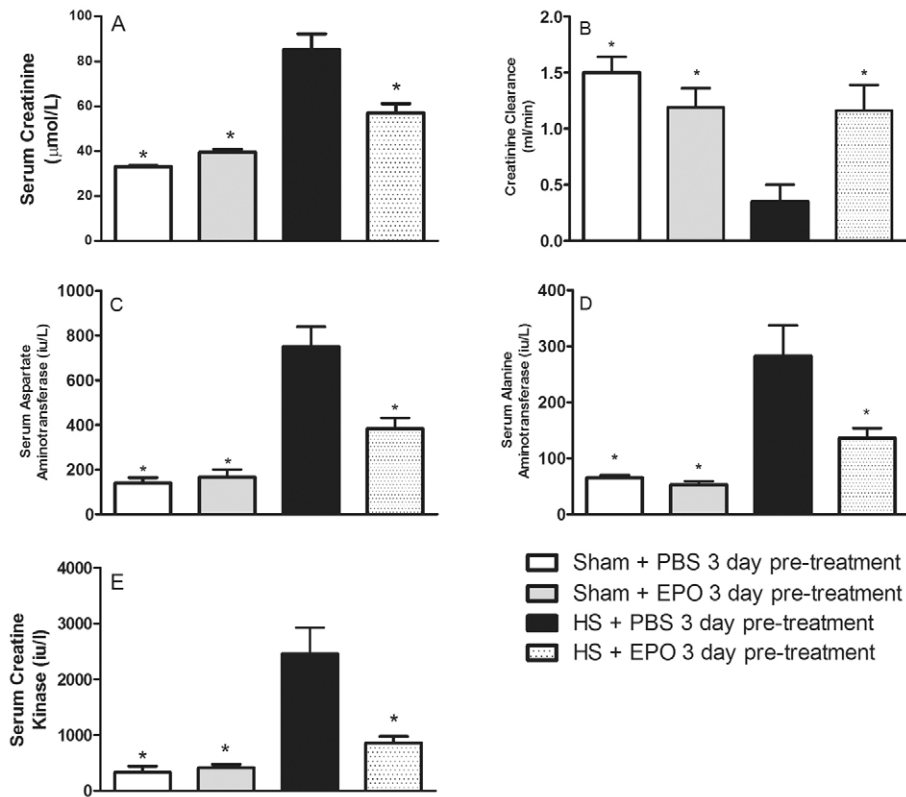
Fig. 2. Effect of EPO pre-treatment on the organ injury and dysfunction induced by hemorrhagic shock. (A-E) Effect of daily EPO pre-treatment over 3 days on: (A) serum creatinine level as a measure of renal function; (B) creatinine clearance as a measure of glomerular function; (C) serum aspartate aminotransferase and (D) alanine aminotransferase levels as a measure of liver injury; and (E) serum creatine kinase level as a measure of neuromuscular injury. Rats were subjected to sham operation on the fourth day (Sham + PBS 3-day pre-treatment, n=4; Sham + EPO 3-day pre-treatment, n=4) or hemorrhagic shock on the fourth day (HS + PBS 3-day pre-treatment, n=12; HS + EPO 3-day pre-treatment, n=12). Data represent mean ± s.e.m. for n observations; *P<0.05 versus HS + PBS 3-day pre-treatment.

2009) and in patients (Corwin et al., 2007); however, this is often associated with an increased incidence of thrombotic events (Corwin et al., 2007). Here, EPO pre-treatment did cause a slight increase in the hematocrit, although the observed increase was not statistically significant.

As the half-life of EPO is ~10 hours in the rat when injected intraperitoneally (Gorio et al., 2005), one could argue that EPO has accumulated in the plasma so that significant plasma levels of EPO are still present when the hemorrhage or injury occurs. However, this was not the case because, even with repetitive injections of 1000 IU/kg of EPO per day, the serum concentration of recombinant human EPO (i.e. injected EPO) prior to the onset of hemorrhage was less than 0.1 IU/ml (~6 IU/kg). Although this value is within the plasma concentration range of EPO that is thought to afford tissue protection (Brines and Cerami, 2008), we believe that this serum concentration of EPO would be insufficient to induce a beneficial effect in our model of HS. In previous studies investigating the therapeutic effects of EPO in the context of HS,

the lowest dose used was 300 IU/kg (Abdelrahman et al., 2004; Wu et al., 2010), which would result in a plasma concentration of ~5 IU/ml when injected intravenously. This is 50-fold higher than the serum concentration measured following 3 days of pre-treatment with EPO. Taking these studies into account, we would assume that due to the complex nature of ischaemia-reperfusion injury and inflammation that occurs in animal models of HS, serum concentrations lower than 5 IU/ml would be inadequate to exert a tissue-protective effect.

There is good evidence that EPO acts via the classical EPO-R present on EPCs to promote their mobilization from the bone marrow in response to hypoxia in order to induce angiogenesis. Additionally, EPCs are known to secrete paracrine proteins, which might contribute to the protective effects we observe in this study (Heeschen et al., 2003). Repetitive dosing with EPO has been reported to increase the proportion of circulating EPCs (defined using combinations of the following markers: CD34, CD45, CD133, sca-1 and flk-1) in experimental animals (Heeschen et al., 2003) and in patients (Bahlmann et al., 2004). In this study, EPO pre-treatment over a period of 3 days significantly increased the proportion of circulating CD34^{+}/flk-1^{+} cells. This is approximately a 100% increase, which is similar to the magnitude of the effect described by Heeschen and colleagues in mice treated with EPO (1000 IU/kg/day for 3 days prior to cell analysis); although the proportion of positive-staining cells appears to be lower in PBS pre-treated mice than in rats (Heeschen et al., 2003). The therapeutic potential of EPCs has been assessed in patients with ST-elevated myocardial infarction; patients received a cell infusion directly into the left anterior descending coronary artery 5 days following percutaneous coronary intervention. An average of

Table 1. Effect of EPO pre-treatment on the hematocrit and serum EPO concentration

Parameter	PBS pre-treated rats	EPO pre-treated rats	n	P-value
Hematocrit (%)	38.0±1.8	44.5±2.0	6	>0.05
Serum rhEPO concentration (i.u./ml)	0±0	0.0847±0.0231	8	<0.0001

Effect of daily EPO pre-treatment on hematocrit and serum rhEPO concentration in rats treated for 3 days with either PBS or EPO. Measurements were taken 24 hours after the final dose. Data represent mean ± s.e.m. for n observations.
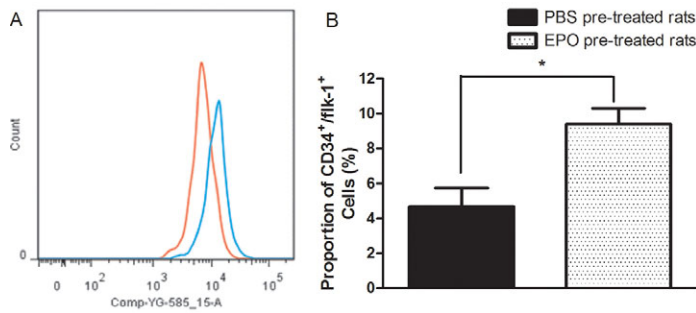
Fig. 3. Effect of EPO concentration on the percentage of circulating CD34+/flk-1+ cells. (A) Representative histogram from flow cytometry measurements of circulating CD34+/flk-1+ cells; the red line represents PBS pre-treated rats and the blue line represents EPO pre-treated rats. (B) Effect of 3-day EPO pre-treatment on the percentage of circulating CD34+/flk-1+ cells in rats treated for 3 days with either PBS (n=3) or EPO (n=3) and sacrificed 24 hours after the last injection.

4.92×10^9 peripheral blood mononuclear cells were injected, of which 0.05% were positive for CD34 and VEGFR2. Cellular infusion resulted in improvement of left ventricular contractile function during the 6-month follow-up period, presumably as a result of increased angiogenesis in the infarcted heart (Tatsumi et al., 2007). Studies using cellular infusions are difficult to conduct because large numbers of cells are required in order to ensure sufficient delivery of cells to the diseased tissue. Therefore, the potential of agents such as EPO (Yip et al., 2011) or granulocyte-macrophage colony-stimulating factor (Subramaniyam et al., 2009) to promote mobilization of cells from the bone marrow might be a more attractive option.

EPO is known to enhance the survival of erythroid progenitor cells (by reducing their apoptosis) by interacting with the classical EPO-R to activate several intracellular signaling pathways, in particular JAK/STAT, PI3K/Akt and MAPK pathways (reviewed by Chateauvieux et al., 2011). Because the EPO-R is expressed in many non-hematopoietic tissues including the endothelium (Anagnostou et al., 1990), brain (Tan et al., 1992), kidneys (Westenfelder et al., 1999) and heart (Depping et al., 2005), it is hypothesized that EPO can activate these pathways in other cells in order to induce a similar anti-apoptotic effect. Therefore, we investigated the potential effect of EPO pre-treatment on the activation of these pathways in the livers of rats subjected to HS and pre-treated with either PBS or EPO. Akt (also known as protein kinase B), which is a member of the phosphoinositide 3-kinase signal transduction enzyme family, regulates cellular activation, inflammatory responses, chemotaxis and apoptosis. When phosphorylated by its upstream regulator, phosphoinositide-dependent kinase, Akt modulates cell survival and growth (Cantley, 2002). In this study, HS caused a decline in Akt phosphorylation and, hence, a decline in activation, which was significantly attenuated by pre-treatment with EPO. Ruscher and colleagues demonstrated a similar effect in the brain; where pre-treatment with EPO up to 48 hours prior to an ischemic insult protected rat cortical neurons from apoptosis by an Akt-dependent mechanism (Ruscher et al., 2002).

GSK-3β is a serine-threonine kinase that was originally recognized as a kinase that phosphorylates glycogen synthase. Unlike most other kinases, GSK-3β is active in a resting cell state and is inactivated by phosphorylation of Ser9. GSK-3β is regulated by multiple signaling pathways including the Akt pathway, which inactivates it by causing Ser9 phosphorylation (Cross et al., 1995).
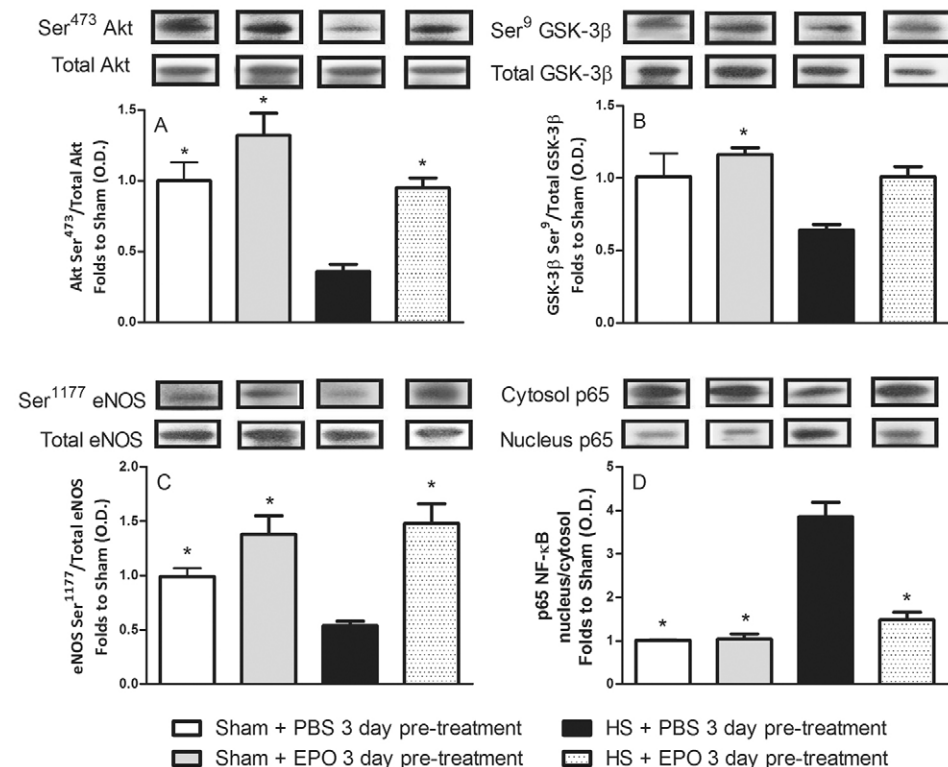
Fig. 4. Effect of EPO pre-treatment on the phosphorylation of Akt, GSK-3β and eNOS, and the nuclear translocation of p65 in the livers of rats subjected to hemorrhagic shock. (A-C) Effect of daily EPO pre-treatment over 3 days on the phosphorylation of Ser473 on Akt (A), Ser9 on GSK-3β (B) and Ser1177 on eNOS (C) in the liver. (D) Nuclear translocation of NF-κB in the livers of rats subjected to sham-operation on the fourth day (Sham + PBS 3-day pre-treatment, n=3; Sham + EPO 3-day pre-treatment, n=3) or hemorrhagic shock on the fourth day (HS + PBS 3-day pre-treatment, n=3; HS + EPO 3-day pre-treatment, n=3). Data represent mean ± s.e.m. for n observations; *P<0.05 versus HS + PBS 3-day pre-treatment.

Here, the HS-induced decline in Akt phosphorylation corresponds to a decline in GSK-3β phosphorylation, which was also significantly attenuated by EPO pre-treatment. This effect has also been confirmed *in vitro* where EPO-induced phosphorylation of Ser9 on GSK-3β was attributable to its anti-apoptotic effect in cardiomyocytes (Ohori et al., 2008). GSK-3β has many downstream targets; however, it is able to interact with the transcription factor NF-κB (Schwabe and Brenner, 2002). NF-κB 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). The p65 subunit of NF-κB is thought to have four phosphorylation sites for the action of GSK-3β, and specific inhibition of GSK-3β has been shown to directly inhibit NF-κB-dependent gene transcription (Schwabe and Brenner, 2002). Also, GSK-3β might inhibit the activation of NF-κB by phosphorylating and degrading IκBα, which is required to prevent NF-κB nuclear translocation (Takada et al., 2004). In this study, HS caused a significant rise in the nuclear translocation of the p65 subunit, which was significantly attenuated by pre-treatment with EPO. A similar effect was observed when EPO was administered 24 hours prior to induction of myocardial infarction; here EPO pre-treatment resulted in a significant reduction in infarct size and a decrease in NF-κB activation in the left ventricle (Xu et al., 2005).

Another downstream effect of Akt activation is the phosphorylation and activation of eNOS, which results in an increased production of nitric oxide (NO) in the endothelium. NO induces local vasodilation, inhibits adhesion of platelets and neutrophils, and regulates angiogenesis (Luque Contreras et al., 2006). Here, the phosphorylation of eNOS at the Akt phosphorylation site; Ser1177, was significantly reduced by HS and this decline was attenuated by EPO pre-treatment. Su and colleagues demonstrated that EPO is able to activate eNOS in endothelial cells by interacting with the βcR and causing Akt-dependent phosphorylation of eNOS (Su et al., 2011).

The JAK/STAT pathway is also activated by the interaction of EPO with the EPO-R. This is a type I cytokine receptor but it does not possess any tyrosine kinase ability, therefore it phosphorylates JAK proteins, which recruit and activate STAT proteins. They are then able to dimerize and translocate to the nucleus in order to influence gene expression (Chateauvieux et al., 2011). STAT3 and STAT5 are particularly implicated in EPO's mechanism of action; however, in this study both HS and EPO pre-treatment had no effect the phosphorylation of STAT3. EPO is also thought to interact indirectly with a host of mitogen-activated protein kinases (MAPKs) through adapter proteins. In particular, activation of ERK1 and ERK2 promotes cellular stress responses such as proliferation, differentiation and production of pro-inflammatory cytokines and occurs in response to injurious stimuli (Donnahoo et al., 1999; Fukudome et al., 2010). However, in this study there was no effect on the phosphorylation of ERK1 and ERK2 in PBS or EPO pre-treated rats subjected to HS.

Pre-treatment of sham-operated rats with EPO did not cause a significant change in the expression of any of the aforementioned proteins, which might have been expected over the 3-day treatment period. However, there was a slight non-significant increase in the phosphorylation of Akt, GSK-3β and eNOS that might have been

sufficient to have a physiological effect in the liver and so contribute to the ability of the EPO pre-treated rats to be more resistant to injury.

The expression of flk-1 on the cell surface of EPCs is of particular interest because activation of this receptor is known to lead to interaction with multiple intracellular signaling pathways, which are similar to those activated by EPO. For example, activation of flk-1 results in phosphorylation and activation of Akt, which promotes cell survival by inhibiting the effects of pro-apoptotic proteins (Gerber et al., 1998a; Gerber et al., 1998b). Additionally, flk-1-mediated phosphorylation of Akt results in the subsequent phosphorylation and activation of eNOS (Dimmeler et al., 1999; Gerber et al., 1998b), which is very similar to the proposed mechanism of anti-apoptotic action of EPO in endothelial cells (Su et al., 2011). Flk-1 is also able to interact with ERK1 and ERK2 to promote cell proliferation (Takahashi et al., 2001). It is possible that in combination with the ability of EPO to activate pro-survival signaling pathways, the mobilization of flk-1^{+} cells is able to contribute to the tissue-protective effects of EPO by potentiating the action of these pathways.

The correlation between EPO-induced EPC mobilization and the beneficial effects of EPO is well documented in the literature. Several authors have demonstrated the ability of EPO to mobilize EPCs in animal models (Bahlmann et al., 2003; Heeschen et al., 2003). This effect has also been observed in stroke patients, where EPO treatment resulted in an increased proportion of circulating EPCs, which was associated with a significant and independent reduction in the incidence of a major adverse neurological event within 90 days of the initial insult (Yip et al., 2011). Ferrario and colleagues also demonstrated a similar correlation between endogenous EPO production and EPC mobilization in patients following acute myocardial infarction (Ferrario et al., 2007). Although the direct inhibition of EPO-induced mobilization of EPCs has not been investigated, it is possible to inhibit EPC mobilization in animals using pharmacologic agents, for example with vascular endothelial growth inhibitor (Tian et al., 2009) or dopamine (Chakroborty et al., 2008). Prevention of the EPC mobilization afforded by EPO by these interventions could allow us to differentiate between the EPC-dependent and EPC-independent effects of EPO. Even if it were possible to block the mobilization of EPCs afforded by EPO, it is still possible that EPO causes tissue protection by directly activating pro-survival signaling pathways.

EPO can activate the above signaling pathways downstream of its molecular target, which is thought to be a complex consisting of a single monomer of EPO-R and CD131 (also known as the βcR). The βcR is a signal-transduction domain that is also present in the receptor complexes for granulocyte-macrophage colony stimulating factor, IL-3 and IL-5. The expression of the βcR-EPO-R receptor complex is known to increase following an injurious stimulus and it is often described as the tissue-protective receptor. Brines and colleagues reported that the beneficial effects of EPO are mediated by this receptor, as demonstrated by the finding that mice lacking the gene for βcR lost the protection exerted by EPO against spinal cord injury whereas wild-type mice subjected to the same procedure did not (Brines et al., 2004). Therefore, it is possible that the protective effects of EPO pre-treatment observed in this study are mediated by this receptor complex.

In conclusion, we report that daily pre-treatment with EPO prior to hemorrhage attenuates the early organ injury and dysfunction in rats subjected to severe HS. Pre-treatment with EPO restored phosphorylation and, hence, activation of Akt, which in turn resulted in inhibition of GSK-3β (secondary to phosphorylation on Ser9) and inhibition of the activation of NF-κB. Activation of Akt also resulted in phosphorylation and activation of eNOS. There is now very good evidence that therapeutic strategies that enhance the activation of Akt and reduce the activation of GSK-3β enhance the resistance of organs to noxious stimuli (including ischemia) and reduce inflammation via inhibition of NF-κB (Takada et al., 2004). Due to the lack of effect on the basal blood pressure, hematocrit and serum EPO concentration, we propose that the mechanism behind the protective effects exerted by EPO in this study involve the mobilization of $CD34^+/flk-1^+$ cells from the bone marrow, resulting in activation of the Akt-eNOS survival pathway, which causes a significant reduction in tissue injury and inflammation by the inhibition of NF-κB. The pre-treatment regimen used in this study could provide new therapeutic options for clinical situations where blood loss is foreseeable; for example, prior to major surgery. Pre-treatment with EPO also offers an alternative to cell-based therapies because it can induce the mobilization of EPCs, which can exert further protection. However, the safety aspects of long-term treatment with EPO need to be considered carefully as there is a significant risk associated with increased hematocrit.

MATERIALS AND METHODS
This study was approved by the ethics committee of Queen Mary University of London and the UK Home Office (PPL: 70/6525) and all procedures were performed strictly under the United Kingdom Animals (Scientific Procedures) Act 1986.

Hemorrhagic shock and quantification of organ injury and dysfunction
This study was carried out on 32 male Wistar rats (Charles River Ltd, Margate, UK) weighing 283±6 g and receiving a standard diet and water *ad libitum*. An additional six rats were used for the isolation of peripheral blood mononuclear cells. All data from rats that had died during the experiment were excluded from data analysis hence the numbers (*n*) presented represent the 'survivors' of the entire experimental protocol.

Rats were anesthetized using sodium thiopentone (120 mg/kg i.p. maintained using ~10 mg/kg i.v.) and cannulation of the trachea, femoral artery, carotid artery, jugular vein and bladder was performed. Blood was withdrawn via a cannula inserted in the right carotid artery in order to achieve a fall in mean arterial pressure (MAP) to 35±5 mmHg within 10 minutes. From this point onwards, MAP was maintained at 35±5 mmHg for a period of 90 minutes, either by further withdrawal of blood during the compensation phase or by administration of Ringer's lactate i.v. during the decompensation phase. The average volume of blood withdrawn during hemorrhage was 10.45±0.19 ml (*n*=24, across all hemorrhaged groups). At 90 minutes after initiation of hemorrhage, i.v. resuscitation was performed with 20 ml/kg Ringer's lactate over a period of 10 minutes and then half the shed blood mixed with 100 IU/ml heparinized saline over a period of 50 minutes. At the end of 1 hour of resuscitation, an i.v. infusion of Ringer's lactate (1.5 ml/kg/hour) was started as fluid replacement and maintained

throughout the experiment for a further 3 hours, at which point 1.2-ml blood samples were collected via the carotid artery into S/1.3 tubes containing serum gel (Sarstedt, Numbrecht, Germany), after which the heart was removed to terminate the experiment. The blood was centrifuged (9000 *g* for 3 minutes) to separate serum. Creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase were measured within 24 hours (IDEXX Laboratories, West Yorkshire, UK). Urine was collected from the cannula inserted into the bladder and creatinine measured for the estimation of creatinine clearance (IDEXX Laboratories). Additionally, liver samples were taken and stored at −80°C.

Experimental design
Rats were randomly allocated into the following groups: (i) sham + PBS 3-day pre-treatment (*n*=4); (ii) sham + EPO 3-day pre-treatment (*n*=4); (iii) HS + PBS 3-day pre-treatment (*n*=12); and (iv) HS + EPO 3-day pre-treatment (*n*=12). Rats were treated with either 1 ml/kg PBS or 1000 IU/kg recombinant human EPO i.p. daily for 3 days. On day 4, rats were subjected to sham operation (surgical procedure only) or HS.

Measurement of hematocrit
The hematocrit was measured prior to hemorrhage in rats treated with either PBS or EPO for 3 days. At 24 hours after the final dose, ~100 μl blood was withdrawn from the cannula inserted in the right carotid artery and analyzed using an ABL77 v1.41 analyzer (Radiometer, Brønshøj, Denmark).

Measurement of the serum concentration of recombinant human EPO concentration
The serum concentration of injected recombinant human EPO (rhEPO) was measured prior to hemorrhage in rats treated with either PBS or EPO for 3 days. Approximately 500 μl of blood was withdrawn 24 hours after the final dose from the cannula inserted in the right carotid artery. Serum was obtained and analyzed as per manufacturer's instructions using a Human EPO Immunoassay (R&D Systems Europe, Abingdon, UK).

Flow cytometry
The proportion of circulating EPCs was measured in rats treated with either PBS or EPO for 3 days. Approximately 3 ml blood was withdrawn 24 hours after the final dose from the cannula inserted in the right carotid artery. Peripheral blood mononuclear cells (PBMNCs) were extracted using density gradient centrifugation at 400 *g* for 30 minutes at 4°C (Histopaque-1077, Sigma-Aldrich, Dorset, UK). PBMNCs were re-suspended in 10 mls Hank's balanced salt solution (HBSS) and centrifuged at 250 *g* for 10 minutes at 4°C; the resultant supernatant was aspirated and the pellet re-suspended in 100 μl HBSS. Cells ($3×10^6$) were incubated for 30 minutes at room temperature with 21 μl of mouse anti-CD34 antibody conjugated with fluorescein (FITC) and 24 μl of mouse anti-flk-1 antibody in 80 μl of HBSS or with HBSS alone. Excess antibody was removed by washing with 1 ml HBSS, centrifuging at 330 *g* for 5 minutes at room temperature and then aspirating the supernatant. The cells were re-suspended in 5 μl of goat anti-mouse IgG-phycoerythrin (PE) and incubated for a further 30 minutes at room temperature. The wash step was then repeated

and cells were re-suspended in 500 µl of HBSS. Analysis was performed using a Fortessa analyzer (Beckman Coulter, High Wycombe, UK) and data analyzed using FlowJo v7.6.5 (Ashland, OR).

Western blot analysis
Western blots were carried out as previously described (Collino et al., 2006). Three separate experiments of western blot analysis were performed for each marker, and tissues were done separately for each western blot experiment. Briefly, rat liver samples were homogenized and centrifuged at 4000 *g* for 5 minutes at 4°C. Supernatants were removed and centrifuged at 15,000 *g* at 4°C for 40 minutes to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer. The suspensions were centrifuged at 15,000 *g* for 20 minutes at 4°C. The resulting supernatants containing nuclear proteins were carefully removed and the protein content determined using a bicinchoninic acid (BCA) protein assay following the manufacturer's directions. Proteins were separated by 8% SDS-PAGE and transferred to a polyvinyldenedifluoride (PVDF) membrane, which was then incubated with a primary antibody (rabbit anti-total Akt, dilution 1:1000; mouse anti-pAkt Ser473, dilution 1:1000; rabbit anti-total GSK-3β, dilution 1:200; goat anti-pGSK-3β Ser9, dilution 1:200; rabbit anti-total eNOS, dilution 1:200; goat anti-peNOS Ser1177, dilution 1:200; rabbit anti-NF-κB p65, dilution 1:400). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1:10,000) and developed using the ECL detection system. The immunoreactive bands were visualized by autoradiography. The membranes were stripped and incubated with β-actin monoclonal antibody (dilution 1:5000) and subsequently with an anti-mouse antibody (dilution 1:10,000) to assess gel-loading homogeneity. Densitometric analysis of the bands was performed using Gel ProAnalyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD) and expressed as relative optical density (O.D.). Optical density analysis was expressed as fold-increase versus the sham group. In the sham group, the immunoreactive bands of the gel were measured and normalized against the first immunoreactive band (standard sham sample) and the results of all the bands belonging to the same group were expressed as mean ± s.e.m. For the sham group, a value of 1 is relative to the first immunoreactive band. The membranes were stripped and incubated with β-actin monoclonal antibody and subsequently with an anti-mouse antibody to assess gel-loading homogeneity. Relative band intensity was assessed and normalized against parallel β-actin expression. The data for each group was then adjusted against corresponding sham data to establish relative protein expression compared with sham animals.

Materials
Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich (Dorset, UK). All stock solutions were prepared using non-pyrogenic saline (0.9% w/v NaCl; Baxter Healthcare, Norfolk, UK). Ringer's lactate was purchased from Baxter Healthcare. Antibodies for western blot analyses were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Antibodies for flow cytometry were purchased from Insight Biotechnology (Middlesex, UK). NeoRecormin (rhEPO) was manufactured by Roche (Welwyn Garden City, UK).

Statistical analysis
All values described in the text and figures are expressed as mean ± s.e.m. for *n* observations. Each data point represents measurements obtained from up to 12 separate animals. Statistical analysis was carried out using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA). Data without repeated measurements were assessed by one-way ANOVA followed by Dunnett's post hoc test. Data with repeated measurements were assessed by two-way ANOVA followed by Bonferroni's post hoc test. Data with comparison of only two groups were assessed by unpaired, two-tailed *t*-test. A *P*-value of less than 0.05 was considered to be significant.

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