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

Kiran K. Nandra1, Massimo Collino2, Mara Rogazzo2, Roberto Fantozzi2, Nimesh S.A. Patel1, Christoph Thiemermann1

1 The William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, U.K.

2 Department of Drug Science and Technology, University of Turin, Turin, Italy

Correspondence and requests for reprints should be directed to: Dr Nimesh S.A. Patel, Centre for Translational Medicine & Therapeutics, The William Harvey Research Institute, Charterhouse Square, London, EC1M 6BQ, UK. Tel: +44 (0)20-7882-8180, Fax: +44 (0)20-7900-3870, e-mail: n.s.patel@qmul.ac.uk

Running title: EPO pre-treatment in hemorrhagic shock

Keywords: erythropoietin, hemorrhagic shock, organ injury, tissue-protection, pre-treatment, endothelial progenitor cells.
Summary

Pre-treatment with erythropoietin (EPO) has been demonstrated to exert tissue protective effects against ‘ischemia-reperfusion’ type injuries. This protection may be mediated by mobilization of endothelial progenitor cells (EPCs) from the bone marrow, 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 for 3 days and 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 3 day pre-treatment with EPO in rats and was significantly increased when compared to rats pre-treated with PBS. EPO pre-treatment significantly attenuated organ injury and dysfunction (renal, hepatic, 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 may protect against the organ injury and dysfunction induced by HS, by a mechanism that may 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 United States of America (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 which contributes to apoptosis and tissue necrosis leading to further organ injury (Rushing et al., 2008). In patients with trauma, failure of more than four organs is linked to certain mortality, therefore 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-kilodalton glycoprotein secreted by the kidneys which 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 which are beneficial in various ischemia-reperfusion type injuries including acute kidney injury (Sharples et al., 2004), myocardial infarction (Brunner et al., 2009), stroke (Siren et al., 2001) and HS (Abdelrahman et al., 2004). In the majority of studies EPO is administered either on or prior to reperfusion, however there are also several studies which 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 injury 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, 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 in (Holmes et al., 2007)). Taken together 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 in (Chateauvieux et al., 2011)), or 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, which is administered prior to the onset of an ischemia-reperfusion type injury.
However, many ischemic insults occur spontaneously and cannot be predicted, for example trauma due to road traffic accident, and the preferred treatment time point 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 may be a viable therapeutic option. EPO is already clinically used as 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 EPO’s ability to mobilize EPCs and its effects on the activation of various cellular signaling pathways [in particular phosphorylation of Akt on Ser$^{473}$, phosphorylation of glycogen synthase kinase-3β (GSK-3β) on Ser$^{9}$, phosphorylation of eNOS on Ser$^{1177}$ 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**

When compared with sham-operated rats, rats subjected to hemorrhage exhibited a significant decline in MAP (P<0.05, Fig 1). Consequent resuscitation increased MAP but this was still significantly lower than 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, when compared with 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**

When 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), and the creatinine clearance (P>0.05, Fig 2B) in sham-operated rats treated with PBS or EPO.
Effect of EPO pre-treatment on the hematocrit and serum EPO concentration

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

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

When compared with PBS pre-treated rats, pre-treatment with EPO significantly increased the percentage of CD34+/flk-1+ cells present within the mononuclear cell component of the blood (P<0.05, Fig 3B); this data is also illustrated 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-R. In particular, EPO can act on the Akt survival pathway which may confer tissue-protective and anti-inflammatory effects; therefore we investigated the effect of EPO pre-treatment on the activation of Akt, and of proteins that interact with Akt such as GSK-3β (Cross et al., 1995) and eNOS (Dimmeler et al., 1999). When 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. When 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 the nuclear translocation of the p65 NF-κB subunit, in the livers of sham-operated rats pre-treated with EPO when 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; Siren 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 min 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/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 may 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., 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 in hematocrit was not statistically significant.

As the half-life of EPO is approximately 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/injury occurs. However, this was not the case as – 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 (approximately 6 iu/kg). Although this value is within the plasma concentration range of EPO that is thought to afford tissue-protection (Brines et al., 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 is 300 iu/kg (Abdelrahman et al., 2004;Wu et al., 2010); which would result in a plasma concentration of approximately 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 may 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 foetal liver kinase-1; flk-1 (murine homolog of VEGFR2)] 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 4.92 x 10⁹ peripheral blood mononuclear cells was 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 as 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 may 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 in (Chateauvieux et al., 2011)). As the EPO-R is expressed in many non-hematopoietic tissues including the endothelium (Anagnostou et al., 1990), the brain (Tan et al., 1992), the kidneys (Westenfelder et al., 1999) and the 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, 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 h 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 Ser\(^9\). GSK-3β is regulated by multiple signaling pathways including the Akt pathway, which inactivates it by causing Ser\(^9\) phosphorylation (Cross et al., 1995). 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 Ser\(^9\) 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 known that it is able to interact with the transcription factor; NF-κB (Schwabe et al., 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 et al., 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 et al., 2002). Also, GSK-3β may inhibit the activation of NF-κB by phosphorylating and degrading IκB\(\alpha\), 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 can induce local vasodilation, inhibit adhesion of platelets and neutrophils, and regulates angiogenesis (Luque Contreras et al., 2006). Here, the phosphorylation of eNOS at the Akt phosphorylation site; Ser^{1177}, was significantly reduced by HS, 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 EPO’s interaction 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). In particular STAT3 and 5 are 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 with a host of mitogen activated protein kinases (MAPKs) indirectly through adapter proteins. In particular, activation of ERK1/2 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 ERK1/2 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 may have been expected over the
three day treatment period. However, there was a slight non-significant increase in the phosphorylation of Akt, GSK-3β and eNOS which may 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 as 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 ERK1/2 to promote cell proliferation (Takahashi et al., 2001). It is possible that in combination with EPO’s ability to activate pro-survival signaling pathways, the mobilization of flk-1+ cells is able to contribute to EPO’s tissue-protective effects by potentiating the action of these pathways.

The correlation between EPO-induced EPC mobilization and EPO’s beneficial effects is well documented in the literature. Several authors have demonstrated EPO’s ability 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.,
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 mobilisation afforded by EPO by these interventions may 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, which 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 have reported that EPO’s beneficial effects are mediated by this receptor as the protection exerted by EPO against spinal cord injury was lost in mice that lacked the gene for βcR when compared to wild-type mice subjected to the same procedure (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, which 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 may involve the mobilization of CD34+/flk-1+ cells from the bone marrow, resulting in activation of the Akt/eNOS survival pathway, which caused a significant reduction in tissue injury and inflammation by the inhibition of NF-κB. The pre-treatment regimen used in this study may 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 as it can induce the mobilization of EPCs, which can exert further protection. Although the safety aspects of long-term treatment with EPO would 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 receiving a standard diet and water ad libitum. An additional 6 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 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 min 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 heparinised saline over a period of 50 minutes. At the end of 1 hour resuscitation,
an i.v. infusion of Ringer’s Lactate (1.5 ml/kg/h) 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 from which creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase were measured within 24 hours (IDEXX Laboratories Ltd., West Yorkshire, UK). Urine was collected from the cannula inserted into the bladder from which creatinine was measured for the estimation of creatinine clearance (IDEXX Laboratories Ltd.). 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 and on day 4 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, approximately 100 µl blood was withdrawn 24 hours after the final dose from the cannula inserted in the right carotid artery and analysed using ABL77 v1.41 analyser (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 blood was withdrawn 24 hours after the final dose from the cannula inserted in the right carotid artery from which serum was obtained and analysed as per manufacturer’s instructions using a Human EPO Immunoassay (R&D Systems Europe Ltd., 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 from which peripheral blood mononuclear cells (PBMCs) were extracted using density gradient centrifugation at 400 g for 30 minutes at 4°C (Histopaque-1077®, Sigma-Aldrich, Dorset, U.K.). PBMCs were re-suspended in 10 ml 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. Three million cells were incubated 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 for 30 min at room temperature. Excess antibody was removed by washing with 1 ml HBSS and centrifuging at 330 g for 5 minutes at room temperature and the supernatant was aspirated. 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 Fortessa...
analyser (Beckman Coulter, High Wycombe, U.K.) and data analysed using FlowJo v7.6.5 (Ashland, Oregon, U.S.A.).

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 4,000 \( \text{g} \) for 5 minutes at 4°C. Supernatants were removed and centrifuged at 15,000 \( \text{g} \) 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 \( \text{g} \) for 20 minutes at 4°C. The resulting supernatants containing nuclear proteins were carefully removed, and protein content was determined using a bicinchoninic acid (BCA) protein assay following the manufacturer's directions. Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane, which was then incubated with a primary antibody (rabbit anti-total Akt dilution 1:1000; mouse anti-pAkt Ser\(^{473} \) dilution 1:1000; rabbit anti-total GSK-3\( \beta \), dilution 1:200; goat anti-pGSK-3\( \beta \) Ser\(^9 \) dilution 1:200; rabbit anti-total eNOS dilution 1:200, goat anti-peNOS Ser\(^{1177} \) dilution 1:200; rabbit anti-NF-\( \kappa \)B p65 dilution 1:400). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1:10000) and developed using the ECL detection system. The immunoreactive bands were visualised by autoradiography. The membranes were stripped and incubated with \( \beta \)-actin monoclonal antibody (dilution 1:5000) and subsequently with an anti-mouse antibody (dilution 1:10000) to assess gel-loading homogeneity. Densitometric analysis of the bands was performed using Gel Pro®Analyzer 4.5, 2000 software (Media
Cybernetics, Silver Spring, MD, USA) and expressed as relative optical density (O.D.) and optical density analysis was expressed as fold-increase versus the sham group. In the sham group, the immunoreactive bands of the gel were respectively 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 ± SEM. This provides SEM. for the sham group where 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. Each group was then adjusted against corresponding sham data to establish relative protein expression when compared to sham animals.

Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). All stock solutions were prepared using non-pyrogenic saline (0.9 % [w/v] NaCl; Baxter Healthcare Ltd., Norfolk, UK). Ringer’s Lactate was purchased from Baxter Healthcare Ltd. Antibodies for Western blot analyses were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Antibodies for flow cytometry were purchased from Insight Biotechnology Ltd. (Middlesex, UK). NeoRecormin (rhEPO) was manufactured by Roche (Welwyn Garden City, U.K).
Statistical Analysis

All values described in the text and figures are expressed as mean ± standard error of the mean (SEM) 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, California, USA). Data without repeated measurements was assessed by one-way ANOVA followed by Dunnett’s post hoc test. Data with repeated measurements was assessed by two-way ANOVA followed by Bonferroni’s post hoc test. Data with comparison of only 2 groups was assessed by unpaired, two-tailed t-test. A P value of less than 0.05 was considered to be significant.
Acknowledgements

We would like to thank Dr Guglielmo Rosignoli from The Bart’s Cancer Institute and The William Harvey Research Institute Flow Cytometry Core Facility at Queen Mary University of London, for his assistance and advice with the flow cytometry experiments performed in this study.

Competing interests

There are no financial or competing interests to declare.

Author contributions

KKN, NSAP and CT were involved in the conception, hypotheses delineation, and design of the study; KKN, MC, MR, RF, NSAP and CT were involved in the acquisition of the data or the analysis and interpretation of such information, and KKN, MC, NSAP and CT were involved in writing the article or substantial involvement in its revision prior to submission.

Funding

This work is supported by the British Heart Foundation (FS/10/57/28485), Kidney Research UK (PDF4/2009) and 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).
References


Figure Legends

Figure 1: Effect of EPO pre-treatment on the circulatory failure caused by hemorrhagic shock. Effect of daily EPO pre-treatment over 3 days on mean arterial pressure (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 ± SEM for n observations, *P<0.05 Sham + PBS 3 day pre-treatment vs. HS + PBS 3 day pre-treatment.

Figure 2: Effect of EPO pre-treatment on the organ injury and dysfunction induced by hemorrhagic shock. Effect of daily EPO pre-treatment over 3 days on renal function (A); serum creatinine level, glomerular function (B); creatinine clearance, liver injury (C + D); serum aspartate aminotransferase and alanine aminotransferase levels; and neuromuscular injury (E); serum creatine kinase level, 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 ± SEM for n observations, *P<0.05 vs. HS + PBS 3 day pre-treatment.

Figure 3: Effect of EPO concentration on the percentage of circulating CD34+/flk-1+ cells. Effect of 3-day EPO pre-treatment on the percentage of circulating CD34+/flk-1+ cells (B) in rats treated for 3 days with either PBS (n=3) or EPO (n=3) and sacrificed 24 hours after the last injection. Representative histogram (A) the red line represents PBS pre-treated rats and the blue line represents EPO pre-treated rats.
Figure 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. Effect of daily EPO pre-treatment over 3 days on the phosphorylation of Ser\(^{473}\) on Akt, Ser\(^{9}\) on GSK-3β and Ser\(^{177}\) on eNOS in the liver; and the 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) 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 ± SEM for n observations, *P<0.05 vs. HS + PBS 3 day pre-treatment.

Table Legends

Table 1: Effect of EPO pre-treatment on the hematocrit and serum EPO concentration. 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 ± SEM for n observations.
**Translational Impact**

**Clinical Issue:** Worldwide, trauma is the cause of approximately 6 million deaths every year and the associated blood loss or hemorrhage is responsible for 40% of all trauma deaths. Gunshot and stab wounds, injuries sustained in road traffic accidents and invasive surgery can all result in hemorrhage. Despite management of hemorrhage with fluid resuscitation and blood transfusions the development of organ injury and dysfunction is still prevalent as the combination of hemorrhage and resuscitation results in a global ischemia-reperfusion type injury. The failure of 4 organs is associated with certain mortality; therefore highlighting the need for pharmaceutical interventions that limit the damage associated with resuscitation.

Erythropoietin (EPO) is a hormone which controls the production of red blood cells by regulating the proliferation of erythroid progenitor cells in the bone marrow. In addition to its erythropoietic effects, EPO can exert anti-apoptotic and anti-inflammatory effects in many models of ischemia-reperfusion type injuries including hemorrhagic shock. Interestingly EPO can also exert these effects when administered as a treatment prior to the ischemic insult, however, the mechanism by which EPO exerts this tissue priming effect is unclear. It is known that EPO is a stimulus for endothelial progenitor cell (EPC) mobilization, these cells promote angiogenesis but they may also exert direct or paracrine effects on tissues within the body which may be tissue-protective.

**Results:** The authors demonstrate that daily treatment of rats with EPO (1000 iu/kg/day i.p.) for 3 days prior to hemorrhage and resuscitation significantly attenuated the renal (glomerular) dysfunction, liver and neuromuscular injury when compared with rats pretreated with vehicle. They report that treating rats for 3 days with EPO resulted in
mobilization of EPCs (defined as CD34+/flk-1+). Additionally, they report that in rats subjected to hemorrhage and resuscitation and pre-treated with EPO there is activation of the Akt/eNOS pro-survival pathway. It is possible that the mobilization of EPCs may contribute to tissue-protective effects observed with EPO either by secretion of paracrine proteins and/or by direct interaction with the Akt/eNOS pro-survival pathway.

**Implications and future directions:** Pre-treatment with EPO prior to foreseeable hemorrhage, for example, before surgery, may be a therapeutic option in order to limit the tissue injury associated with hemorrhage and resuscitation. The mechanism of action reported here suggests that dosing with EPO is an alternative to using cell based therapies as it is able to mobilize EPCs which can potentiate EPO’s tissue-protective effects.
Ser^{473} Akt
Total Akt

Ser^{9} GSK-3β
Total GSK-3β

A

B

Akt Ser^{473}/Total Akt
Folds to Sham (O.D.)

GSK-3β Ser^{9}/Total GSK-3β
Folds to Sham (O.D.)

Ser^{1177} eNOS
Total eNOS

Cytosol p65
Nucleus p65

C

D

eNOS Ser^{1177}/Total eNOS
Folds to Sham (O.D.)
p65 NF-κB
nucleus/cytosol
Folds to Sham (O.D.)

- Sham + PBS 3 day pre-treatment
- Sham + EPO 3 day pre-treatment
- HS + PBS 3 day pre-treatment
- HS + EPO 3 day pre-treatment
<table>
<thead>
<tr>
<th></th>
<th>PBS pre-treated rats</th>
<th>EPO pre-treated rats</th>
<th>n</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>38.0 ± 1.8</td>
<td>44.5 ± 2.0</td>
<td>6</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum rhEPO concentration (iu/ml)</td>
<td>0 ± 0</td>
<td>0.0847 ± 0.0231</td>
<td>8</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>