Novel Translational Strategies to Treat Cardiac Injury and Dysfunction

A thesis presented by

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I dedicate this thesis to both my parents. My late dear father, Ismail, who is greatly missed. His children were his life and he believed the sky is the limit when it comes to education. My mother Nabiha for being my rock and a great role model who always reminds me nothing is ever impossible!

Abstract

There is ample evidence of the crucial role of PI3K/Akt dependent signalling in cardiac function, cellular growth and cell apoptosis. The PI3K/Akt pathway mediates cardioprotective effects in experimental models of cardiovascular disease. For example, activation of this pathway ameliorates the sepsis-induced cardiac dysfunction, whereas its activation in myocardial ischaemia/reperfusion (I/R) limits cardiac injury.

This thesis investigates the role of two drugs, which activate the PI3K/Akt-pathway, namely the haematopoietic cytokine erythropoietin and the anti-malarial drug artesunate, in a mouse animal model of experimental sepsis-induced cardiac dysfunction and in a rat model of regional myocardial I/R injury, respectively.

Using a clinically relevant model of caecal ligation and puncture in mice, I demonstrated that aged (8 months) C57BL/6 mice (receiving fluid resuscitation and antibiotic therapy) developed significant cardiac dysfunction (within 24 h), while younger mice (2 months) did not. Erythropoietin attenuated the impaired systolic contractility (in vivo and ex vivo) caused by endotoxaemia (lipopolysacchride 9 mg kg^{-1}; young mice) and sepsis (aged mice). These beneficial effects were associated with activation of Akt and endothelial nitric oxide synthase survival pathways and inhibition of the glycogen synthase kinase 3β, nuclear factor-κB and interleukin 1β pro-inflammatory pathways, secondary to activation of the β-common receptor.

A single bolus administration of artesunate at the start of reperfusion in a rat model of myocardial I/R significantly attenuated the infarct size. This effect was mediated via activation of pro-survival pathways (PI3K/Akt and ERK 1/2 and STAT-3) and inhibition of the glycogen synthase kinase 3β and nuclear factor-κB pro-inflammatory pathways.

Thus, in this thesis I have demonstrated that pharmacological activation of the PI3K/Akt pathway by erythropoietin and artesunate in sepsis and myocardial I/R, respectively, plays a vital role in the amelioration of cardiac dysfunction and injury.

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Publications

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Abstracts

Khan AI, Coldewey SM, Shintani Y, Patel NS, Thiemermann C. (2009). Erythropoietin Attenuates Cardiac Dysfunction Caused by Endotoxaemia in Mice. British Pharmacological Society, London, England. December 2009 (Oral presentation).

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Abbreviations

AAR	area at risk
ABX	antibiotics
ARS	artesunate
ART	artemisinin
ATP	adenosine 5’triphosphate
BCA	bicinchoninic acid
βcR (also called CD131)	β-common receptor
CD	cyclodextrin
CLP	caecal ligation and puncture
DHA	dihydroartemisinin
DMSO	dimethylsulphoxide
ECG	electrocardiogram
EF	ejection fraction
eNOS	endothelial nitric oxide synthase
EPO	erythropoietin
EpoR	erythropoietin homodimer
ERK	extracellular signal-regulated kinase
FAC	fractional area of change
FS	fractional shortening
G	gauge
GM-CSF	granulocyte colony stimulating factor
GSK-3β	glycogen synthase kinase-3β

IL-1β	interleukin-1beta
IL-3/5	interleukin-3/5
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneally
IPC	ischaemic preconditioning
I/R	ischaemia/reperfusion
JAK	janus kinase
KHB	Krebs-Henseleit buffer
KO	knock-out
LAD	left anterior descending coronary artery
LV	left ventricle
LVDP	left ventricular developed pressure
LVEDV	left ventricular end-diastolic volume
LVIDD	left ventricular internal dimension-diastole
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
mPTP	mitochondrial permeability transition pore
NBT	p-nitroblue tetrazolium
NF-κB	nuclear factor kappa B
NO	nitric oxide
PI3K	phosphatidylinositide 3-kinases
PVDF	polyvinyldenedifluoride
rhEPO	recombinant human EPO

RISK	reperfusion injury signalling kinase
SAFE	survivor activating factor enhancement
Ser	serine
SDS-PAGE	sodium dodecyl sulphate-PAGE
STAT3	signal transducer and activator of transcription 3
TLR	toll like receptor
TNF-α	tumour necrosis factor-α
WT	wild type

Measurements and units

HR	heart rate
IU	international unit
MAP	mean arterial pressure
mmHg	millimetre of mercury
PRI	pressure rate index
s.c.	subcutaneously

Miscellaneous terms

ANOVA	analysis of variance
SEM	standard error of the mean

Chapter 1

General Introduction

1.1 Sepsis

Sepsis is a severe, debilitating clinical condition that has gained momentum as becoming the leading cause of death in critically ill patients (Hotchkiss & Karl, 2003; Martin, 2012), predominantly as a consequence of multiple organ failure (MOF) (Rudiger & Singer, 2007). The increase in incidence of sepsis is affected by various patient-specific factors ranging from age to co-morbid medical conditions (Martin, 2012) to race and gender (Martin *et al.,* 2003; Esper *et al.,* 2006; Mayr *et al.,* 2010). Sepsis disproportionately affects the elderly with an average age being in early to mid-60s with older age being an independent risk factor for death from sepsis (Martin *et al.,* 2003). Co-morbid conditions such as cancer and diabetes which alter the immune system result in a significantly elevated risk for developing sepsis and may also contribute to succumbing to contracting nosocomial sepsis due to their increased interaction with the healthcare systems (Danai *et al*., 2006). Likewise, the increased use of invasive procedures and immunosuppressive drugs also plays a part in the increased incidence, with a study showing that sepsis among patients immunosuppressed prior to the onset of sepsis were associated with higher mortality than in immunocompetent patients (Poutsiaka *et al*., 2009). Additionally, other factors contributing to the increased incidence of sepsis include transplantation, and prosthetic implants and devices, as well as the increasing problem of antimicrobial resistance (LaRosa, 2010). Despite technical developments in intensive care units (ICU) and advanced supportive treatment, sepsis still affects approximately 700,000 people annually with a resulting death rate of 210,000 per year in the United States (Angus *et al*., 2001). A recent study explored the impact of evidence-based treatments for severe sepsis in critical care units across England, Wales and Northern Ireland between 1995 and 2005 and revealed not only a rise in admissions from 23.5 % to 28.7 %, but also an increase in the total number of deaths from 9,000 to 14,000 (Harrison *et al*., 2006).

Sepsis is defined as a systemic inflammatory response to known or suspected infection (i.e. by positive culture) manifested by two or more of the conditions of the systemic inflammatory response syndrome (SIRS) (Table 1.1). Severe sepsis refers to sepsis that is accompanied by evidence of hypoperfusion abnormalities (lactic acidosis, oliguria, and acute alterations in mental state) or dysfunction of at least one organ system. When severe sepsis is accompanied by hypotension (systolic arterial pressure < 90 mmHg, mean arterial pressure < 60 mmHg or a reduction in systolic blood pressure of >

40 mmHg from baseline) or the need for vasopressors, despite adequate fluid resuscitation, the term 'septic shock' applies (Bone *et al.*, 1992, Levy *et al*., 2003). The development of hypotension is mediated in part by the enhanced formation of NO by iNOS, which leads to excessive vasodilatation resulting in a reduced response of the vasculature to vasopressor therapy with catecholamines (vascular hyporeactivity) (Julou-Schaeffer *et al*., 1990; Thiemermann & Vane, 1990; Szabo *et al*., 1993; Hollengberg *et al.*, 2000) Progression of this continuum from sepsis to severe sepsis and septic shock is associated with increased morbidity and mortality, whereby mortality rates are seen to rise from 25-30% for severe sepsis up to 40-70% for septic shock (Lever & Mackenzie, 2007).

Table 1.1 Defining criteria of ACCP/SCCM named conditions

Criterion	**Value**
SIRS	Core body temperature >38 ^{0}C or < 36 ^{0}C; Heart rate >90 beats per minute; Respiratory rate >20 breaths per minute or $PaCO_2$ <32 mm Hg; White blood cell count >12 x 10^9 cells/L or <4 x 10^9 cells/L, or 10 % immature bands
Sepsis	At least two SIRS criteria caused by known or suspected infection
Severe sepsis	Sepsis with acute organ dysfunction (including hypoperfusion and hypotension) caused by sepsis
Septic shock	Sepsis with persistent or refractory hypotension or tissue hypoperfusion despite adequate fluid resuscitation

ACCP: American College of Chest Physicians; SCCM: Society of Critical Care Medicine (Bone *et al*., 1992).

1.1.2. Pathophysiology

There has recently been a shift in the spectrum of responsible microorganisms from predominantly Gram-negative bacteria in the late 1970s, to a higher incidence of Gram-positive bacteria at present, with the odd cases of sepsis caused by viruses, fungi, and to a lesser extent, parasites (Riedemann *et al.*, 2003). A 2009 data review of more than 11,000 patients from 37 countries demonstrated i) the cause of the infections was

Gram-negative in 57 %, Gram-positive in 44 % and fungal in 11 %; ii) the primary source of infection was 47 % in the lung, 23 % in the abdomen or 8 % in the urinary tract and iii) a significant proportion of patients presented with co-morbidities including 24 % with diabetes and 14 % with congestive heart failure (Martin *et al*., 2009). Sepsis develops when what is otherwise an expected and appropriate inflammatory response to an infectious process becomes amplified and gradually dysregulated. A continuum exists from a low grade systemic inflammatory response associated with a self-limited infection to a marked systemic response with solitary or multi-organ dysfunction (Cinel & Opal, 2009).

Bacterial recognition and signalling are dynamically modulated during sepsis, serving as essential functions of cells of the innate immune system (Salomão *et al.*, 2008). Several bacterial components such as lipopolysaccharide (LPS) of Gram-negative bacteria and peptidoglycan (PepG) primarily of Gram-positive bacteria induce septic shock in animals. The initiation of the host response during sepsis involves a family of pattern recognition receptors which, recognise unique cellular motifs found on pathogens, referred to as pathogen-associated molecular patterns (Janeway & Medzhitov, 1998).

Host recognition of microbial components leading to the initiation of the septic response is highly dependent on the presence of LPS-binding protein (LBP) and the opsonic receptor cluster of differentiation 14 (CD14) (Wright *et al*., 1990). This receptor which can be found in two forms: a membrane bound form (mCD14) or one that is free in the circulation soluble CD14 (sCD14) not only is involved in the activation of monocytes by LPS and PepG (Pugin *et al.*, 1994), but it also mediates macrophage apoptosis (Devitt *et al*., 1998) and is involved in the shuttling of LPS between serum proteins such as LBP (Yu *et al*., 1997). The importance of CD14 in sepsis is highlighted by two findings i) serum levels of CD14 rise in sepsis (Landmann *et al*., 1995) and ii) administration of an anti-CD14 antibody conferred protection in primates against lethal endotoxin shock (Leturcq *et al*, 1996).

LPS is shed from the bacterial cell wall and binds to CD14 however, initiation of cellular activation cannot take place without the association of the LPS/CD14 complex with a family of transmembrane receptors known as toll-like receptors (TLR's) (Cohen, 2002). Thus, TLR4 is known to recognise LPS, whilst TLR2 is predominantly responsible for the recognition of Gram-positive cell-wall structure (Takeuchi *et al.*, 1999). Intracellular signalling relies on binding of the intracellular TLR domain, TIR (Toll/IL-1

receptor homology domain), to IRAK (IL-1 receptor-associated kinase), a process facilitated by two adapter proteins, MyD88 (myeloid differentiation protein 88) and TIRAP (TIR domain-containing adapter protein) (Doyle & O'Neill, 2006). An effector response is achieved through one of two pathways; an MyD88-dependent pathway, which relies on the use of tumour necrosis factor (TNF)-α receptor associated factor (TRAF)-6, or an MyD88-independent pathway, which employs the use of the inducing interferon- (TRIF) adaptor protein (Doyle & O'Neill, 2006).

As a result of the host-microbial interaction, there is activation of the innate immune response with a purpose to orchestrate a defensive response incorporating both the humoral and cellular components of the immune system (Cohen, 2002). The transcriptional regulatory factor NF-κB is a central player in modulating the expression of various immunoregulatory mediators (e.g. IL-1β, TNF-α and nitric oxide synthase) that are involved in cellular responses to infection and participate in inflammatory reactions leading to organ dysfunction and death in patients with sepsis (Hiscott *et al*., 1993; Udalova *et al*., 1998; Abraham, 2000; Tak & Firestein, 2001; Abraham, 2003). Signalling pathways initiated by engagement of TLR's by bacterial products or cytokine receptors lead to nuclear accumulation of NF-κB and enhanced transcription of the above genes (Tak & Firestein, 2001; Zhang & Ghosh, 2001; Senftleben *et al*., 2002). Cytokines such as IL-1 and TNF- α that are under the regulatory control of NF-κB can induce further activation of this transcriptional factor, leading to potentiation of inflammatory responses in the septic host (Abraham, 2003). Exposure of animals to LPS or bacteria results in the activation of NF- κB in various organs and cell populations (Blackwell *et al*., 2000; Carlsen *et al*., 2002). Similarly, NF- κB activation is enhanced in patients with sepsis (Böhrer *et al*., 1997; Arnalich *et al*., 2000) and sepsis-induced acute lung injury (Schwartz *et al*., 1996; Moine *et al*., 2000). Of note, the degree of activation of this transcriptional factor was a better predictor of outcome than physiological based scores in patients with sepsis (Böhrer *et al*., 1997) with a similar pattern reported for pulmonary and circulating levels of IL-1β and TNF-α showing persistent elevation correlating with a worse clinical outcome (Meduri *et al.*, 1995).

During the first 30-90 min post exposure to LPS, mononuclear cells release the prototypic inflammatory cytokines such as TNF-α and interleukin 1β (IL-1β) which, mediate various immunopathological features of LPS (Dinarello, 1997 Consequently, a secondary level of inflammatory response is activated, involving many lipid mediators,

reactive oxygen species (ROS), cytokines, and up-regulation of cell adhesion molecules which, facilitate the migration of inflammatory cells, namely leukocytes (Cohen, 2002). The up-regulation of adhesion molecules both on neutrophils and endothelial cells sees extravasation of neutrophils into the surrounding tissue which, not only aides in the killing of microorganisms but also leads to injury of the endothelium by the release of mediators which increase vascular permeability with the eventual flow of protein-rich oedema fluid into tissues (Russell, 2006). In addition, the activation of endothelial cells results in the release of nitric oxide (NO), a potent vasodilator and key mediator of septic shock (Russell, 2006). Inducible nitric oxide (iNOS) a cytokine-induced enzyme involved in immune defence (Nathan & Hibbs, 1991), is found in macrophages (Stuehr *et al*., 1991), and various other cells (Scott-Burden *et al*., 1992; Charles *et al*., 1993) is known to produce large amounts of NO over a long period of time (Cho *et al.,* 1992). This enzyme is normally not active under normal conditions but the activation of macrophages and various other cells with proinflammatory cytokines or endotoxin leads to expression of iNOS (Thiemermann, 1997).Induction of iNOS by endotoxin in vitro and in vivo is a secondary process to the release of pro-inflammatory cytokines such as IL-1 and TNF-α, which alone, in concert with other cytokines, or with platelet-activating factor activate cells to express iNOS protein and activity (Thiemermann, 1997). The enhanced formation of NO by iNOS in the vasculature has been implicated in the pathophysiology of septic shock, as evidenced by the fact serum levels of NO oxidative products are elevated in animals (Cunha *et al*., 1994) and humans (Barthlen *et al*., 1994; Goode *et al*., 1995) undergoing septic shock. Circulatory shock is associated with an enhanced formation of NO because of the early activation of eNOS and the delayed induction of iNOS activity in various cell types such as macrophages and cardiac myocytes (Szabo *et al*., 1993). Studies demonstrating inhibition of NO synthesis reduces the hypotension induced by LPS (Thiemermann & Vane, 1990), whilst mice lacking iNOS are resistant to endotoxin-induced mortality (Wei *et al.,* 1995) and impairment of vascular contraction (Gunnett *et al.,* 1998). Likewise, in large animal models, selective iNOS inhibitors attenuated the organ dysfunction resulting from endotoxaemia (Matejovic *et al.,* 2001) or bacterial sepsis (Matejovic *et al*., 2004; Enkhbaatar *et al*., 2006; Matejovic *et al*., 2007). Furthermore, the adaptive immune system becomes activated involving the activation of B-cells. Invading microorganisms become opsonised and are presented to natural killer (NK cells). If the infection is cleared a resolution phase ensues. However, if the infection

persists, amplification of the inflammatory response can be detrimental to the host often leading to organ dysfunction (Russell, 2006).

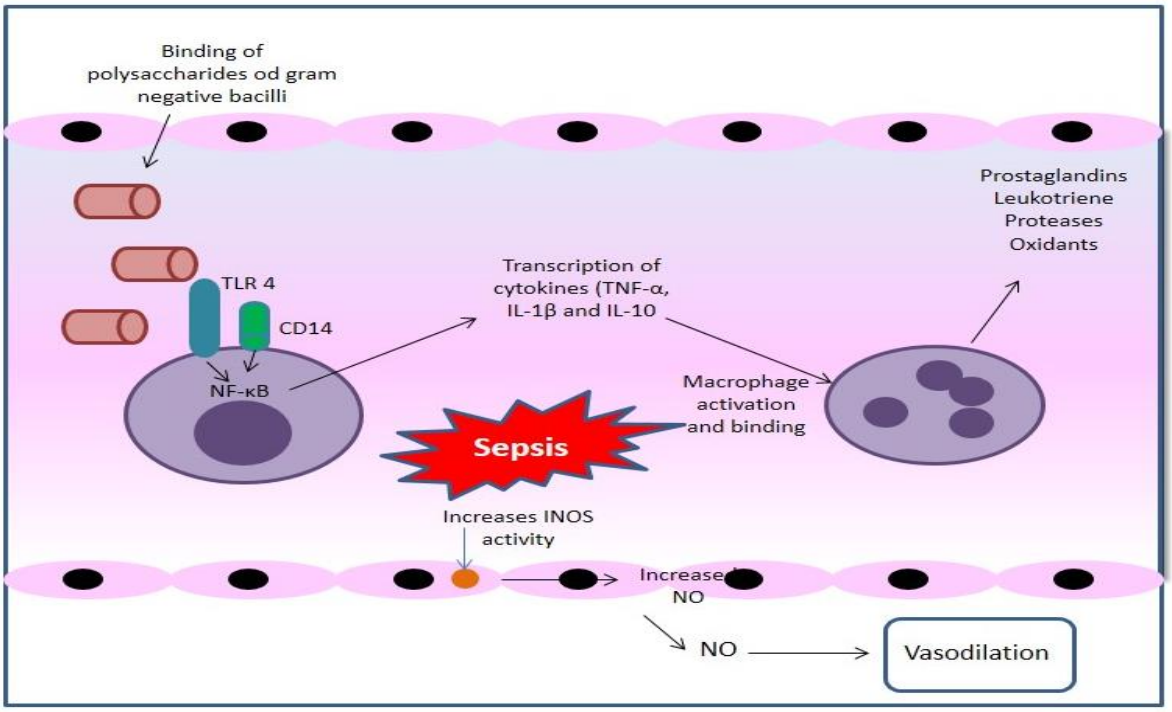


Figure 1.1.1 The orchestration of the inflammatory response to sepsis. Binding of LPS to TLR4 and PepG to TLR2 activate intracellular signal-transduction pathways which leads to the activation and translocation of cytosolic NF-κB to the nucleus resulting in the initiation of gene transcription of various pro-inflammatory cytokines such as (TNF-α and IL-1β) and anti-inflammatory cytokines such as (IL-10). Release of proteases, oxidants and prostaglandins secondary to activation of neutrophils, macrophages and monocytes injure the endothelium resulting in increased permeability and vasodilation. Activation of endothelial cells by the action of cytokines induces the production of NO, a potent vasodilator, by inducible nitric oxide synthase (Russell, 2006).

1.1.3. MOF

MOF is defined as 'the presence of altered organ functions in an acutely ill patient such that homeostasis cannot be maintained without intervention (Bone *et al.*, 1992). Septic shock is the main cause of MOF in the ICU, and the severity of MOF is correlated directly to mortality (Blanco *et al.*, 2008). It has been shown to be the main cause of death

in patients with severe sepsis, accounting 43.1% of patients (Vincent *et al*., 2011). Patients that survive the initial septic insult may ultimately succumb to widespread organ dysfunction that can either be acute, due to hyper-inflammatory response, or more prolonged due to immune dysfunction and infection (Sauaia *et al*., 1996: Baue, 2006).

The pathogenesis of organ dysfunction is multifactorial, comprising of endothelial dysfunction, tissue hypoperfusion, and/or mitochondrial dysfunction and excessive inflammation. It is evident that the persistent microvascular alteration in sepsis is associated with the development of organ dysfunction and death (Chierego *et al.,* 2006), with the endothelium recognised as central in the pathogenesis of microvascular dysfunction and sepsis physiopathology (Aird, 2003). The association between endothelial cell signalling activation and the subsequent severity of organ dysfunction was highlighted by a recent study demonstrating a strong correlation between circulating levels of biomarkers of endothelial activation [e.g. soluble E-selectin, soluble fms-like tyrosine kinase-1 (sFlt-1)] and sepsis severity (Shapiro *et al*., 2010). sFlt-1 which, contributes to endothelial cell activation and correlates with inflammatory cascade activation (Shapiro *et al*., 2008), exhibited the strongest association in patients with the Sequential Organ Failure Assessment (SOFA) score, a measure of quantifying the severity of patient illness based on the degree of organ dysfunction (Vincent *et al*., 1996). In addition, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), an endothelial cell membrane protein that recognises endotoxin and pathogenic microorganisms (Chen *et al*., 2002), mediates endothelial dysfunction, induces superoxide production and increases endothelial adhesiveness to leukocytes and chemokine production. Inhibition of LOX-1 reduced leukocyte adherence in intestinal submucosal venules of endotoxaemic rats (Landsberger *et al*., 2010).

Furthermore, tissue hypo-perfusion and hypoxia are thought to be central factors to the pathogenesis of organ dysfunction (Cohen, 2002). There is however, an ongoing debate as to the relative contributions of microvascular dysfunction and bioenergetic derangements leading to the development of MOF. The mechanisms which are thought to impair blood flow and, thus, result in hypo-perfusion include: i) widespread fibrin deposition as a result of an IL-6-induced up-regulation of tissue factor in conjunction with a TNF-α-induced suppression of natural anticoagulants, leading to microvascular occlusion, ii) development of tissue exudates compromising adequate oxygenation, and

iii) disorders of microvascular homeostasis resulting from elaboration of vasoactive substances such as histamine and prostanoids (Cohen, 2002).

In addition to hypoxia, cells may be dysoxic in that they are unable to properly utilise available oxygen. Whilst Ince and Sinaasappel suggest the sepsis-associated microcirculatory distress leading to tissue hypoxia is associated with microvascular flow redistribution (Ince & Sinaasappel, 1999), others have shown increased tissue oxygen tension in organs of animals (Rosser *et al*., 1995) and patients (Boekstegers *et al.*, 1991), suggesting that maybe the defect in sepsis lies in the cellular oxygen uptake (tissue dysoxia) rather than in oxygen delivery. Indeed, recently Brealey and Colleagues demonstrated an association between NO overproduction, antioxidant depletion, mitochondrial dysfunction and reduced concentrations of adenosine 5' triphosphate, correlate with organ failure and eventual outcome. In addition, to tissue hypo-perfusion/microvascular flow redistribution, this observation highlights that bioenergetics failure could be a central mechanism underlying multi-organ dysfunction (Brealey *et al*., 2002).

Indeed microvascular shunting can result in regional areas of tissue hypoxia and thus reduced generation of adenosine triphosphate (Spronk *et al*., 2004), whilst reduction in mitochondrial respiration can be attributable to a variety of factors such as i) decreased expression of mitochondrial proteins and ii) direct inhibition/damage from reactive nitrogen and oxygen species, despite adequate oxygenation (cellular dysoxia) (Singer *et al*., 2004). Increased venous oxygen saturations seen in resuscitated sepsis could be explained by a combined effect of shunting and reduced mitochondrial utilisation, whilst a maintained/elevated tissue oxygen tension that have been demonstrated in various organ beds (Rosser *et al.*, 1995; VanderMeer *et al*., 1995) may be a result of a reduction in metabolic activity to match/exceed the reduction in energy supply related to a decreased oxygen delivery or mitochondrial dysfunction (Abraham & Singer, 2007).

1.1.4. Cardiac Dysfunction

Myocardial dysfunction is a recognized manifestation of sepsis and septic shock, with myocardial depression occurring in almost 40 % - 50 % of patients (Fernandes & Assuncao, 2012; Rudiger & Singer, 2007). Patients with myocardial dysfunction have significantly higher mortality (70%) compared to 20 % in septic patients without

cardiovascular impairment (Parillo & Parker, 1990). Contractile dysfunction is characterised by a reversible reduction in ejection fraction, biventricular dilatation, blunted blood pressure response to intravenous fluids, and a diminished ability to augment cardiac output despite increased levels of circulating catecholamines (Hoesel *et al*., 2007). Cardiovascular dysfunction has over the years gained platform to becoming a prevalent problem in the management of critically ill patients, associated with MOF morbidity and mortality (Muller-Werdan *et al*., 2006).

What are the characteristics of septic myocardial dysfunction? Clinically, before the introduction of the pulmonary arterial catheter, two distinct clinical profiles of septic shock were described (MacLean *et al*., 1967), one characterised by warm, dry skin and a bounding pulse despite hypotension ('warm' shock) and the other associated with hypotension, clammy skin and low volume pulse ('cold' shock) (Rabuel & Mebazaa, 2006). Subsequently, it was concluded that patients in septic shock went through an initial early hyperdynamic phase following onset of illness and ultimately either recovered or deteriorated in heart failure or myocardial depression related to sepsis, leading to hypodynamic shock and death (Clowes *et al*., 1966). The latter phase was further confirmed by animals studies demonstrating septic shock characterised by reduced cardiac output (CO) and high systemic vascular resistance (SVR), resulting in animal death (Weil *et al*., 1956; Postel & Schloerb, 1977). However, a later study by Wilson and colleagues were the first to provide a description of septic shock in humans as associated primarily with normal or elevated CO (with elevated SVR) and very rarely with low CO (Packman & Rackow 1983). With the recognition of inadequate volume resuscitation of patients with septic shock, subsequent studies employing the use of pulmonary artery catheter consistently demonstrated that adequately volume-resuscitated patients typically exhibit a hyperdynamic circulatory state associated with a high CO, reduced SVR, normal stroke volume and high heart rate (Wilson *et al*., 1965; Weisel *et al*., 1977; Winslow *et al*., 1973; Krausz *et al*., 1977; Parker *et al*., 1987; Ahmed *et al*., 1991), which also consistent with non-survivors (Parker *et al.,* 1987).

Evidence of myocardial depression in septic shock was demonstrated by Calvin and colleagues who were first to show myocardial dysfunction in septic patients adequately resuscitated, characterised by a decreased ejection fraction (EF) and an increased end diastolic volume index (Calvin *et al*., 1981). Extending this observation, Parker and colleagues demonstrated that i) septic survivors showed an increased end-

diastolic volume index with reduced EF, which was not seen in non-survivors as they exhibited normal cardiac volumes, and ii) the acute changes in end-diastolic volume index and EF were reversible even after persisting for several days (Parker *et al*., 1984). Since then, a number of studies have confirmed the development of cardiac dysfunction, ranging from isolated cardiomyocytes (Ren *et al.,* 2002), to isolated hearts (McDonough *et al*., 1998; Merx *et al*., 2004), to *in vivo* animal models (Natanson *et al*., 1986; Stahl *et al*., 1990; Merx *et al*., 2005), and in human subjects (Jafri *et al*., 1990; *et al*., 1998; Poelaert *et al*., 1997). All of these studies support the view that decreased contractility and impaired myocardial compliance are causative factors in sepsis-induced myocardial dysfunction.

Furthermore, myocardial depression has also been explored with respect to its prognostic value. Parker and colleagues upon reviewing septic patients on initial presentation and at 24 h, found that a heart rate (HR) of < 106 beats per min (bpm) predicted a favourable outcome (Parker *et al.,* 1987). Presenting at 24 h with a systemic vascular resistance index of > 1529 dyne. s^{-1}. cm^{-5}. m^{-2}, a HR of < 95 bpm, and a cardiac index of > 0.5 L. min^{-1}. m^{-2} was also associated with survival. In addition, common biomarkers of cardiovascular disease, troponin I and T have also been evaluated as prognostic markers of sepsis: Troponin levels correlate positively with left ventricular dysfunction (Fernandes *et al*., 1999; ver Elst *et al.,* 2000; Mehta *et al.* 2004), with intensity of vasopressor therapy (Turner *et al*., 1999), duration of hypotension (Arlati *et al*., 2000) and severity of sepsis, all predicting a poor short-term outcome (Amman *et al.,* 2001; Mehta *et al*., 2004). More recently, Rudiger and colleagues investigated using a long-term rat model of faecal peritonitis, whether depressed myocardial function could be detected early in sepsis. They demonstrated i) significant differences in stroke volume and heart rate assessed 6 h after insult could predict a 3-day mortality with positive and negative predictive values of 93 % and 80 %, respectively and ii) presence of intrinsic myocardial depression with poor-prognosis animals tolerating less fluid compared to good-prognosis or sham animals (Rudiger *et al.,* 2013). The predicted mortality was found to be associated with significant differences in transcripts of genes expressing proteins related to TLR2/MyD88 and JAK/STAT inflammatory pathways.

1.1.4.1 Aetiology of cardiac dysfunction

A number of mediators have been implicated in myocardial depression in sepsis (Figure 1.1.2). These include mitochondrial dysfunction, inflammatory signalling, and alterations in cellular homeostasis. One of the early notions based on animal studies of cardiac depression was that myocardial dysfunction resulted from ischaemia, a result of an inadequate coronary blood supply. However, this was later disputed by two studies evaluating coronary haemodynamics in patients with septic shock. Patients were found to have similar or greater coronary flow when compared to control subjects (Cunnion *et al*., 1986; Dhainaut *et al.,* 1987). These findings disregarded ischaemia as a key factor in sepsis-induced cardiac dysfunction.

Various sepsis studies have highlighted the importance of mitochondrial dysfunction (Gallerich *et al.,* 2002; Crouser, 2004), linking it to severity and outcome (Brealey *et al.,* 2002). Mitochondria comprise approximately 30 % of the myocardial volume (Kayar *et al*., 1987). Cardiomyocyte mitochondrial ultrastructural damage has been shown in both animal (Schumer *et al.,* 1971; Watts *et al*., 2004; Suliman *et al*., 2004) and clinical studies of sepsis (Cowley *et al*., 1979; Soriano *et al*., 2006). Disturbed mitochondrial respiratory function characterised by reduced oxygen consumption is often seen in the late stages of sepsis (Schumer *et al*., 1971). Reduced activities of mitochondrial electron transport chain enzyme complexes are reported in septic hearts *in vivo*, resulting in part, from inhibitory effects of reactive oxygen and nitrogen species on the ATP production and oxidative phosphorylation (Trumbeckaite *et al*., 2001; Levy *et al*., 2004; Suliman *et al*., 2004). Furthermore, the mitochondrial permeability transition pore (mPTP) has been shown to play a role in the development of mitochondrial dysfunction since its inhibition has been shown to improve cardiac function *ex vivo* and reduce mortality in septic mice (Larche *et al*., 2006).

The existence of a myocardial depressant factor (MDF) dates back as early as 1947, as seen in an experimental model of haemorrhagic shock (Wiggers *et al.,* 1947) to 1970's, using blood of dogs subjected to endotoxic shock (Lefer & Martin, 1970) and lastly to 1979 modelled using coronary ostia of dogs infused with plasma of septic patients (McConn *et al.,* 1979). Many substances have been proposed as the MDF, including TNF-α and IL-1β. Indeed, these cytokines constitute key mediators of myocardial depression in sepsis (Fernandes Jr & Cesar de Assuncao, 2012). Recently,

TNF-α was demonstrated to trigger cardiac apoptosis in an LPS model of endotoxaemia, where TNF-α-dependent caspase activation was induced thereby, contributing to the development of cardiac dysfunction (Carleson *et al*., 2005). Caspase activation is a crucial step which leads to apoptotic cell death, a process which has been implicated in the pathophysiology of human cardiovascular diseases such as ischaemic heart disease (reviewed in Haunstetter & Izumo, 1998). Likewise various investigators have shown the part played by caspase in role of apoptosis in the cardiac dysfunction in sepsis, whereby inhibition of caspase with the broad spectrum benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.fmk) ameliorated the endotoxin-induced cardiac apoptosis and contractile dysfunction (Fauvel *et al*., 2001; Nevière *et al*., 2001; Carelson *et al*., 2005). Furthermore, not only do TNF-α and IL-1β individually exert cardio depressant effects, but they can also synergistically depress contractile function, an effect which could be reversed by inhibition of NO synthase (Cain *et al*., 1999). Additionally, further evidence of the involvement of NO in the TNF-α-mediated myocardial depression effects were seen whereby TNF-α inhibited contraction of isolated hamster papillary muscles, which was reversed following the addition of L-NG-monomethyl-L-arginine, a non-selective inhibitor of NO synthase (Finkel *et al.*, 1992). These data strongly implicate myocardial NO synthase in the negative inotropic effects of cytokines such as TNF-α and IL-1β.

Moreover, over the last 15 years, the effect of NO as a potential mediator of myocardial depression in sepsis is becoming increasingly established. The impact of NO on cardiac function is multifaceted, as it has an effect on systemic vascular tone, thus impacting preload, afterload, and coronary vascular tone (Fernandes & Assuncao, 2012). In septic shock, the increased production in NO via the action of inducible NO synthase (iNOS) is an important contributor to circulatory collapse and possibly organ/dysfunction and host defence (Thiemermann & Vane, 1990). Ullrich and colleagues demonstrated that iNOS (NOS2) is required for the development of systolic and diastolic dysfunction in mice challenged with endotoxin (Ullrich *et al*., 2000). Infusion of methylene blue, a non-specific NOS inhibitor, to septic patients prevented the stroke volume, left ventricular stroke work from falling and reduced the need for inotropic support (Kirov *et al*., 2001). However, these effects did not lead to improvements in patient outcome. Other studies in animals (Meyer *et al*., 1994) and human subjects (Hussein *et al*., 1999; Bakker *et al*., 2004; Watson *et al*., 2004) have explored reversing the hypotension associated with septic

shock using analogues of L-arginine such as monomethylarginine and nitroarginine methyl ester as inhibitors of NOS. Although these studies demonstrate an increase in arterial pressure following NOS inhibition, some animal studies have indicated increased mortality (Young, 2004). Following the phase II clinical studies (Bakker *et al*., 2004; Watson *et al*., 2004) testing the safety and efficacy of 546C88 (NGmethyl- L-arginine hydrochloride; NOS inhibitor) in patients with septic shock, a phase III study was undertaken to extend the study of safety and efficacy of this inhibitor, with the primary efficacy objective being survival at 28 days (Lopez *et al*., 2004). However, this trial was stopped due to an increase in mortality observed in the treatment group. Increases in pulmonary hypertension, cardiac failure, and reduced cardiac output was seen in these patients (Lopez et al., 2004). A possible reason for the disparity between the above studies was attributed to the use of a higher dose of 546C88, whilst a post-hoc analysis suggested that low doses of this drug (i.e. $\leq$ 5 mg kg^{-1} hr-1)may have provided an overall survival benefit (Lopez *et al*., 2004). This could be explained in part by the fact that widespread blockade of NO synthesis could lead to excessive vasoconstriction and a fall in cardiac output due to the inhibition of not only excessive NO production (from iNOS) but also basal production from endothelial or neuronal NOS (constitutive NOS) (Young, 2004). Therefore, a more targeted approach to inhibition of nitric oxide synthesis may be warranted in the future.

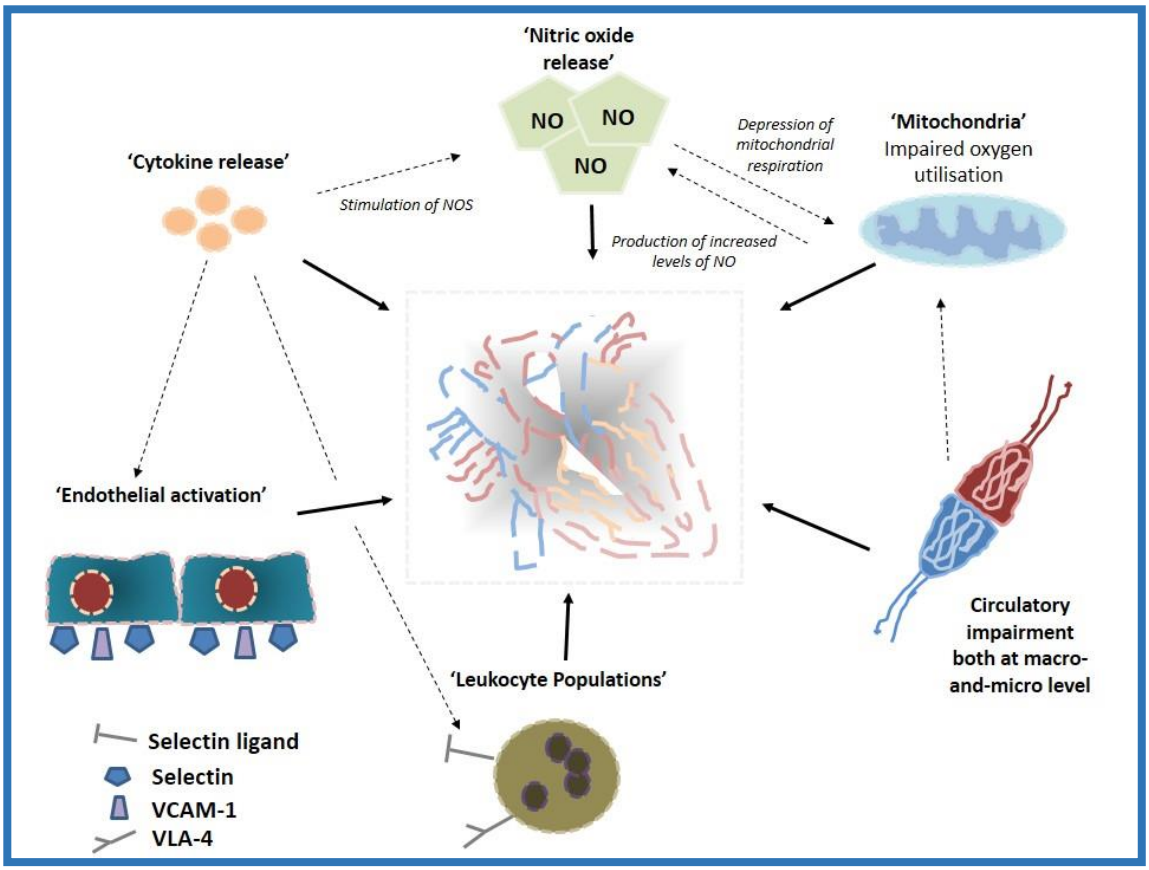


Figure 1.1.2. Illustration showing how some of the potential underlying mechanisms contribute to myocardial dysfunction and also effect on one another. Cytokines such as tumour necrosis factor α (TNF- α) an early mediator of endotoxin-induced shock is derived from various cells including cardiac myocytes in response to sepsis. It leads to release of interleukin 1 β (IL-1β), which depresses cardiac contractility via stimulation of nitric oxide synthase (NOS). High levels of nitric oxide (NO) contributes to cardiac dysfunction through reactive oxygen species (ROS) generation. Circulating myocardial depressant substances (MDF) such as IL-1β found in increased concentrations in sepsis also exerts cardiodepressive effects. Furthermore, mitochondria produce increased levels of NO and ROS, which lead to inhibition of oxidative phosphorylation and decreased production of adenosine triphosphate (ATP), a process leading to 'cytopathic hypoxia'. Endothelial cells undergo structural as well as functional changes 'activation', which result in increased up-regulation and thus expression of cell adhesion molecules such as selectins, immunoglobulins (vascular cell adhesion molecule 1; VCAM-1). This subsequently leads to leukocyte adhesion, followed by traversing of endothelium and finally migration extravascularly. *VLA-4* alpha4beta1 integrin (very late antigen-4). Picture redrawn from Merx, 2007.

1.1.5. Current treatments

Over the years there has been considerable progress of our understanding of both pathophysiology of sepsis and of the fundamental principles governing pathogen-host interactions. Indeed, many studies have reported novel interventions, which appear to improve outcome in animal models of sepsis. These findings fuelled the hope that these discoveries may ultimately result in a reduction in mortality in septic patients. Over the past three decades, the general consensus regarding the current management of sepsis has mainly focused on supportive measures, consisting of the administration of broad-spectrum antibiotics, haemodynamic resuscitation and appropriate support of organ function (Osuchowski *et al*., 2009).

Early goal directed therapy (EGDT) with targeted fluid resuscitation in conjunction with measures of oxygen delivery has been shown to improve survival in patients presenting with septic shock in a randomised, controlled, single-centre study (Rivers *et al*., 2001). This protocol was shown to be effective when it was initiated during the first 6 h in the emergency department, an effect seen to reduce 28-day mortality rate (Rivers *et al.*, 2001).

In addition, if the patient demonstrates lack of response to resuscitation or if there is evidence of volume overload, the stimulation of the depressed cardiovascular system can be achieved by means of vasopressor therapy. The recommended first choice agent for septic shock is norepinephrine, which results in potent peripheral arterial vasoconstriction without significant increases in heart rate or cardiac output (Dellinger *et al*., 2008; Dellinger *et al*., 2013). Clinical trials have shown that when compared with dopamine, norepinephrine was associated with improvement in in-hospital and 28-day mortality in septic patients (Vasu *et al*., 2011) and a decreased risk of death and lower incidence of arrhythmic events (De Backer *et al.*, 2012). However, it is suggested that dopamine should be used only in highly selected patients, such as when there is low risk of tachyarryhthmias or bradycardia (Dellinger *et al*., 2013). Furthermore, second-line agents for patients who have persistent hypotension despite maximal doses of norepinephrine/dopamine include epinephrine and vasopressin (Dellinger *et al.*, 2013). Vasopressin has been proposed for use in septic shock due to it being an endogenous peptide with potent vasoactive effects in addition to its depressed circulating levels in septic shock (Landry *et al*., 1997). Low doses of vasopressin may be effective in raising blood pressure in patients, refractory to other vasopressors and may exhibit other potential

physiologic benefits such as improvement in renal function (Holmes *et al*., 2001; Patel *et al*., 2002; Lauzier *et al*., 2006). However, according to the 2012 Surviving Sepsis Campaign guidelines, since high doses of vasopressin have been associated with cardiac, digital, and splanchnic ischaemia, it should not be the single initial vasopressor but should be reserved for salvage therapy, when failure to achieve an adequate MAP with other vasopressors is not possible (Dünser *et al*., 2003; Dellinger *et al*., 2013). Following first-line treatment, 0.03 U/min of vasopressin may be added to norepinephrine with an anticipated effect equivalent to that of norepinephrine alone (Dellinger et al., 2008; Dellinger *et al.*, 2013).The Vasopressin and Septic Shock Trial (VASST) demonstrated that although vasopressin administration reduced the requirement for norepinephrine, mortality rates were not significantly lower in patients receiving vasopressin in addition to norepinephrine when compared to patients receiving norepinephrine alone (Russell *et al*., 2008).

Moreover, in the case of inotropic support for septic patients, dobutamine has been recommended as the first choice inotrope for patients with measured or suspected reduced cardiac output, in the presence of adequate left ventricular filling pressure (or clinical assessment of sufficient fluid resuscitation) and adequate MAP (Dellinger *et al*., 2013). A dose of up to 20 $\mu g\ kg^{-1}\ min^{-1}$ is recommended by the 2012 Surviving Sepsis Campaign, in the presence of myocardial dysfunction or persistent hypoperfusion despite adequate fluid resuscitation and adequate MAP (Dellinger *et al.*, 2013). In addition, in patients who continue to be hypotensive following fluid resuscitation who may exhibit low, normal or increased cardiac outputs, treatment with a combined inotrope/vasopressor such as norepinephrine is suggested (Dellinger *et al*., 2013). Inotropic therapy is not recommended for increasing cardiac index to supranormal levels (Dellinger *et al.*, 2008; Dellinger *et al*., 2013), since a study showed increased mortality rates in patients with sepsis who were maintained on high levels of oxygen delivery (Hayes *et al*., 1994).

The use of corticosteroids in patients with sepsis is controversial: Many studies have demonstrated that high-dose corticosteroid therapy is not effective in patients with severe sepsis or septic shock (Sprung *et al*., 1984; Bone *et al.*, 1987; Cronin *et al.*, 1995), while others suggest that corticosteroid therapy is associated improvement in the shock state and a survival benefit in patients with vasopressor-unresponsive septic shock (Bollaert *et al*., 1998; Briegel *et al.*, 1999; Annane *et al*., 2002). It should be noted, that a

benefit with corticosteroid therapy was associated with a greater risk of nosocomial infection and recurrent sepsis (Sprung *et al*., 2008).

Furthermore, many studies have focused on targeting inflammatory and coagulation pathways, which have shown promising results in animal studies, but have either failed to be translated into a clinical setting or were associated with significant adverse effects. For instance, recombinant human activated protein C (APC), which plays a role in regulating blood clotting and inflammation, was shown to reduce absolute mortality by 6.1 % and relative mortality by 19.4 % in a randomized study of 1690 patients (Ely *et al.,* 2003). In contrast, a later analysis of six randomised clinical trials including 6307 adults and 474 paediatric patients with either high or low risk of death found no evidence that APC reduced the risk of death in adults or children with severe sepsis or septic shock, and instead was associated with increased risk of bleeding (Marti-Cavajal *et al*., 2012). The more recent PROWESS-SHOCK trial evaluated the effects of dotrecogin alfa (Xigris) in 1697 septic shock patients and showed no beneficial effect on the 28-day mortality, which resulted in the subsequent removal of this product from the market (Ranieri *et al*., 2012).

Thus, there is still an unmet clinical need for the discovery of specific therapeutic interventions for septic shock.

1.2. Myocardial Infarction

Despite major advances in prevention and treatment, ischaemic heart disease remains the leading cause of morbidity and mortality in the Western world, and is rapidly gaining its leading position in the developing world (Bolli *et al*., 2004). Restoration of early blood flow to the ischaemic myocardium with thrombolytic therapy is presently the most effective therapy to limit infarct size and, thus, preserve cardiac function and geometry following myocardial infarction (MI) (Fibrinolytic therapy trialists, 1994; Cannon *et al*., 1994). However, reperfusion alone is inadequate to salvage the damaged myocardium, as complications resulting from loss of viable myocardium are still common post MI. For instance, reperfusion of previously ischaemic tissue may result in 'myocardial ischemia/reperfusion (I/R) injury', whereby reperfusion itself leads to additional cell death and increase in infarct size (Braunwald & Kloner, 1985). Various interventions aimed at reducing myocardial I/R injury, although proven successful in experimental studies, have failed to translate into clinical settings (Yellon & Hausenloy, 2007). One reason for this is translational failure is that many of these interventions are given prior to or during the ischaemic insult in animals, while this strategy cannot be applied clinically (Bolli *et al*., 2004; Arslan *et al*., 2008). One other reason is that animal studies are carried out in young, healthy male animals without common underlying diseases that so often are found in patients with MI (Byrne *et al*., 2012). Thus, there continues to be a growing need to uncover novel translatable interventions that could lessen the global impact of this disease on society.

1.2.1. Ischaemic Injury

Myocardial ischaemic injury results from severe impairment of the coronary artery blood supply that is inadequate to meet the myocardial oxygen requirements, usually as a result of thrombosis or other acute alterations of coronary atherosclerotic plaques. (Buja *et al*., 2005). During the early stages of ischaemic injury, the loss of oxygen leads to the cessation of mitochondrial oxidative phosphorylation and, thus, depletion in adenosine triphosphate (ATP) production for energy metabolism. To compensate there is an increase in anaerobic glycolysis for ATP production which, results in the accumulation of hydrogen ions (H^+) and lactate, and consequently intracellular acidosis and inhibition of glycolysis as well as mitochondrial fatty acid and residual

energy metabolism. Alterations in ion transport in the sarcolemma and organelle membranes contribute to the impaired contraction despite the persistent electrical activity resulting in ventricular arrhythmias (Buja *et al*., 2005).

Progression to an advanced stage of cardiomyocyte injury is characterised by an altered metabolic milieu and constant increase in cytosolic calcium ion (Ca^{2+}) which, results in phospholipase activation and phospholipid degradation with release of lysophospholipids and free fatty acids, all of which, culminate in impairment of cell membrane function (Buja *et al*., 2005). Furthermore, changes including i) generation of toxic oxygen species and free radicals-induced peroxidative damage to fatty acids of membrane phospholipids by ischaemic myocytes as well as ischaemic endothelial cells and activated leukocytes and ii) cleavage of cytoskeletal filaments by activated proteases, collectively lead to progressive increase in membrane permeability, severe derangements of intracellular electrolytes and ATP exhaustion (Buja *et al*., 2005).

1.2.2. Reperfusion Injury

Whilst clinically early and successful myocardial reperfusion is the most effective strategy for reducing the size of a myocardial infarct and thus improving clinical outcome, the process of restoring blood flow to the ischaemic myocardium can itself induce injury (Yellon & Hausenloy, 2007), with some animal studies demonstrating its accounts up to 50 % of the final size of an MI (Simonis *et al*., 2012). This paradox termed myocardial reperfusion injury, which was first postulated in 1960 by Jennings and colleagues paradoxically reduces the beneficial effects of myocardial reperfusion (Jennings *et al*., 1960).

The injury to the myocardium during reperfusion can be characterised by four types of cardiac dysfunction: i) 'myocardial stunning' which describes the mechanical dysfunction persisting after the reperfusion despite the absence of irreversible damage and restoration of normal or near normal coronary flow (Braunwald & Kloner, 1982), ii) 'no-reflow phenomenon', is defined as the inability to reperfuse a previously ischaemic region (Krug *et al*., 1966) due to impedance encountered by the microvascular blood flow (Ito *et al*., 2006), iii) 'reperfusion arrythmias' which, although can be potentially harmful, can be controlled with effective interventions (Manning *et al*., 1984) and iv) 'lethal reperfusion injury', is thought to be an independent mediator of cardiomyocyte death, distinct from ischemic injury (Yellon & Hausenloy, 2007).

Lethal reperfusion injury as a separate entity has been a matter of debate. Some investigators suggest that reperfusion can exacerbate the necrotic component of cell death following a fixed period of ischaemia, as evidenced by an extension in infarct size (Matsumura *et al*., 1998; Zhao *et al*., 2000a). Other investigators on the other hand, demonstrate that the sudden metabolic changes and the existence of oxidative stress accompanying reperfusion can itself initiate injury irrespective of ischaemia (Hearse, 1977; Vanden Hoek *et al.,* 1996). Nevertheless, a means of validating the existence of lethal reperfusion injury has been demonstrated by the ability to modify myocyte death by interventions administered at the time of reperfusion.

The reperfusion phase sees the ischaemic myocardium become subjected to various rapid biochemical and metabolic changes, which augment the changes generated during the ischaemic period. These changes include the generation of ROS, rapid restoration of physiological pH and intracellular Ca^{2+} overload and immune activation, all of which, converge to mediate cardiomyocyte death through the opening of the mPTP. ROS generation through xanthine oxidase and the reduced nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate oxidase system, mediates myocardial injury by damaging the cell membrane by lipid peroxidation, acting as a neutrophil chemoattractant and mediating dysfunction of the sarcoplasmic reticulum, contributing to intracellular Ca^{2+} overload (Yellon & Hausenloy, 2007).

Disruption in the normal mechanism regulating Ca^{2+} in the cardiomyocyte sees an abrupt increase in intracellular Ca^{2+} which, is secondary to sarcolemmal-membrane damage and oxidative stress-induced dysfunction of the sarcoplasmic reticulum, a phenomenon termed the calcium paradox (Piper *et al*., 1998). Restoration of perfusion results in the transport of H^+ in exchange with Na^+, to normalize the pH. Increased intracellular levels of Na^+ activates the sarcolemmal Na^+/Ca^{2+} exchanger which, leads to Ca^{2+} overload in the cell with subsequent hypercontracture of cardiomyocyte and mitochondrial permeability transition pore opening, eventually resulting in death (Yellon & Hausenloy, 2007).

Furthermore, several hours into reperfusion sees the influx of neutrophils into the infarcted myocardium in response to the release of chemoattractants. Subsequently, up-regulation of cell adhesion molecules such as integrin beta-2 and intracellular adhesion molecule 1 facilitates the migration of neutrophils into myocardial tissue where they

cause vascular plugging, release of degradative enzymes and generation of ROS, leading to cardiomyocyte death (Vinten-Johansen, 2004).

1.2.3. Cardiac Cell Death

It is a recognised fact that cell death following I/R occurs via both necrosis and apoptosis **(**Kajstura *et al*., 1996; Gill *et al*., 2002; Chiong *et al*., 2011). Necrosis is a degenerative process, characterised by loss in cellular integrity and release of cytosolic contents which provokes an inflammatory response (Hamacher-Brady *et al*., 2006). It is found to predominate within the ischaemic myocardium (Hamacher-Brady et al., 2006) and is fundamental to I/R injury and oxidative damage (Vaseva *et al*., 2012). In contrast, the energy-dependent mechanism, apoptosis, (Gill *et al*., 2002) is characterised by a pattern of nuclear pyknosis, cytoplasmic condensation and cell shrinkage, without provoking an inflammatory response (Buja *et al*., 1993), and is known to dominate in the ischaemic border and non-ischaemic regions (Hamacher-Brady et al., 2006).

Although studies performed on both animal models and tissue from human subjects implicate both apoptosis and necrosis in myocardial I/R (Gottlieb *et al*., 1994; Kajstura *et al*., 1996; Zhao *et al*., 2000b; Freude *et al*., 2000), the exact contribution of these two forms of cell death in the setting of I/R injury is unclear, as are the factors that determine which death pathway is initiated (Hausenloy & Yellon, 2004). However, taking all experimental evidence into account (Gottlieb *et al*., 1994; Freude *et al*., 2000; Zhao *et al*., 2000b) and the fact that ATP the main driver of apoptosis is depleted during ischaemia, one can conclude that the reperfusion phase either triggers or accelerates the apoptotic component of cell death. Furthermore, the relationship between apoptosis and necrosis is also unclear, with some suggestions that there may be overlap in terms of early signalling events between the two pathways, as shown by various investigators (Hausenloy & Yellon, 2004). A study by Zhao and colleagues demonstrate that both necrosis and apoptosis occur simultaneously during reperfusion, with necrotic cell death peaking after 24 h post-reperfusion and apoptotic cell death increasing up to 72 h of reperfusion (2001).Others have shown that pharmacological inhibition of apoptotic signalling during reperfusion can attenuate both the apoptotic and necrotic components of cell death (Yaoita *et al*., 1998; Holly *et al*., 1999; Mocanu *et al*., 2000; Zhao *et al*., 2003), which suggest that the apoptotic process can evolve into necrotic cell death. suggest that targeting the reperfusion-induced apoptotic component of cell death can

impact on both process of cell death leading to not only a reduction in infarct size but also an improvement in contractile function (Zhao *et al*., 2003)

1.2.3.1. Mitochondrial Mediated Apoptosis

Apoptosis is mediated by two pathways; the intrinsic pathway, primarily mediated by the mitochondria, and the extrinsic pathway which involves activation of death receptors (Whelan *et al*., 2010). The intrinsic pathway begins with the permeabilisation of the mitochondrial outer membrane which is thought to either be, mPTP dependent, or independent (Green & Kroemer, 2004).

Signals transmitted to the mitochondria lead to the release of pro-apoptotic proteins such as cytochrome c from its intermembrane space to the cytosol which in conjunction with other proteins forms a complex known as an apoptosome (Rodriguez & Lazebnik, 1999). This complex promotes the activation of caspase 9, which in turn activates effector caspases that collectively orchestrate the execution of apoptosis. In contrast, the pore independent pathway is regulated by the B-cell lymphoma (Bcl)-2 family which is sub-categorised into anti-apoptotic members such as Bcl-2 and B-cell lymphoma-extra-large and pro-apoptotic components (Green & Kroemer, 2004). The actions of the pro-apoptotic components activate apoptosis via two ways; i) disruption of function of anti-apoptotic Bcl-2 family members (Letai *et al*., 2005), or ii) formation of channels in the outer mitochondrial membrane to aide in the release of apoptogenic proteins from the intermembranal space (Korsmeyer *et al*., 2000).

1.2.3.2. Death Receptor Pathway of Apoptosis

The receptor-mediated pathway is initiated by the binding of a death ligand (CD95/Fas ligand or TNF-α) to its cognate cell surface death receptor (CD95/Fas or TNFα receptor 1) (Ashkenazi *et al.,* 1998). Subsequently, death adapter molecules such as Fas-associated death domain (FADD) and TNF receptor-associated death domain form homotrimers which become recruited to the cytoplasmic tail of the death receptor, through interactions between death domains found present in both proteins (Thorburn *et al.,* 2004). Consequently, procaspase 8 is recruited by FADD to form the death inducing signalling complex. Once activated, caspase 8 precipitates the apoptotic cascade via

processing of downstream effector caspases such as caspase 3 (Hamacher-Brady *et al*., 2006), leading to the death of the cell.

Studies of experimental models of myocardial infarction have demonstrated an enhanced expression of Fas ligand (Kajstura *et al.*, 1996) possibly due to an increased sensitivity of myocytes to Fas-mediated death following I/R injury (Rasper *et al*., 1998). Indeed, under normal conditions Fas is tightly regulated by its inhibitors namely cFLIP which, interacts with FADD to antagonise apoptosis (Irmler et al., 1997). However, this inhibitory protein otherwise abundantly expressed in the heart, is downgraded dramatically following I/R injury (Rasper *et al.*, 1998), rendering cardiomyocytes susceptible to death via the Fas-pathway (Gill *et al*., 2002).

1.2.4. Pro-survival Signalling Pathways

Pharmacological activation of endogenous pro-survival kinase cascades, during the first few minutes of reperfusion, following a lethal ischaemic insult, has been hypothesized to attenuate reperfusion-induced cell death (Yellon & Baxter, 1999). Since it has been identified that apoptosis contributes to myocyte death following reperfusion injury (Gottlieb *et al*., 1994; Freude *et al*., 2000), and that activation of pro-survival kinases protects against apoptotic injury, the ability to manipulate/up-regulate the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways, during early reperfusion may provide a potential approach to limiting reperfusion-induced cell death.

1.2.4.1. The Reperfusion Injury Salvage Kinase Pathway

The RISK pathway refers to a group of protein kinases namely PI3K-Akt and p42/p44 extra-cellular signal regulated kinases (ERK 1/2) that, when specifically activated during the early stages of reperfusion, confer cardioprotection by preventing lethal reperfusion injury (Hausenloy & Yellon, 2004).

A conserved family of signal transduction enzymes, the PI3Ks and their downstream target Akt are known to regulate inflammatory response, cellular proliferation and survival (Cantley, 2002). Akt, exerts its effect via activation of two subsets of downstream substrates: i) the anti-apoptotic substrates such as eNOS (Bell & Yellon, 2003), which, when phosphorylated, are activated stimulating cellular processes

essential for increased survival and ii) the pro-apoptotic substrates such as GSK-3β (Nishihara *et al*., 2006), which, when phosphorylated, become inactive (Franke *et al*., 2003). Additionally, a member of the mitogen-activated protein kinases (MAPKs), ERK 1/2 (p42/p44) is associated with regulation of cell proliferation, differentiation and survival (Widmann *et al.,* 1999). Activation of ERK 1/2 has been shown to mediate cellular protection in settings myocardial I/R (Shimizu *et al*., 1998; Yue *et al*., 2000).

There is extensive preclinical evidence that activation of the RISK pathway by various pharmacological agents such as hydrogen sulphide *ex vivo* (Hu *et al*., 2008), and insulin-like growth factor-1 *in vitro* (Matsui *et al*., 1999) and *in vivo* (Fujio *et al*., 2000) as well as by mechanical interventions such as pre (Li *et al*., 2011) -and post-conditioning (Zhu *et al*., 2006) reduces the myocardial infarct size by up to 50 %. This protection has been demonstrated to be in part due to inhibition of the mPTP (Davidson *et al*., 2006), improved uptake of Ca^{2+} into the sarcoplasmic reticulum (Abdallah *et al*., 2006), and the recruitment of anti-apoptotic pathways (Yellon & Baxter, 1999).

The pharmacological activation of the RISK pathway has been demonstrated to induce cardioprotection through the phosphorylation of a variety of downstream effectors such as Bcl-2-associated promoter (BAD) (Jonassen *et al*., 2001), eNOS (Bell & Yellon, 2003) and GSK-3β (Gross *et al*., 2004). Many of these protective downstream signalling pathways appear to converge on the mitochondria, acting in concert to inhibit the mPTP: i) phosphorylation and inactivation of pro-apoptotic factors such as BAD which otherwise exert their apoptotic effects via opening of the mPTP, ii) phosphorylation and activation of eNOS is expected to inhibit mPTP opening via the release of NO (Brookes *et al*., 2000) and iii) phosphorylation and inhibition of GSK-3β is shown to mediate inhibition of mPTP (Juhaszova *et al*., 2004). Additionally, activation of the ERK 1/2 component of the RISK pathway is shown to form complexes with mitochondrial PKCε (Baines *et al* 2002) which confers cardioprotection through inhibition of mPTP opening (Baines *et al*., 2003).

1.2.4.2. The Survivor Activating Factor Enhancement Pathway

Over the years TNF-α has been recognised to play a vital role in the innate immune system's response to various forms of stress, such as infection (Huang *et al*., 2009; Roman-Campos *et al*., 2009; Lula *et al.,* 2009; Ba *et al*., 2010), trauma and I/R (Thielmann *et al*., 2002; Reil *et al*., 2007; Skychally *et al*., 2007; Heusch *et al*., 2007). However, emerging evidence paradoxically support the notion that i) exogenous TNF-α

could confer cardioprotection against an I/R insult in a time- and dose-dependent manner (Lecour *et al*., 2002; Lecour *et al*., 2005), and ii) activation of this multifunctional cytokine contributes to myocardial adaptation (Valeur *et al.*, 2009) and is required for protection by ischaemic pre-(Deuchar *et al*., 2007) and post-conditioning (Lacerda *et al.*, 2009).

Therefore, how does TNF-α exhibit these differing roles? TNF-α levels in blood and tissue are increased in acute MI with it being implicated as a mediator of adverse remodelling in heart failure (HF) (Mann, 2003). Indeed, mice lacking TNF-α are protected against myocardial I/R injury (Maekewa *et al*., 2002), whilst addition of TNF-α antibodies results in a reduced infarct size in both rats (Gurevitch *et al*., 1997) and rabbits (Belosjorow *et al*., 2003). However, attempts to neutralise TNF-α in patients with HF has led to a worsening effect (Mann, 2002). Likewise, Kurrelmeyer showed mice deficient in both TNF-α receptors developed larger myocardial infarcts, compared with their littermate controls following acute MI (2000). These apparent contradictory findings can depend on which receptor is activated, both of which are found in human and rat cardiac myocytes (Krown *et al*., 1995; Torre-Amione *et al*., 1995). In fact, myocardial TNF-α and TNF receptor activation can be said to exhibit an ambivalent role in myocardial I/R injury and protection from it (Figure 1.2.1). Where excessive expression of TNF-α and subsequent cardiomyocyte TNF receptor type 1 (TNFR1) activation induces contractile dysfunction, hypertrophy, fibrosis and cell death and mediates pro-inflammatory responses. Whilst, a lower TNF-α concentration and subsequent cardiomyocyte TNF receptor 2 (TNFR2) activation mediates signals that promote tissue repair and angiogenesis and confers protection (Luo *et al*., 2006; reviewed in Kleinbongard *et al*., 2011). Additionally, besides TNF-α concentration (Lecour *et al*., 2002; Deuchar *et al*., 2007) and receptor subtype activation, the duration of its exposure and localisation also contributes to the myocardial action of TNF-α (Kleinbongard *et al*., 2011).

Now it has become clear that TNF-α initiates the activation of an alternative and 'RISK free' pathway termed SAFE (Lecour, 2009) which, involves the activation of Janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT-3) (Lecour *et al*., 2005a) (Figure 1.2.2). Briefly, following activation of the membrane receptor (TNFR2) by a ligand (e.g.TNF-α) two adjacent juxtaposed JAK's are transphosphorylated activating STAT proteins (Boengler *et al*., 2008). The subsequent

phosphorylation of STAT proteins results in its dissociation from its receptor and translocation into the nucleus, whereby it binds to specific DNA sequences, regulating the expression of target genes involved in various processes such as inflammation and apoptosis (Boengler *et al.*, 2008). Considering STAT-3 is a transcription factor, its cardioprotective effects do not seem to ensue at a transcriptional level, since the timeframe between its activation and its action is too short. Therefore, it is possible that STAT-3 elicits additional effects via direct phosphorylation of different components (Lecour, 2009). Several targets of STAT-3 implicated in conferring its cardioprotective effects have been identified; i) phosphorylation and inactivation of pro-apoptotic factor Bad (Lecour *et al*., 2005b), ii) phosphorylation and subsequent inactivation of GSK-3β (Gross *et al*., 2006) and iii) induction of mitochondrial ROS (Lacerda et al., 2006). Within the cardiomyocyte, the mitochondria represent the most relevant site for ROS formation (Dröge, 2002; Turrens, 2003; Balaban *et al*., 2005; Murphy, 2009), where generation of free radicals is perceived to be essential to achieve protection by TNF- α (Lecour 2005). It is also perceived to act as a trigger to ischaemic preconditioning (Baines *et al*., 1997; Tritto *et al*., 1997) through the activation of protein kinase C (PKC) (Baines *et al*., 1997). The study by Lacerda demonstrates that TNF- α initiates sequential mitochondrial perturbations mediated by the production of ROS which originates mainly from the mitochondria, in C_2C_{12} murine skeletal muscle cells. Free radical scavengers inhibited the protective effect of TNF-α on cell viability, their respiratory parameters and their inner mitochondrial membrane potential (2006).

It could be said that like TNF-α, ROS exhibits paradoxical effects, which is more dependent its variable concentration. Mitochondrial DNA is highly susceptible to increased oxidative stress damage, whereby excess oxidative stress besides contributing to irreversible myocardial injury also contributes to reversible contractile dysfunction (‘stunning’) and further inducing long-lasting mPTP opening, resulting in cellular dysfunction and cell death (Bolli, 1990; Guth *et al*., 1993; reviewed in Perrelli *et al*., 2011).On the other hand, low levels of ROS is perceived to play a role in modulating cardioprotective signalling pathways such as PKC through redox signalling and also as secondary messengers (reviewed in Perrelli *et al*., 2011; Penna *et al*., 2006; Philipp *et al*., 2006; Zatta *et al*., 2006; Cohen *et al*., 2008). In fact, ROS formation is seen to result following activation of mitochondrial adenosine triphosphate-dependent potassium (K_{ATP}) channel activation, secondary to activation of PKC (Lecour *et al*., 2002; Lecour *et*

al., 2005). Furthermore, limited ROS production not only inhibits mPTP opening but a transient or short duration opening of mPTP has been suggested to induce a slight transient formation of ROS and various investigators have demonstrated to be relevant for cardioprotection (Bernardi & Petronilli, 1996, Ichas *et al*., 1997; Petronilli *et al.,* 1999; Di Lisa & Ziegler, 2001; Petronilli *et al.,* 2001; Lim *et al*., 2007; Hausenloy *et al*., 2009). In addition, the involvement of redox signalling by transient/reduced formation of ROS could also be another mechanism by which ROS is involved in cardioprotection in this setting of SAFE pathway activation. Studies show that ROS scavengers such as N-acetylcysteine prevents the protective effects of ischaemia or pharmacological post conditioning (Penna *et al*., 2006; Penna *et al*., 2007; Tsutsumi *et al*., 2007; Penna *et al*., 2009; Yao *et al*., 2010). Likewise, the IPC-induced cardioprotective effects are completely abolished when the heart is pre-perfused with N-acetyl cysteine to scavenge ROS, which suggests that redox signalling plays a crucial role in generating survival during myocardial adaptation to ischaemia (reviewed in Das & Maulik, 2003).Furthermore, activation of the RISK or SAFE pathways do not necessarily occur independent of one another, as some studies demonstrate that in fact they can occur concomitantly, in settings of ischaemic preconditioning (Suleman *et al*., 2008), insulin, and opioid-induced cardioprotection (Fuglesteg *et al*., 2008; Gross *et al*., 2008). Such interactions have been attributed to i) p58 regulatory subunit of PI3K may control serine phosphorylation of STAT-3 (Pfeffer *et al*., 1997) and ii) JAK can enhance the phosphorylation of Akt subsequent to binding to the p58 regulatory subunit of PI3K (Zhang *et al.,* 2005).

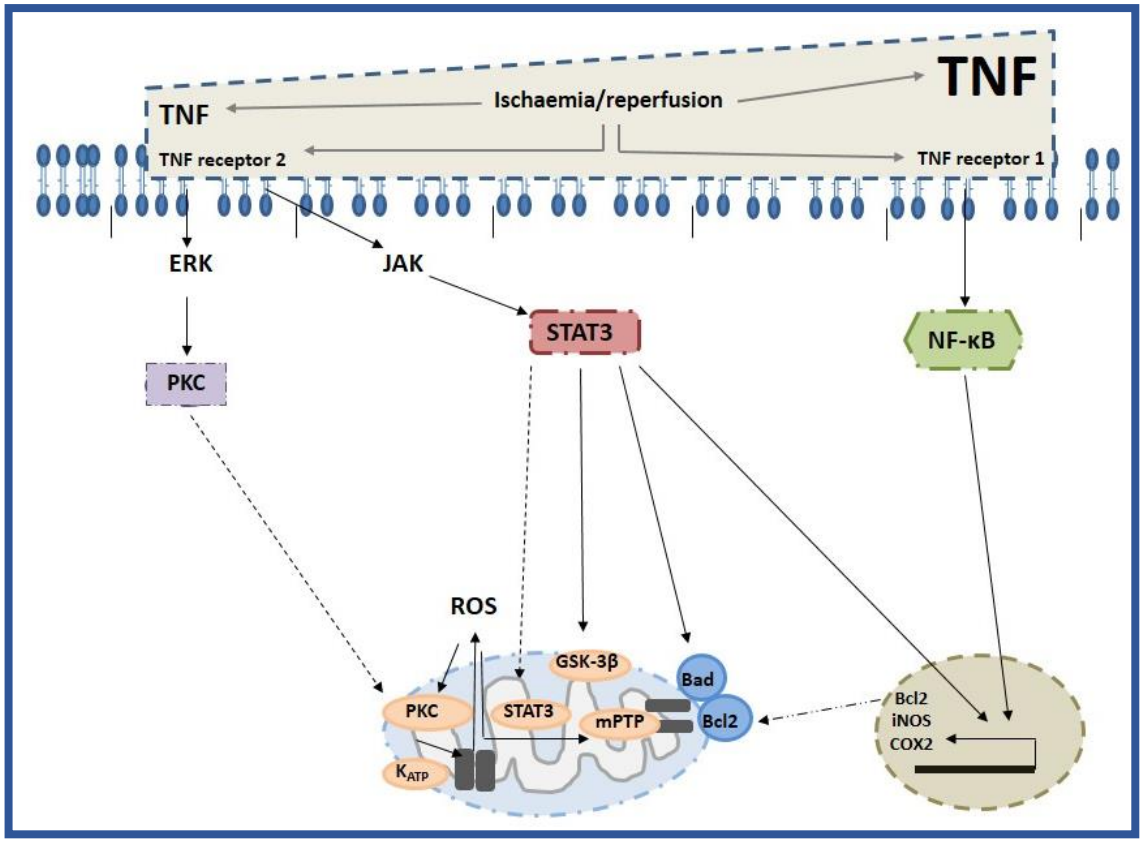


Figure 1.2.1. The protective vs. damaging effects of TNF-α in myocardial ischaemia reperfusion (I/R). Depiction of the differential concentrations of tumour necrosis factor α (TNF-α) along with the subsequently activation of receptor subtype (TNFR1 and TNFR2). Activation of TNFR2 initiates various signalling cascades, which eventually lead to inhibition of mitochondrial permeability transition pore (mPTP) opening. The expression of the anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2) is increased, similarly, the phosphorylation and thus inactivation of the pro-apoptotic factor Bcl-2-associated death promoter (Bad) is increased by TNF-α. Additionally, some of these signalling cascades is also shared with TNFR1, which can induce increased activation of anti-apoptotic proteins via nuclear factor kappa B (NF-κB). Dotted lines depict protein translations. *COX2* cyclooxygenase 2, *ERK* extracellular signal-regulated kinase, *GSK-3β* glycogen kinase synthase 3 β, *iNOS* inducible nitric oxide synthase, JAK Janus kinase, K_{ATP} adenosine triphosphate-dependent potassium channels, PKC protein kinase C, *ROS* reactive oxygen species, *STAT3* signal transducer and activator of transcription. Redrawn from Kleinbongard *et al*., 2011.

1.2.5. Mitochondrial Permeability Transition Pore

Under physiological conditions, the mitochondria provide ATP through oxidative phosphorylation, with its inner membrane acting as a selective barrier to metabolites and ions (Halestrap *et al*., 2004). The mitochondria possess an innate mechanism, that is mediated by a non-specific pore, namely the mPTP which upon activation i.e. under cellular stress opens (Crompton *et al*., 1999), converting the mitochondria from an organelle that supports the life of the cell to that which actively induces both necrotic and apoptotic cell death (Halestrap *et al*., 2004).

It comes as no surprise that since the mitochondrion plays a fundamental role in the apoptotic death machinery of the cell (Kroemer, 1998) that many of the anti-apoptotic pathways which are activated by pro-survival kinase cascades converge here. It is the role of the mPTP, a non-specific large conductance pore, of the inner mitochondrial membrane that is suggested to be a critical determinant of lethal reperfusion injury (Hunter & Hayworth, 1979; Heusch *et al*., 2010). During ischaemia, the mPTP remains closed, only to open during the first few minutes of reperfusion (Griffiths & Halestrap, 1995), in response to oxidative stress, mitochondrial Ca^{2+} overload and restoration of physiological pH (Griffiths & Halestrap, 1995; Kim *et al*., 2006). Furthermore, the extent of mitochondrial permeability determines the fate of the cell, thus either i) the cell recovers if the mitochondrial permeability is minimal, ii) moderate effect leads to activation of programmed cell death, apoptosis, and iii) if severe, due to the inadequate energy production, die via necrosis (Honda & Ping, 2006). As the mPTP is known to be a critical determinant of lethal reperfusion injury (Crompton *et al*., 1999) , many investigators have demonstrated its pharmacological inhibition at the time of reperfusionto be cardioprotective (Griffiths & Halestrap, 1993; Hausenloy *et al*., 2002; Clarke *et al*., 2002). Indeed, it's been shown that inhibition of mPTP via activation of pro-survival pathways (as mentioned above) is through modulation of several kinases which converge on the mitochondria (Davidson *et al*., 2006).

1.2.6. Ischaemic Preconditioning

A means of protecting the myocardium before occurrence of an acute coronary artery occlusion, is to precondition the myocardium with brief periods of ischaemia. This cardioprotective phenomenon, termed ischaemic preconditioning (IPC), was first discovered by Murry and colleagues by which, brief non-lethal ischaemic episodes (5 to

10 min), followed by periods of reperfusion, confers protection against a subsequent prolonged period of myocardial ischemia (index ischaemia) (Murry *et al.*, 1986). IPC offers two windows of protection in time; 'early' and 'late' preconditioning (Kuzuya *et al*., 1993). Early protection also known as 'classical IPC' lasts for approximately 1 to 2 h following the initial preconditioning (Baxter & Yellon, 1994) and is shown to reduce the infarct size by 75 % (Murry *et al*., 1986). Late protection also termed 'delayed IPC' reappears from approximately 24 to 72 h after the initial preconditioning stimulus (Yellon & Baxter, 1995) and is thought not to be as powerful as the early preconditioning effect, reducing the infarct size by approximately 50 % (Kuzuya *et al.*, 1993).

The amount and duration of the ischaemic phase during IPC influences not only the amount of protection conferred on the myocardium but also the signalling pathways involved, as too many repetitive stimuli might abolish preconditioning (Barbosa *et al*, 1996; Liem *et al*., 2001). Indeed, the duration of the ischaemic phase during IPC as well as the period of reperfusion demonstrate rigid time frames within which protection of the myocardium is seen (Riksen *et al*., 2004). Thus, for periods of ischaemia one cycle of 1.25 min (Barbosa et al, 1996) to five 5-min ischaemia/5-min reperfusion cycles confers protection to the myocardium (Piot *et al.*, 1997). On the other hand, for the duration of reperfusion, the minimum amount to confer protection is usually around 30 sec and 1 min (Alkhulaifi *et al*., 1993), whilst anything more than 1 to 2 h abolishes the infarct-limiting effect (Li *et al*., 1992; Burckhartt *et al*., 1995).

During the brief preconditioning periods of ischaemia and reperfusion, trigger substances are released such as bradykinin, adenosine and opioids (Riksen *et al*., 2004), which in turn activate specific membrane-bound receptors. As a consequence, several complex intracellular signalling cascades notably isoforms of protein kinase C (PKC) (Ping *et al.*, 1997 Liu *et al*., 2001; Meldrum *et al*., 1997) and tyrosine kinases (Ping *et al.*, 1999a) are activated, which converge on mitochondrial end-effectors, including the ATP sensitive potassium channel and the mPTP (Riksen *et al*., 2004). Indeed, the RISK pathway has been identified to be an important target for cardioprotection during IPC (Tong *et al*., 2000; Oldenberg *et al.*, 2004). Studies have shown PKC activation is achieved via activation of PI3K, which in turn activates the serine/threonine kinase Akt. The resulting effect is inactivation of the pro-apoptotic GSK-3β (Riksen *et al*., 2004).

As for ERK 1/2, its involvement in the setting of IPC is still unclear, with some studies showing ERK1/2 phosphorylation following an IPC stimulus (Fryer *et al* 2001;

Mocanu *et al* 2002), and in one instance showing this phosphorylation leading to protection (Fryer et *al* 2001). Furthermore, activation of ERK 1/2 mediates early preconditioning in parallel (Vahlhaus *et al*., 1998) or downstream of PKC (Ping *et al*., 1999b). It is interesting to note that the same kinase cascades are involved in mediating cardioprotection at the time of reperfusion and following an IPC trigger. Strong evidence suggests that the various upstream signalling pathways converge on mitochondrial proteins aimed at limiting in particular the reperfusion injury (Riksen *et al*., 2004). It is possible that the kinase cascades may constitute a common pathway of cardioprotection for both instances. Evidence suggests this might in actual fact be the case; i) agents that possess preconditioning effects such as bradykinin (Strickler *et al*., 1996; Baxter & Ebrahim, 2002) also exert protection when given upon reperfusion (Budde *et al*., 2000; Bell & Yellon, 2003) and ii) agents such as insulin that are cardioprotective when given upon reperfusion have also been demonstrated to precondition the myocardium (Baines *et al.,* 1999; Gordon *et al*., 2003).

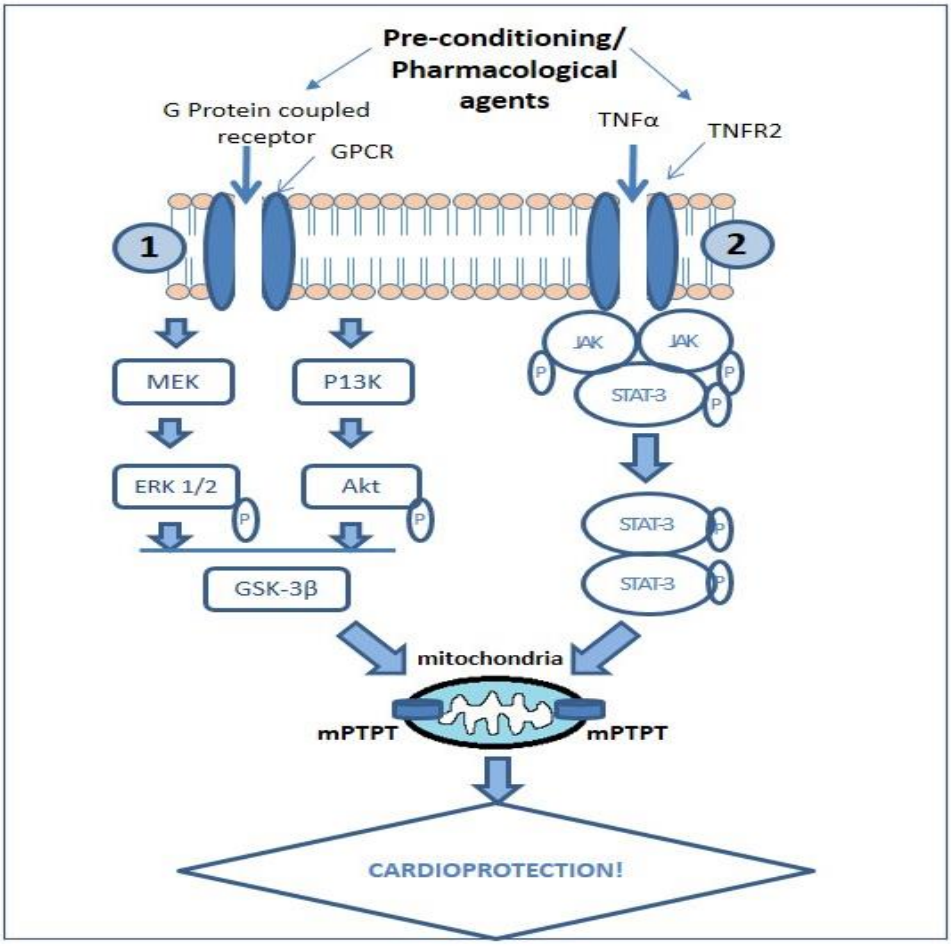


Fig 1.2.2. Activation of survival pathways. Ischaemic preconditioning or administration of pharmacological agents at reperfusion can activate either the RISK pathway (1) or the SAFE pathway (2), to confer protection. Modified from Lacerda *et al.*, 2009

1.3. Erythropoietin

Erythropoietin (EPO) is an acidic glycoprotein of approximately 34 kDa, that is primarily produced by the peritubular fibroblasts in the renal cortex (Wen *et al.,* 1994). EPO's role is to regulate hematocrit, by acting directly on red blood cell progenitors and precursors in the bone marrow, in order to control their proliferation, differentiation and maturation. As a hormone, EPO circulates at concentrations in the low picomolar range (1-7 pmol L^{-1}), where plasma concentrations are maintained by a negative feedback loop in response to hypoxia, via stabilisation of hypoxia-inducible factor-1-alpha. This subsequently leads to the production and release of EPO into the circulation by the kidney (Jelkmann, 2007). Once EPO is in the circulation, it targets the hematopietic stem cells in the bone marrow to stimulate differentiation along the erythroid lineage by acting as a survival factor for erythroid progenitor cells.

EPO binds to a preformed homodimeric EPO receptor (EPOR), which is approximately a 60 kDa transmembrane polypeptide (Jelkmann, 2011). The affinity of this receptor for EPO is approximately 100-200 pmol L^{-1}, and is seen to exhibit peak expression at the colony forming unit-erythroid/proerythroblastic stage followed by an undetectable level at the reticulocytes (Ng *et al*., 2003). Binding of EPO to EPOR results in the homodimerisation of the receptor, followed by the activation of several signal transduction pathways, namely janus activated kinase (JAK2) and G-protein Ras. Subsequently, phospho-JAK-2 activates mutliple intacelullar pathways such as signal transducer and activator of transcription and PI3K/Akt (Figure 1.3.1). This ultimately results in the production of anti-apoptotic proteins and erythrocyte precursor survival (Jelkmann *et al*., 2008).

Recombinant human EPO (RHuEPO) has not only revolutionised the treatment of patients with anaemia of chronic renal failure (CRF), but has also been demonstrated to be effective in correcting anaemia associated with a variety of non-uraemic conditions. Patients with CRF have a deficiency in endogenous erythropoietin production. Clinical studies demonstrated that treatment with EPO corrects the anaemia of CRF, avoids blood transfusion and improves quality of life (Eschbach, 1994). In addition, anaemia is a compliaction commonly encountered in malignancy, especially those of haematological origin, either at presentation or during the course of treatment (Ng *et al*., 2003), as more than half of cancer patients exhibit low serum level of EPO (Miller *et al*., 1990). Anaemia

identified at diagnosis is a poor prognostic factor and may affect the outcome of radiotherapy treatment (Ng *et al*., 2003). Although blood tranfusion remains the mainstay of treatment for symptomatic anaemia, it comes with its own side effect (Hamasaki & Yamamoto, 2000; van Bommel *et al*., 2001; Fernandes *et al*., 2001). Therefore, treatment with RHuEPO for the correction of anaemia either as a supportive (post-cehmotherapy), preventive (pre-treatment), or maintenance (optimisation of haemaglobin while not on treatment) therapy, has been used for various types of cancers (Cazzola *et al*., 1995; Rai *et al*., 1995; Dammaco *et al*., 1998; Demetri *et al*., 1998; Antonadou *et al.*, 2001; Crawford *et al*., 2002) and has proven to have an excellent safety profile (Ng *et al*., 2003). Approximately two thirds of HIV patients have anaemia, especially those that are on treatment with Zidovudine (Ng et al., 2003). Studies have shown that treatment with RHuEPO not only corrects the anaemia but also improves the patient's quality of life and survival (Henry *et al*., 1992; Phair *et al*., 1993; Moore *et al*., 1998). Furthermore, patients in intensive care regularly require blood transfusion since they present with very low endogenous serum EPO (van Iperen *et al.*, 2000). The efficacy of EPO treatment with this subgroup of people has produced conflicting results, with an initial clinical trials demonstrating a reduction in the requirement of blood transfusions and an increase in the haemogblobin concentration (Corwin *et al*., 1999; Corwin *et al*., 2002). This was later challenged when the same group found that trauma patients treated with epoietin alfa showed a lack of reducing the incidence of red-cell transfusion, which is an unexpected finding. It is presumed that this is due to the change in transfusion practice between the studies (Corwin *et al.*, 2007). However, a decrease in mortality in the trauma patients who received epoietin alfa was observed, an effect that they speculate may be attributable to actions of EPO that are distinct to haematopoiesis.

Moreover, the detection of EPOR mRNA, EPO binding, and EPOR signalling in various non-haemtaopoietic tissues such as the heart led to various investigators utilising adminstration of exogenous EPO to confer tissue protection (Sasaki *et al*., 2000; Li *et al*., 2004; Jelkmann & Wagner, 2004) . Specifically, treatment with EPO has been shown to reduce mycardial infarct size, protect against ischaemia reperfusion and promote ventricular remodelling as well as suppress the systemic inflammatory response in experimental animals subjected to coronary ligation and exert anti-apoptotic effects on myocytes and fibroblasts in the heart (Parsa *et al*., 2003; Moon *et al.*, 2003; Narmoneva *et al*., 2004; Parsa *et al*., 2004; Nishiya *et al.*, 2006; Shen *et al*., 2010). This alongside

knowing that i) EPO activates the PI3K/Akt pathway to confer tissue protection (Condorelli, *et al*., 2002; Calvillo *et al*., 2003; Kim *et al*., 2008) and ii) EPO reduced organ damage and improved survival in a model of endotoxic shock (Aoshiba *et al*., 2009), make it a very appealing agent in investigating its potential role in protecting against the sepsis-induced cardiac dysfunction.

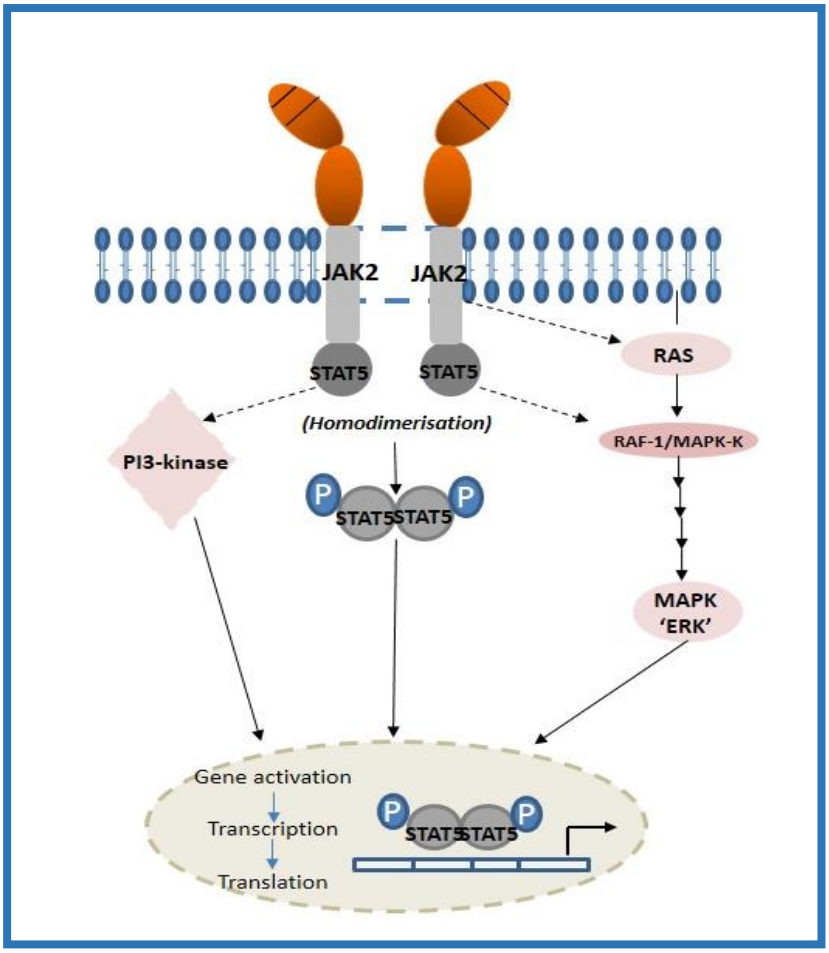


Figure 1.3.1. **Simplified view of the main signal transduction pathways activated by the erythropoietin haematopoietic receptor (EPOR).** Binding of erythropoietin to its receptor (EPOR) induces dimerisation. Janus activated kinase (JAKs) bound via their SH2 domains undergo trans-phosphorylation and subsequently phosphorylate signal transducers and activators of transcription (STATs). Activated STATs translocate to the nucleus whereby they can activate/repress target gene promoters that control proliferation and apoptosis such as the Bcl-2 gene family. In addition, additional signal transduction pathways such as PI3K/Akt and Ras/Raf can also become activated following JAK2 trans-phosphorylation which play a part in apoptosis and cellular growth, respectively. Dotted lines depicts phosphorylation process. Modified from Ng *et al*., 2003

1.3.1 Arestunate

Artesunate (ARS) is a semi-synthetic derivative of artemisinin (*qinghaosu*), the principle active component of the medicinal plant qinghao (*Artemisia annua)* which, has been used as a remedy for fevers and chills over two millennia in China (Krishna *et al*., 2004). Its anti-malarial potential was first demonstrated in 1971 when it was shown to be successful in killing *Plasmodium berghei* in mice (Qinghaosu Antimalaria Coordinating Research Group, 1979; Hien & White, 1993). However, to improve the poor solubility seen with artemisinin in oil and water, many derivatives were introduced which, today comprise a very important class of antimalarials (Figure 1.4) (Krishna *et al*., 2004). These include the water soluble ARS, considered the most clinically useful of the artemisinin class which, is metabolised to dihydroartemisinin (DHA) the active component responsible for exerting antimalarial effects due to its longer elimination half-time (~ 1 h) (Krishna *et al*., 2004).

Although the relatively short half-time for elimination exhibited by DHA confers an advantage that selection for drug-resistant parasites is less likely to occur, this may lead to a higher risk of recrudescence when the artemisinin class of drugs are used as monotherapy (Krishna *et al*., 2004). Thus, quite often this class of drugs is given as combination therapy which consists of two drugs often co-formulated with one artemisinin derivative and another antimalarial drug from a different class such as quinine, to treat uncomplicated *falciparum* malaria (FM). This being said, in endemic areas parenteral administration of ARS has been shown to be superior in antiparasitic efficacy and in lowering mortality due to severe malaria compared to quinine, showing beneficial effects especially in patients with high parasite levels (Dondorp *et al*., 2005; Dondorp *et al*., 2010; Sinclair *et al.*, 2012). Likewise, a retrospective evaluation from a UK centre highlighted the superior efficacy of ARS over quinine for the treatment of severe FM (Eder *et al*., 2012), with recent studies advocating ARS as the preferred choice of first-line treatment in any geographical area (Askling *et al*., 2012; Sinclair *et al*., 2012).

Artemisinins act more rapidly and via mechanisms that are distinct from other anti-malarial drugs, whilst exhibiting a wider range of parasite killing, from tiniest rings to more mature stages of parasites (ter Kuile *et al*., 1993; Angus *et al*., 1997). The peroxide bridge within the pharmacophore of this class of drugs is an essential component

for their anti-malarial activity since compounds lacking a peroxidic oxygen atom are devoid of activity (Krishna *et al*., 2004). Various targets have been implicated in the action of artemisinins, with independent lines of evidence from a range of experimental techniques propose that sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) of *P. falciparum* (PfATP6) might be the primary target of artemisinins (Eckstein-Ludwig *et al*., 2003; Jung *et al*., 2005; Uhlemann *et al*., 2005). Additionally, the involvement of the mitochondria in the mechanism of action of this class of drugs is recognised, with studies implicating the electron transport chain of *P. falciparum* as a potential target for artemisinins (Li *et al*., 2005), and effects on the mitochondrial inner membrane potential seen in neuronal cell cultures (Schmuck *et al*., 2002) as well as in parasites (Krungkrai *et al*., 1999).

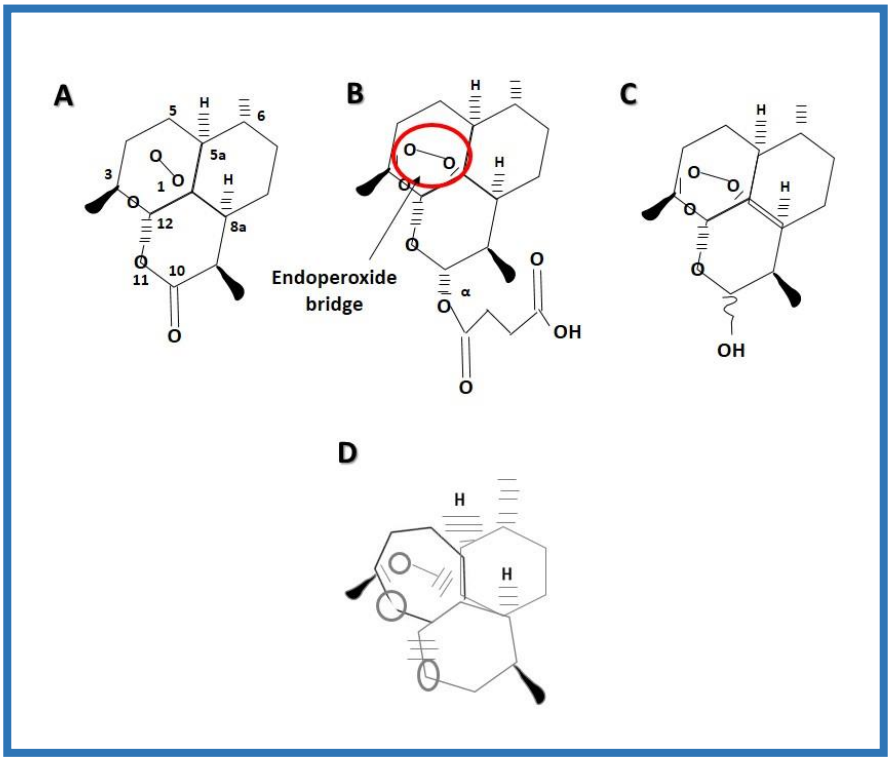


Figure 1.3.2. Chemical structures of members of the artemisnin family (A) Artemisinin and derivatives (B) artesunate (ARS), and (C) dihydroartemisinin (DHA). Artemisinins that lack the endoperoxide bridge such as 1-Carba-10-deoxoartemisinin (D) is devoid of anti-malarial activity due to the replacement of one of the peroxide oxygen atoms with carbon. Redrawn from Krishna *et al*., 2004

Besides its antimalarial effects, ARS has been shown to possess antiviral (Kaptein *et al*., 2006) and anti-inflammatory (Mirshafiey *et al.*, 2006; Xu *et al*., 2007; Cheng *et al*., 2011) to name a few. Of importance however in the context of the potential effects of ARS on the cardiovascular system, one recent study by Sun and colleagues investigating the effect of ARS in a model of I/R of the isolated rat myocardium, demonstrated for the first time that ARS alleviates the myocardial I/R injury in rats possibly via its anti-oxidising effects and scavenging of free radicals (2007). This alongside the recent discovery that ARS has been found to associate with the PI3/Akt pathway (Xu *et al*., 2007; Hou *et al*., 2008; Cheng *et al*., 2011), makes it a promising agent to be investigated further for its cardioprotective effects in a model of myocardial I/R *in vivo.*

1.3. Aims

Activation of cell signalling pathways confer tissue protection and/or inhibit inflammation in various settings of cardiovascular disease. Indeed, the involvement of the PI3K/Akt pathway in different settings of cardiac pathologies is well established. Activation of this pathway has been shown to ameliorate cardiac dysfunction and mortality during sepsis/septic shock. Likewise, activation of PI3K/Akt-dependent signalling protects cardiac myocytes against apoptosis and confers protection on the myocardium from I/R injury.

Therefore, the purpose of this thesis is to investigate whether erythropoietin and artesunate, known activators of the PI3K/Akt pathway are cardioprotective in models of cardiac injury (sepsis-induced cardiac dysfunction) and dysfunction (myocardial I/R injury), respectively. In order to answer these questions, the investigation was divided into three main studies:

Study 1: Development of a Model of Polymicrobial Sepsis (Chapter 2)

As sepsis most frequently occurs in aging patients and the model of caecal ligation and puncture (CLP) mimics various aspects of clinical sepsis, I will set up a model of CLP focusing specifically on the development of cardiac dysfunction. In order to do this I will investigate the effects of sex, strain, severity of the model (altering needle size), fluid resuscitation and antibiotic therapy, and age on the susceptibility to develop consistent organ dysfunction specifically cardiac dysfunction.

Study 2: Sepsis-Induced Cardiac Dysfunction (Chapter 3)

Our understanding of the actions of erythropoietin (EPO), a haematopoietic cytokine has shifted from a belief that the hormone acts exclusively on erythroid progenitor cells to the knowledge that this protein exerts significant protection in various tissues. 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. Protection conferred by EPO in models of doxorubicin-induced cardiotoxicity and myocardial infarction have implicated activation of the PI3K/Akt

pathway. Thus, once I develop a CLP model of cardiac dysfunction, I will investigate the effect of erythropoietin on sepsis induced cardiac dysfunction, specifically:

- the effect EPO has on the impairment of systolic contractility (measured *in vivo* by echocardiography or *ex vivo* in the isolated Langendorff-perfused heart) associated with endotoxaemia and polymicrobial sepsis using wild-type (WT) C57BL/6 mice
- whether the effect of EPO is mediated through the proposed tissue protective receptor, with the use of βcR knock-out (KO) mice
- the mechanisms underlying the observed beneficial effects of EPO including the phosphorylation of Akt on Ser^{473}, phosphorylation of glycogen synthase kinase-3β (GSK-3β) on Ser^{9}, phosphorylation of endothelial nitric oxide synthase (eNOS) on Ser^{1177}, activation of nuclear factor (NF)-κB (measured as nuclear translocation of p65) and expression of interleukin-1beta (IL-1β).

Study 3: Myocardial Ischaemia/Reperfusion Injury (Chapter 4)

The demand to discover novel therapies for treating myocardial infarction can be said to correlate with its ever increasing mortality and morbidity rates. Artesunate (ARS), an anti-malarial drug has over the past few years shown to affect various cellular biochemical processes such as cellular apoptosis and oxidative stress. Additionally, various studies have demonstrated ARS to modulate inflammatory and survival pathways.

In a model of regional I/R in the rat, I will investigate whether:

- ARS attenuates the injury associated with myocardial I/R by measuring infarct size as an end point.
- The mechanism underlying the observed beneficial effect, focusing on the activation of well-known survival pathways; i) RISK pathway (pharmacological inhibition of PI3K and ERK 1/2 signalling, phosphorylation of Akt on Ser^{473}), ii) SAFE pathway (phosphorylation of STAT3 on Tyr^{705}), and the downstream mediators of these pathway (eNOS; GSK-3β; NF-κB).

Chapter 2

Modification of a Model of Polymicrobial Sepsis – Caecal Ligation and Puncture

2.1. Introduction

Sepsis is a heterogeneous condition, which consists of a dysregulated host response to an infection, subsequently ensuing in haemodynamic, cardiovascular, metabolic, inflammatory, and innate and adaptive immune alterations (Levy *et al*., 2003; Namas *et al*., 2012). The increasing prevalence, mortality rates and lack of effective therapy underscore the need for further research and highlights the importance for understanding the fundamental pathophysiological processes involved, to aide in recognizing new therapeutic targets.

The development of experimental sepsis models to elucidate the progression and pathophysiology of clinical sepsis go back as far as the 1930's, where studies focused primarily in exploiting models of intra-abdominal sepsis with the administration of endotoxin and intravenous or peritoneal infusion of live organisms. The 1960's saw a shift in focus from models of endotoxaemia to models of bacteraemia (Deitch *et al*., 2005). These included injection of live bacteria, inoculation of faeces, and the intramuscular, intraperitoneal or subdermal implantation of faeces-containing capsules. The path of discovery led to various models involving ischaemia and bowel perforation like the most frequently used sepsis model caused by CLP (reviewed in Mai *et al*., 2012).

The CLP model of intra-abdominal sepsis was developed by Wichterman and colleagues is today regarded as the gold standard for modelling polymicrobial sepsis (Wichterman *et al*., 1980), as it closely resembles the development and features of human sepsis (Dejager *et al*., 2011). Multiple aspects of the CLP procedure address the complicated clinical course of sepsis: these include tissue trauma as a result of a laparotomy, necrosis instigated by ligation o;f the cecum, and infection as a consequence of the leakage of peritoneal microbial flora into the peritoneum. The latter process eventually leads to septic shock via activation of the inflammatory response, secondary to translocation of enteric bacteria into the bloodstream (Dejager *et al*., 2011). Accordingly, polymicrobial sepsis is characterised by an early hyperdynamic phase, subsequently followed by a late hypodynamic phase (Rittirsch *et al*., 2009). Animals subjected to CLP appear healthy in the initial stages post procedure, but at 12 h after the procedure, they begin to demonstrate the clinical signs of sepsis including malaise, fever,

chills, pilorection, generalised weakness and dampened gross motor activity, with lethality ensuing at about 18-24 h post CLP (Rittirsch *et al*., 2009).

Despite its clinical relevance and widespread utilisation in sepsis research, one of the major concerns of the CLP model is controlling the magnitude of the septic challenge across different laboratories (Dejager *et al*., 2011). On the basis of the characteristics of the model, the outcome post CLP is strongly influenced by various factors during the procedure: i) the percentage of cecum ligated (Singleton & Wischmeyer, 2003) and, hence, the amount of necrosis that is induced; ii) the microbial amount entering the peritoneum, which, is dependent on the number and size of puncture and pressure exerted on the caecal end; iii) sex, age and strain of the animals; and iv) supportive treatment such as fluid resuscitation and antibiotic regimen (Dejager *et al*., 2011). Nevertheless, the variability of this procedure confers on it an advantage by which the model can be tailored to induce sepsis with a range in severity, and allows to investigate acute as well as chronic sepsis (Dejager *et al.*, 2011).

Therefore, I sought to establish a model of CLP, which results in a consistent cardiac dysfunction as assessed *in vivo* by echocardiography. Specifically, I investigated the effect of i) gender differences; ii) differing strains (inbred C57BL/6 and outbred CD-1) in a time course-dependent manner; iii) alterations in fluid resuscitation and antibiotic therapy; iv) needle size; and v) age of C57BL/6 mice, on the development of cardiac dysfunction. The preference to utilise mice over rats was because the long term aim was to be able to potentially test a compound in a clinical model of CLP (Chapter 3). As most genetically manipulated knockout mice are on a C57BL/6 background, this mouse strain was the preferred choice.

2.2 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 Stationary Office and the Guide for the Care and Use of Laboratory Animals of the National Research Council (PPL: 70/6525).

Animals and Quantification of Organ Dysfunction

This study was performed on a total of 60 C57BL/6 inbred mice [12 female, 19.38 ± 0.28 g;46 male (2 months, 26.68 ± 0.51 g; 5 months, 32.78 ± 1.10 g; 8 months, 32.15 ± 1.74 g) Harlan Laboratories, Wyton, UK], and a total of 43 CD-1 outbred mice (23 female, 28.46 ± 0.50 g; 20 male, 38.27 ± 2.15 g, Harlan Laboratories, Wyton, UK), , receiving a standard diet and water *ad libitum.*

At the end of each experiment, mice were anaesthetised with a 1.5 ml kg^{-1} of ketamine (100 mg ml^{-1}) and xylazine (20 mg ml^{-1}) mixture (2:1; 1.5 ml kg^{-1} i.p.) before being sacrificed. Approximately 1 ml of blood was collected via cardiac puncture into serum gel S/1.3 tubes (Starstedt, Nümbrecht, Germany). Blood was centrifuged for 3 min at 9000 g at room temperature, to separate serum. Serum samples were transferred to plain eppendorfs and immediately packaged and sent off at room temperature to a contract laboratory for veterinary clinical chemistry (IDEXX Laboratories, Wetherby, UK) and analysed within 24 h of collection. The following marker enzymes were evaluated as biochemical indicators of multiple organ injury/dysfunction, using a kinetic ultraviolet test on a Beckman Coulter analyser (Beckman Coulter Ltd, High Wycombe, UK):

- ✓ Renal function was evaluated by measuring the rise in serum levels of urea (indicator of impaired excretory function of the kidney and/or increased protein and amino acid catabolism) and creatinine (indicator of reduced glomerular filtration rate, and hence, renal dysfunction (Thiemermann *et al*., 1995).
- ✓ Hepatic injury was assessed by measuring the rise in serum levels of alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury), and aspartate aminotransferase (AST, a non-specific marker for hepatocellular injury) (Thiemermann *et al*., 1995).

Caecal Ligation and Puncture

I followed the original CLP protocol introduced by Wichterman and colleagues (1980) with slight modifications including analgesia (buprenorphine; 0.5 mg kg^{-1}) and antibiotic therapy (Imipenem/Cilastatin; 20 mg kg^{-1}). This specific combination of imipenem and cilastatin was chosen due to the fact it is a broad spectrum beta-lactam antibiotic, known for its role in inhibiting bacterial cell wall biosynthesis (Birnbaum *et al*., 1985). Additionally, it appears to be a preferred choice of antibiotics, employed by various other laboratories in the mouse CLP model (Wang *et al*., 1998; Ulloa *et al*., 2002) and has been associated with increased survival when compared with triple antibiotic therapy (Newcomb *et al.*, 1998). A 20-gauge (G) needle was used with the double puncture technique for the studies involving the different strains, sex and altered antibiotics therapy, which in some cases went for a maximum period of 5 days. For the experiments of increased severity which were carried in aged C57BL/6 mice, an 18-G (i.e. larger size and hence, a more severe insult) needle was used with a double puncture technique, in order to generate cardiac dysfunction during the early phase of sepsis (24 h).

Briefly, anaesthesia was achieved with a ketamine (100 mg ml^{-1})/ xylazine (20 mg ml^{-1}) mixture (2:1; 1.5 ml kg^{-1}) injected i.p. Buprenorphine was administered additionally to provide adequate analgesia. A temperature of 37 ^{0}C was maintained by placing the animals on a homeothermic blanket and performing surgery in a temperature-controlled room. Surgery was performed under sterile conditions (i.e. use of sterile drapes, sterilised equipment) and began with the opening of the peritoneum via a 1.5 cm midline incision in order to exteriorise the caecum. The caecum was ligated just below the ileocaecal valve and punctured at both opposite ends. After a small amount of faecal matter was extruded from both ends to ensure patency, the caecum was placed back in its anatomical position. The peritoneum was sutured; 1 ml of Ringer's lactate solution was given for resuscitation subcutaneously and the mice were placed back in their cages. Analgaesia was administered i.p. and fluid resuscitation and antibiotics therapy were administered subcutaneously 6 h after surgery and every 12 h after that for a maximum

of 3 days, unless otherwise stated. Sham-operated animals underwent the same surgical procedures (i.e. laparotomy and fluid resuscitation and antibiotic therapy), the caecum was exteriorised and then replaced but was neither ligated nor punctured.

Experimental Design

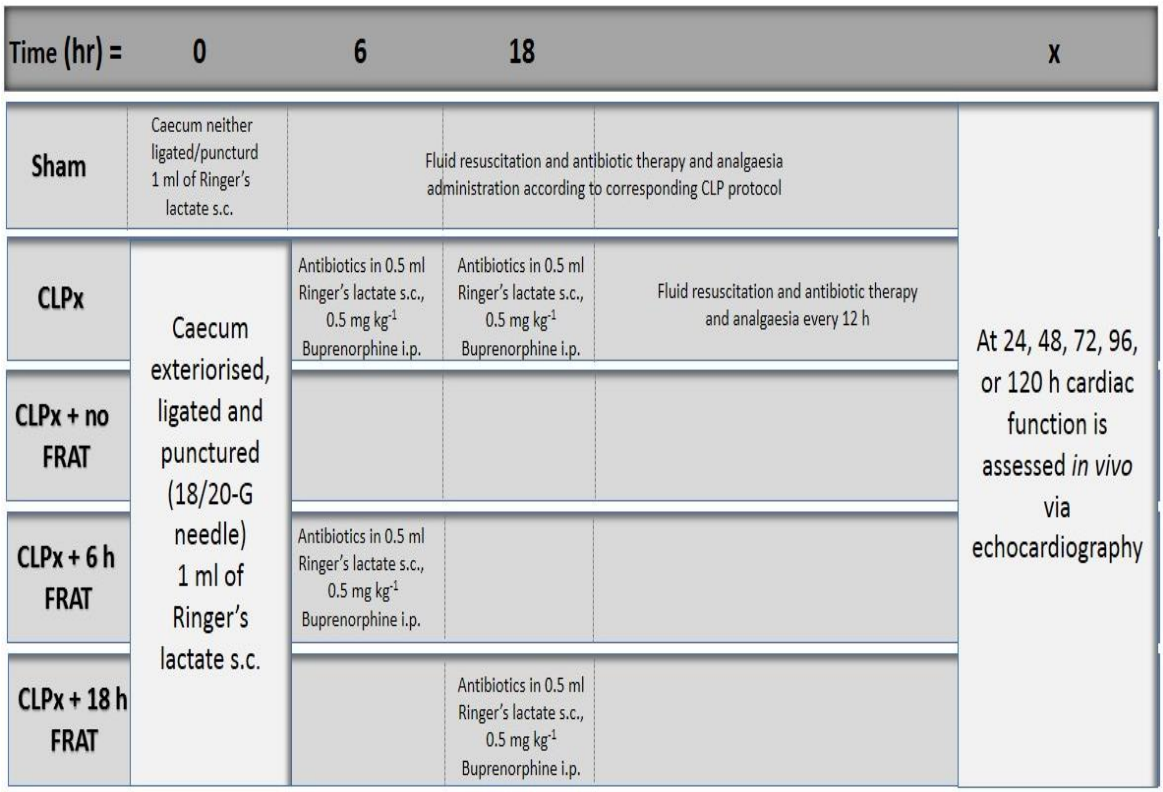
Animals were randomised into 4 study groups, subjected to CLP surgery, received relevant fluid resuscitation and antibiotics therapy, and assessed for cardiac function via echocardiography. A summary of the 4 study protocols is given in Table 2.2.1 with a detailed description treatments of animals given in Figure 2.2.1.

Table 2.2.1 Experimental groups of C57BL/6 and CD-1 male and female mice.

Study	Strain/ Sex	Age (Months)	Group	Protocol (h)	Needle	*n*
1	**Male C57BL/6**	2	Sham	N/A	N/A	5
		2	CLP24	24	20-G	4
		2	CLP48	48	20-G	3
		2	CLP72	72	20-G	3
	Female C57BL/6	2	Sham	N/A	N/A	4
		2	CLP24	24	20-G	3
		2	CLP48	48	20-G	5
2	**Male CD-1**	2	Sham	N/A	N/A	4
		2	CLP24	24	20-G	4
		2	CLP48	48	20-G	3
		2	CLP72	72	20-G	2
		2	CLP96	96	20-G	4
		2	CLP120	120	20-G	3
	Female CD-1	2	Sham	N/A	N/A	3
		2	CLP24	24	20-G	4
		2	CLP48	48	20-G	4
		2	CLP72	72	20-G	3
		2	CLP120	120	20-G	3
3	**Male C57BL/6**	2	CLP24 + no FRAT	24	20-G	4
		2	CLP24 + 6 h FRAT	24	20-G	14
		2	CLP24 + 18 h FRAT	24	20-G	3
	Female CD-1	2	CLP24 + 6 h FRAT	24	20-G	2
		2	CLP72 + 6 h FRAT	72	20-G	2
4	**Male C57BL/6**	2	CLP 2 months	24	18-G	5
		5	CLP 5 months	24	18-G	5
		8	CLP 8 months	24	18-G	9

FRAT, fluid resuscitation and antibiotic therapy; **N/A**, not applicable; **G**, gauge

Figure 2.2.1. Experimental procedure and animal treatment. FRAT, fluid resuscitation and antibiotic therapy



Assessment of Cardiac Function *in vivo*

Left ventricular (LV) function was assessed in mice by echocardiography *in vivo* as reported previously (Khan *et al.*, 2013). At 24 h subsequent to CLP surgery, mice were anesthetized with 3 % isoflurane and placed in a supine position, atop a heating pad embedded with ECG leads, to maintain body temperature and measure heart rate. Fur was removed with hair removal cream from the neckline to mid chest level, pre-heated ultrasound gel was applied to the chest and a steady state sedation level was maintained thereafter with 0.5 – 0.7 % isoflurane for the duration of the procedure. Mice were allowed to stabilize for approximately 10 mins after which, the heart was imaged using a Vevo-770 imaging system (VisualSonics, Toronto, Canada). Percent fractional area change (FAC) was assessed with a two-dimensional (B-mode) view along the parasternal short axis mid-ventricular papillary muscle level. Subsequently, measurements of LV dimensions and contractility such as ejection fraction (EF) and fractional shortening (FS) were obtained using an M-mode tracing.

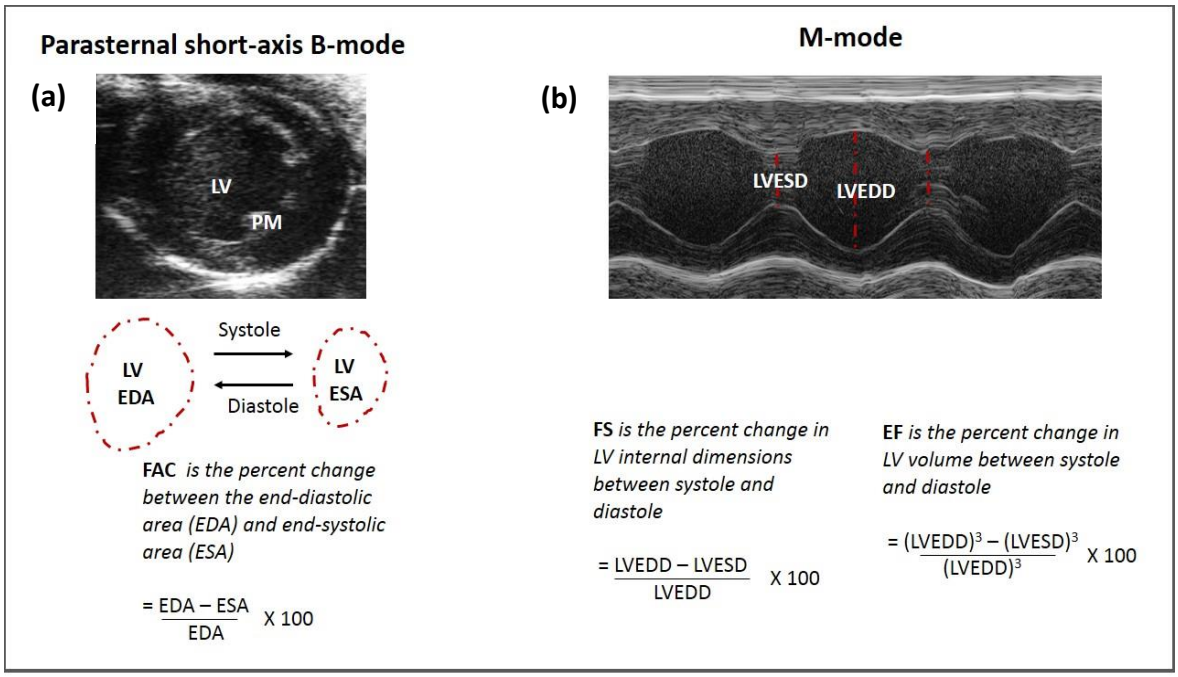


Figure 2.2.2. Echocardiographic assessment showing (a) parasternal short-axis view producing a transverse cut of the left ventricle (LV) at the papillary muscle (PM) level at which the fractional area change (FAC) can be determined, (b) M-mode tracing used to display LV cavity and dimensions is used to determine ejection fraction (EF) and fractional shortening (FS). LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter.

Materials

Unless otherwise stated, all compounds in this study were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

Statistical Analyses

All values described in the text and figures are presented as mean ± standard error of the mean (SEM) of n observations, where n represents the number of animals studied. Statistical analysis was performed using GraphPad Prism 5.0d (GraphPad Software, San Diego, California, USA). Data without repeated measurements were assessed by a one-way ANOVA followed by a Dunnet's post-hoc test.

2.3 Results

Heart rate measurements in C57BL/6 and CD-1 mice following caecal ligation and puncture

Study 1- Heart rates of male and female C57BL/6 mice that have underwent CLP surgery, when compared to sham operated animals treated with vehicle (Sham), male C57BL/6 mice subjected to CLP surgery demonstrated no significant changes in heat rate (HR) ($P > 0.05$, Table 2.3.1), at 24, 48 and 72 h post-surgery. In contrast, when compared to sham operated animals treated with vehicle (Sham), female C57BL/6 mice subjected to CLP surgery demonstrated a significant change in HR at 24 (608.20 ± 15.74 vs. 418.00 ± 43.47; $P < 0.05$, Table 2.3.1) and 48 hr (608.20 ± 15.74 vs. 440.00 ± 31.54; $P < 0.05$, Table 2.3.1) post-surgery.

Study 2- Heart rates of male and female CD-1 mice that underwent CLP surgery, when compared to sham operated animals treated with vehicle (Sham), male CD-1 mice subjected to CLP surgery demonstrated no significant change in HR ($P > 0.05$, Table 2.3.1), up to 120 h post-surgery. Similarly, when compared to sham operated animals treated with vehicle (Sham), female CD-1 mice subjected to CLP demonstrated no significant change in HR at 24, 48, 72 and 120 h ($P > 0.05$, Table 2.3.1) post-surgery.

Study 3- Heart rates of mal C57BL/6 and female CD-1 mice that underwent CLP surgery with subsequent alteration in fluid resuscitation and antibiotic therapy, when compared to sham operated animals treated with vehicle (Sham), male C57BL/6 mice subjected to CLP surgery for 24 h and administered no fluid resuscitation and antibiotic therapy (FRAT) demonstrated a significant change in HR (608.20 ± 15.74 vs. 451.25 ± 44.39; $P < 0.05$, Table 2.3.1). Whereas, animals subjected to CLP surgery for 24 h and administered either fluid resuscitation and antibiotic therapy only at 6 h or 18 h post-surgery demonstrated no significant changes in HR ($P > 0.05$, Table 2.3.1) when compared to Sham animals.

Moreover, when compared to sham operated animals treated with vehicle (Sham), female CD-1 mice subjected to CLP surgery and administered fluid resuscitation and antibiotic therapy only at 6 h 24 h or 72 h post-surgery demonstrated no significant changes in HR ($P > 0.05$, Table 2.3.1).

Study 4- Heart rates of aging male C57BL/6 mice that underwent CLP surgery, when compared to sham operated animals treated with vehicle (Sham), 2, 5 and 8 months-old male C57BL/6 mice subjected to CLP surgery demonstrated significant changes in HR (2 months, 608.20 ± 15.74 vs. 497.40 ± 46.09; $P < 0.05$, Table 2.3.1; 5 months, 608.20 ± 15.74 vs. 476.40 ± 25.18; $P < 0.05$, Table 2.3.1; 8 months 608.20 ± 15.74 vs. 469.00 ± 12.81; $P < 0.05$, Table 2.3.1).

Table 2.3.1. Heart rate of C57BL/6 and CD-1 male and female mice

Study	Strain/ Sex	Age (Months)	Group	Protocol (h)	*n*	Needle	Heart rate (bpm)
1	**Male C57BL/6**	2	Sham	N/A	5	N/A	608.20 ± 15.74
		2	CLP24	24	4	20-G	474.50 ± 56.74
		2	CLP48	48	3	20-G	475.33 ± 57.74
		2	CLP72	72	3	20-G	447.33±38.74
	Female C57BL/6	2	Sham	N/A	4	N/A	575.75 ± 15.11
		2	CLP24	24	3	20-G	418.00 ± 43.47 ★
		2	CLP48	48	5	20-G	440.00 ± 31.54 ★
2	**Male CD-1**	2	Sham	N/A	4	N/A	524.25 ± 25.16
		2	CLP24	24	4	20-G	450.50 ± 42.27
		2	CLP48	48	3	20-G	610.67 ± 61.91
		2	CLP72	72	2	20-G	485.00 ± 12.00
		2	CLP96	96	4	20-G	552.75 ± 29.66
		2	CLP120	120	3	20-G	459.33 ± 45.73
	Female CD-1	2	Sham	N/A	3	N/A	506.00 ±69.30
		2	CLP24	24	3	20-G	519.00 ± 21.94
		2	CLP48	48	4	20-G	580.50 ± 59.89
		2	CLP72	72	3	20-G	515.67 ± 31.52
		2	CLP120	120	3	20-G	478.67 ± 45.50

Table 2.3.1. Heart rate of C57BL/6 and CD-1 male and female mice continued

Study	Strain/ Sex	Age (Months)	Group	Protocol (h)	*n*	Needle	Heart rate (bpm)
3	**Male C57BL/6**	2	CLP24 + no FRAT	24	4	20-G	451.25 ± 44.39 #
		2	CLP24 + 6 h FRAT	24	14	20-G	495.57 ±22.32
		2	CLP24 + 18 h FRAT	24	3	20-G	526.00 ± 73.75
	Female CD-1	2	CLP24 + 6 h FRAT	24	2	20-G	588.00 ± 37.00
		2	CLP72 + 6 h FRAT	72	2	20-G	522.50 ± 75.50
4	**Male C57BL/6**	2	CLP 2 months	24	5	18-G	497.40 ± 46.09 #
		5	CLP 5 months	24	5	18-G	476.40 ± 25.18 #
		8	CLP 8 months	24	9	18-G	469.00 ± 12.81 #

FRAT, fluid resuscitation and antibiotic therapy; **N/A**, not applicable; **G**, gauge

Heart rate measurements in C57BL/6 and CD-1 mice. Data are expressed as means ± SEM. for n number of observations. ★ $P < 0.05$ vs. Female C57BL/6 Sham; # $P < 0.05$ vs. Male C57BL/6 Sham, (one-way ANOVA followed by a Dunnett's post-hoc test).

Study 1 - Assessment of cardiac function in male and female C57BL/6 mice that were subjected to CLP over time

The first step in establishing a model of CLP was to investigate the effects of sex on the response of mice to a septic insult and, thus, the susceptibility to develop cardiac dysfunction. Figure 2.3.1A shows representative M-mode echocardiograms of Sham, CLP24, CLP48 and CLP72 in male C57BL/6 mice. When compared to Sham mice, mice subjected to CLP surgery demonstrated no significant alterations in EF, FS, and FAC at 24, 48, nor 72 h post-surgery ($P > 0.05$, Figures 2.3.1B-D).

Figure 2.3.2A shows representative M-mode echocardiograms of sham, CLP24 and CLP48 in C57BL/6 female mice. When compared to sham mice, mice subjected to CLP surgery demonstrated no significant alterations in EF, FS, and FAC 24 or 48 h post-surgery ($P > 0.05$, Figures 2.3.2B-D).

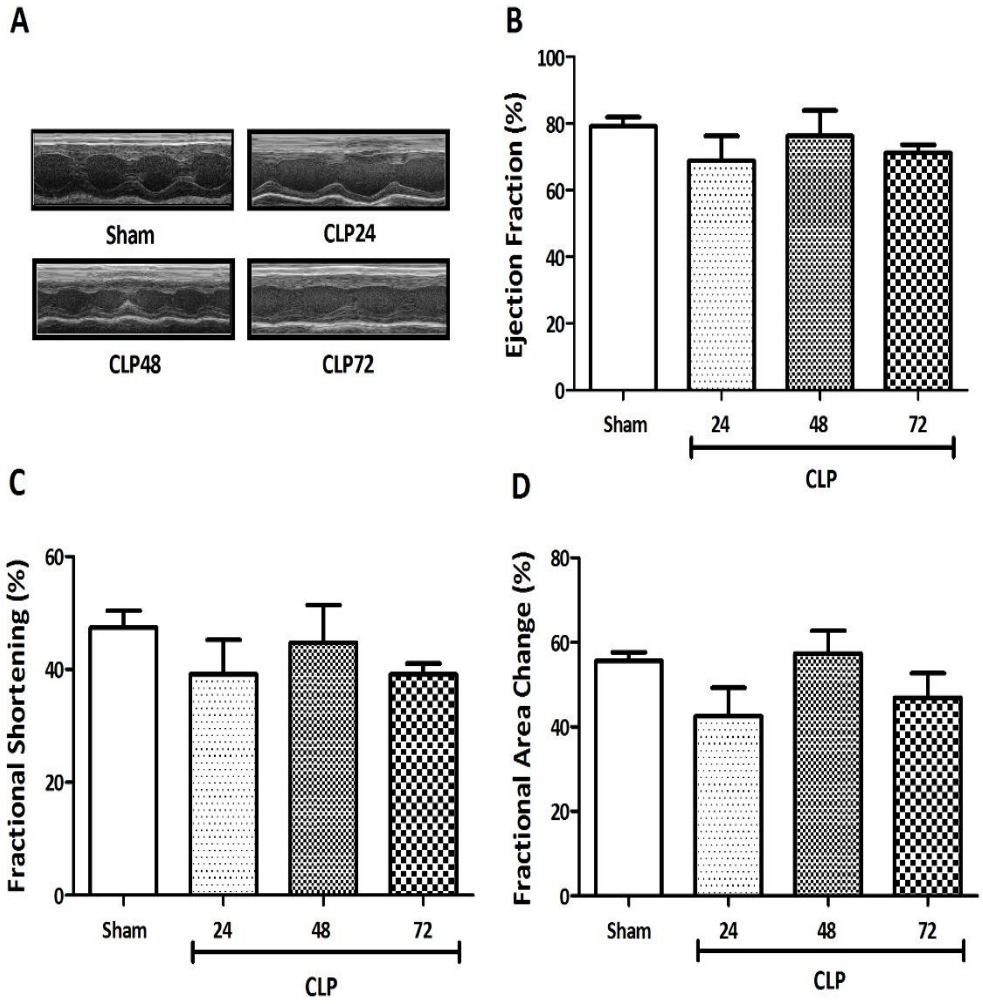


Figure 2.3.1. Cardiac function in male C57BL/6 mice that underwent CLP. Representative M-mode echocardiograms (A), percentage ejection fraction (B), fractional shortening (C) and fractional area change (D) in mice randomised to undergo either: sham operation (Sham, $n = 5$), or CLP surgery with double puncture technique, and left for either 24 h (CLP24, $n = 4$), 48 h (CLP48, $n = 3$) or 72 h (CLP72, $n = 3$). Data are expressed as means ± SEM. for n number of observations.

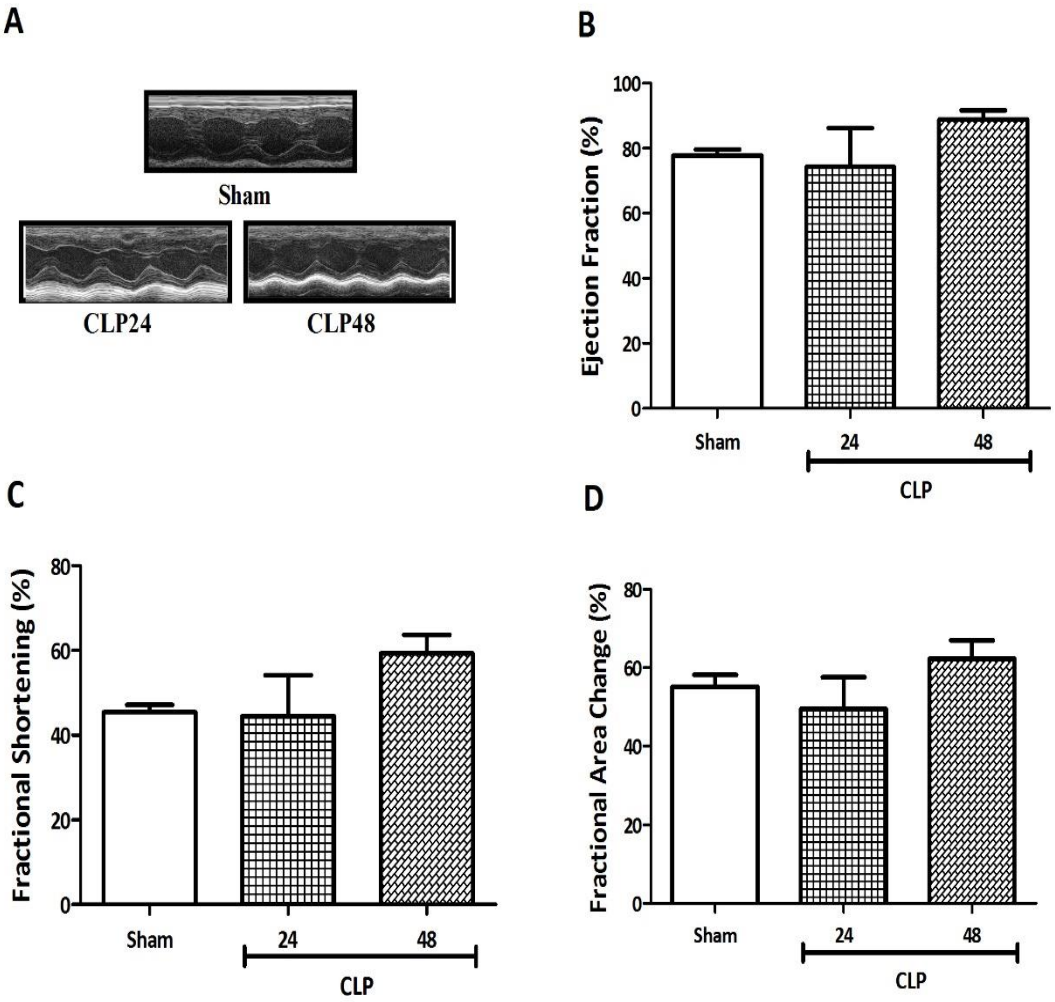


Figure 2.3.2. Cardiac function in female C57BL/6 mice that underwent CLP. Representative M-mode echocardiograms (A), percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) in mice randomised to undergo either: sham operation (Sham, *n* = 4), or CLP surgery with double puncture technique, and left for either 24 h (CLP24, *n* = 3), or 48 h (CLP48, *n* = 5). Data are expressed as means ± SEM. for *n* number of observations.

Study 2 - Assessment of cardiac function in male CD-1 mice subjected to CLP over time

As CLP survival and the physiologic response to sepsis are greatly affected by genetic background (Godshall *et al*., 2002, in addition to investigating the response of inbred C57BL/6 mice to a septic insult, I sought to investigate the susceptibility of outbred CD-1 mice to develop cardiac dysfunction. Figure 2.3.3A shows representative M-mode echocardiograms of sham, CLP24, CLP48, CLP72, CLP96, and CLP120 in CD-1 male mice. When compared to sham mice, mice subjected to CLP surgery demonstrated no significant alterations in EF, FS, and FAC over a 5-day period ($P > 0.05$, Figures 2.3.3B-D).

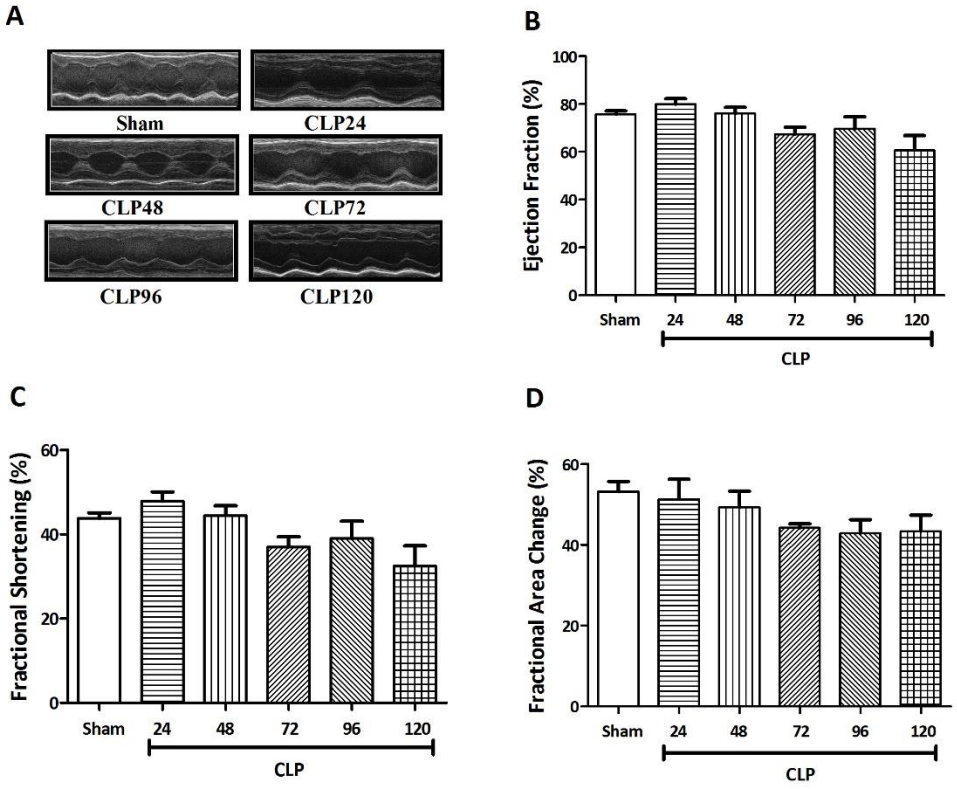


Figure 2.3.3. Cardiac function in male CD-1 mice that underwent CLP. Representative M-mode echocardiograms (A), percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) in mice randomised to undergo either: sham operation (Sham, $n = 4$), or CLP surgery with double puncture technique, and left for either 24 h (CLP24, $n = 4$), 48 h (CLP48, $n = 3$), 72 h (CLP72, $n = 2$), 96 h (CLP96, $n = 4$), or 120 h (CLP120, $n = 3$). Data are expressed as means ± SEM. for n number of observations.

Assessment of cardiac function in female CD-1 mice subjected to CLP over time

Figure 2.3.4A shows representative M-mode echocardiograms of sham, CLP24, CLP48, CLP72, and CLP120 in female CD-1 mice. When compared to sham mice, mice subjected to CLP surgery demonstrated no significant alterations in EF, FS, and FAC at 24, 48, 72 and 120 h post-surgery ($P > 0.05$, Figures 2.3.4B-D).

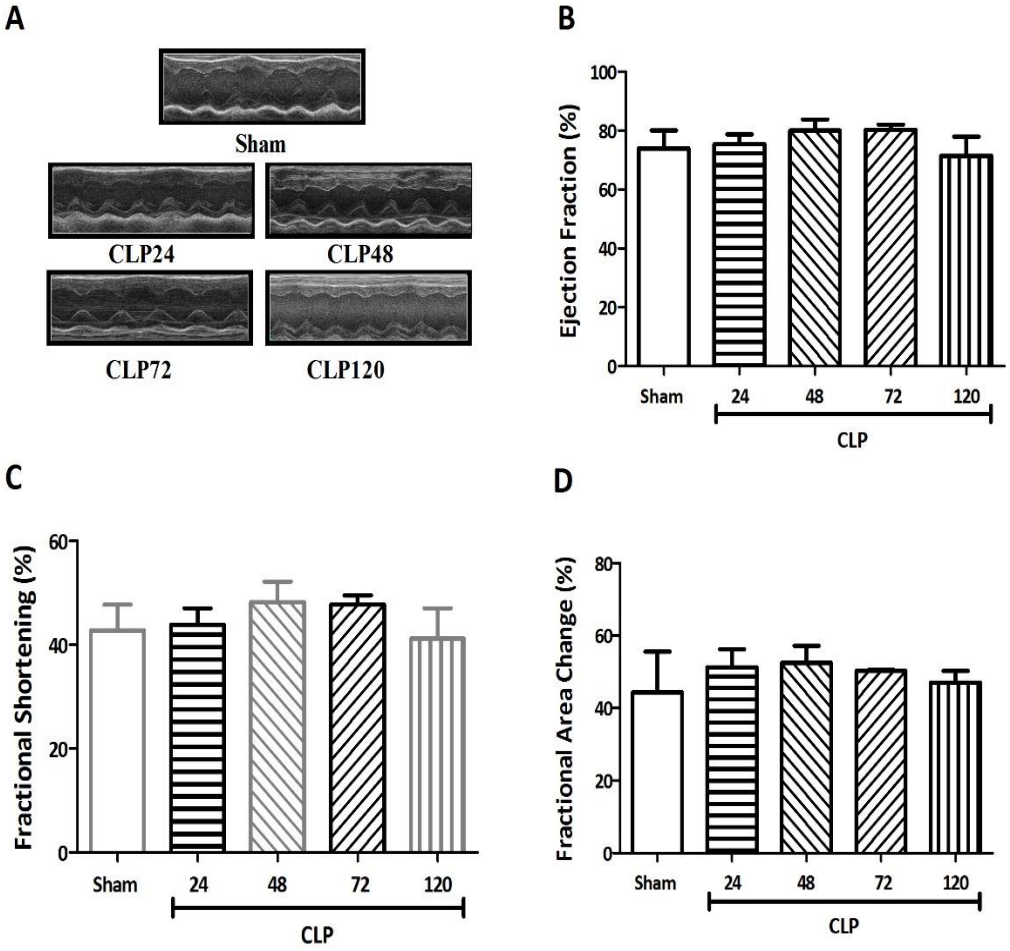


Figure 2.3.4. Cardiac function in female CD-1 mice that underwent CLP. Representative M-mode echocardiograms (A), percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) in mice randomised to undergo either: sham operation (Sham, $n = 3$), or CLP surgery with double puncture technique for either 24 h (CLP24, $n = 4$), 48 h (CLP48, $n = 4$), 72 h (CLP72, $n = 3$), or 120 h (CLP120, $n = 3$). Data are expressed as means ± SEM. for n number of observations.

Study 3- Assessment of cardiac function in male C57BL/6 and female CD-1 mice subjected to CLP with subsequent alterations in fluid resuscitation and antibiotic therapy

Although animal models which do not receive supportive care (i.e. fluid resuscitation and antibiotic therapy) are not representative of the clinical situation and are likely to demonstrate high mortality rates, I wanted to alter the fluid resuscitation and antibiotic therapy (FRAT) frequency to determine whether this might alter the susceptibility of C57BL/6 and CD-1 mice to succumbing to develop cardiac dysfunction. Figure 2.3.5A shows representative M-mode echocardiograms of sham, CLP24, CLP24 + No FRAT, CLP24 + 6 h FRAT, and CLP24 + 18 h FRAT in C57Bl/6 male mice. When compared to sham mice, mice subjected to CLP surgery demonstrated no significant alterations in EF, FS, and FAC ($P > 0.05$, Figures 2.3.5B-D) 24 h post-surgery. In addition, alterations in fluid resuscitation and antibiotic therapy which, included administering no FRAT following CLP surgery, giving FRAT only at 6 h post CLP surgery, or giving FRAT only at 18 h post-surgery resulted in no significant alterations in EF, FS, and FAC ($P > 0.05$, Figures 2.3.5B-D).

Figure 2.3.6A shows representative M-mode echocardiograms of sham, CLP24, CLP24 + 6 h FRAT, and CLP72, and CLP72 + 6 h FRAT in CD-1 female mice. When compared to sham mice, mice subjected to CLP surgery demonstrated no significant alterations in EF, FS, and FAC ($P > 0.05$, Figures 2.3.6B-D) 24 h post-surgery. Altering the frequency of dosing of fluid resuscitation and antibiotic therapy by administering only one dose of FRAT at 6 h post CLP surgery resulted in no significant alterations in EF, FS, and FAC ($P > 0.05$, Figures 2.3.6B-D) 24 h post-surgery.

In addition, when compared to sham mice, mice subjected to CLP surgery demonstrated no significant alterations in EF, FS, and FAC ($P > 0.05$, Figures 2.3.6B-D) 72 h post-surgery. Altering the fluid resuscitation and antibiotic therapy by administering only one dose of FRAT at 6 h post CLP surgery resulted in no significant alterations in EF, FS, and FAC ($P > 0.05$, Figures 2.3.6B-D) 72 h post-surgery.

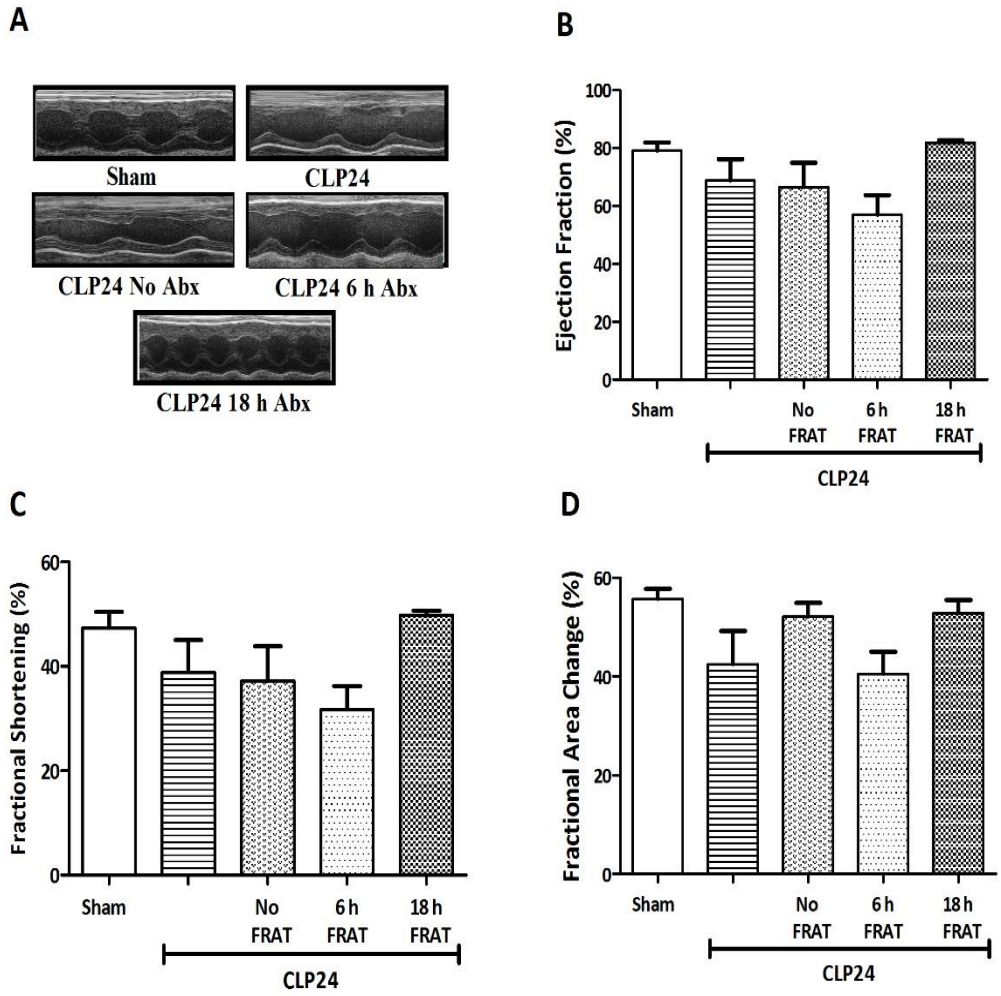


Figure 2.3.5. Cardiac function in male C57BL/6 mice that underwent CLP with subsequent alterations to fluid resuscitation and antibiotic therapy (FRAT). Representative M-mode echocardiograms (A), percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) in mice randomised to undergo either: sham operation (Sham, *n* = 5), or CLP surgery with double puncture technique and given either a normal FRAT therapy (CLP24, *n* = 4), no FRAT (CLP24 + o FRAT, *n* = 4), FRAT only at 6 h (CLP24 + 6 h FRAT, *n* = 14) or FRAT only at 18 h (CLP24 + 18 h FRAT, *n* = 3) 24 h post-surgery. Data are expressed as means ± SEM. for *n* number of observations.

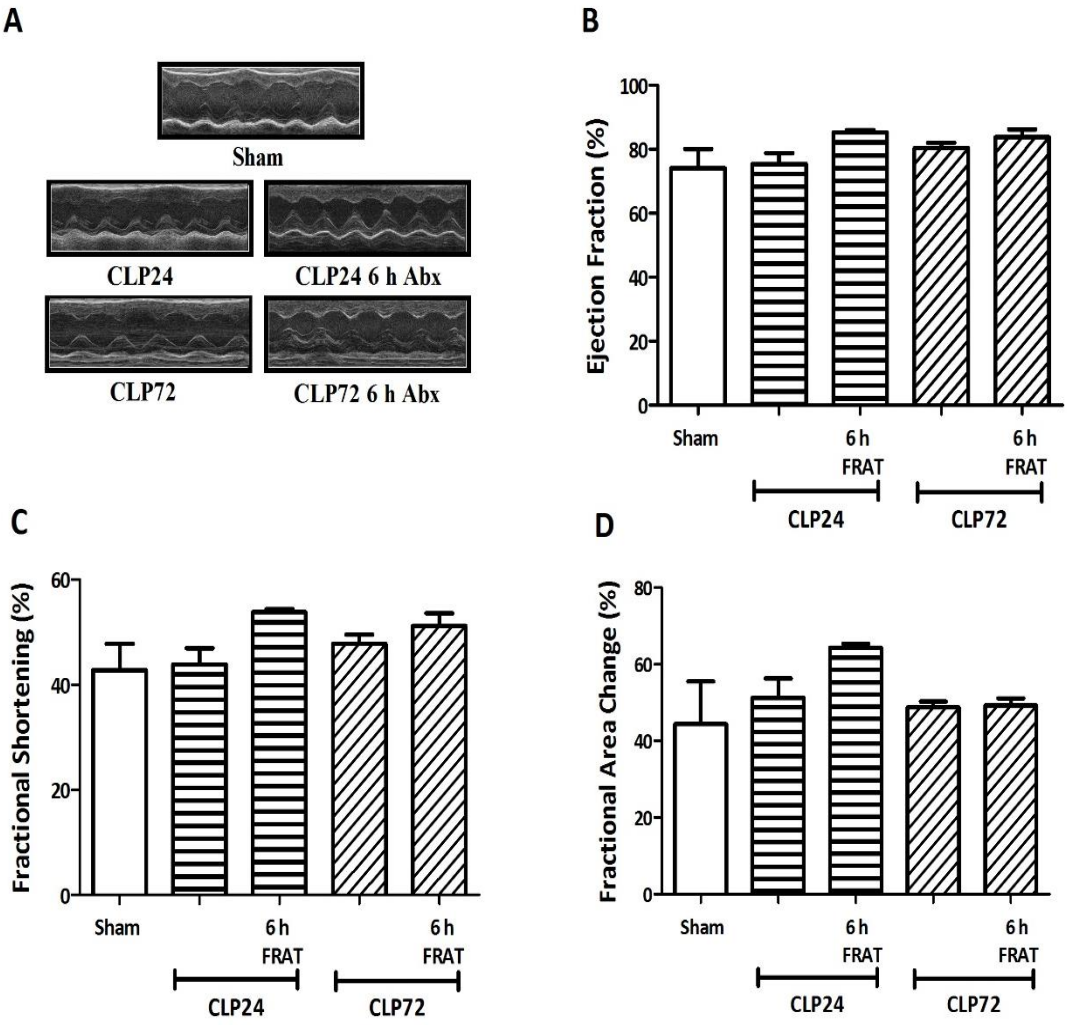


Figure 2.3.6. Cardiac function in female CD-1 mice that underwent CLP with subsequent alterations to fluid resuscitation and antibiotics therapy (FRAT). Representative M-mode echocardiograms (A), percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) in mice randomised to undergo either: sham operation (Sham, *n* = 4), or CLP surgery with double puncture technique and given either normal FRAT (CLP24, *n* = 4) or FRAT only at 6 h post-surgery (CLP24 + 6 h FRAT, *n* = 2) 24 h post-surgery, or CLP surgery with double puncture technique and given either normal FRAT therapy (CLP72, *n* = 3) or FRAT at only 6 h post-surgery (CLP72 + 6 h FRAT, *n* = 2) 72 h post-surgery. Data are expressed as means ± SEM. for *n* number of observations.

Study 4 - The development of cardiac dysfunction in aging male C57BL/6 mice subjected to CLP

Although sepsis is more frequently observed in aging patients (Destarac & Ely, 2002; Girard *et al*., 2005), the majority of preclinical studies utilise young mice as their model system. I sought to determine the effect that age has on the susceptibility of C57BL/6 mice to develop cardiac dysfunction. For this study I used a larger needle size, and thus a more severe model of polymicrobial sepsis and assessed cardiac function 24 h following CLP surgery. Figure 2.3.7A shows representative M-mode echocardiograms of Sham 2 months, CLP 2 months, CLP 5 months, and CLP 8 months, in C57BL/6 male mice. When compared to sham operated animals treated with vehicle (Sham), 2, 5, and 8 months old mice subjected to CLP surgery developed an age-dependent decrease in systolic contractility, characterized by a significant decrease in EF ($P < 0.05$, Figure 2.3.7B), FS ($P < 0.05$, Figure 2.3.7C), and FAC ($P < 0.05$, Figure 2.3.7D) 24 h post-surgery.

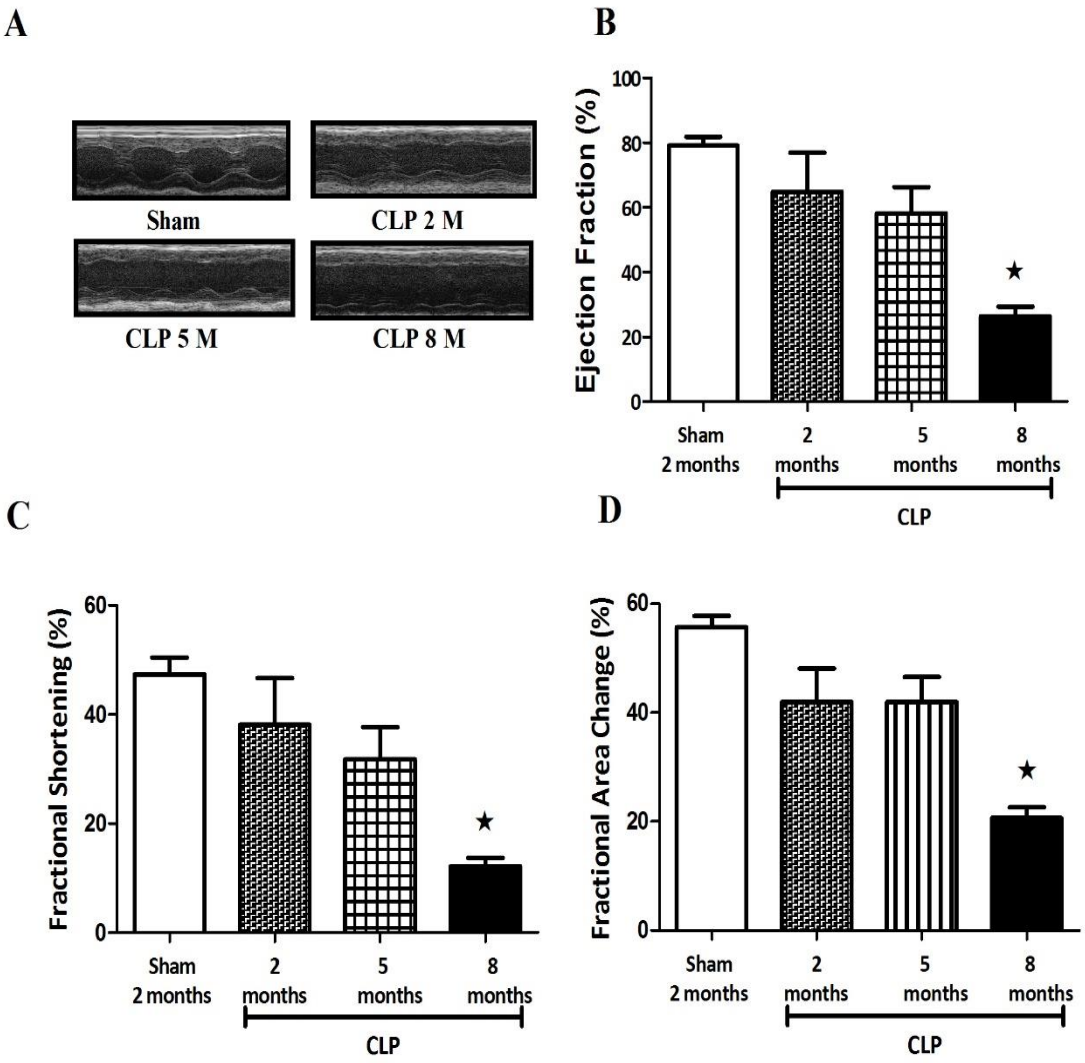


Figure 2.3.7. Cardiac function in aging male C57BL/6 mice that underwent CLP. Representative M-mode echocardiograms (A), percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D), in mice randomised to undergo either: sham operation (Sham, n = 5), or CLP surgery with double puncture technique in mice of at ages 2 months (n = 5), 5 months (n = 5), 8 months (n = 9) 24 h post-surgery. Data are expressed as means ± SEM. for n number of observations. ★ $P < 0.05$ vs. Sham 2 months, (one-way analysis of variance with Dunnett's post-hoc test)

Serum levels of markers of organ injury/dysfunction in C57BL/6 and CD-1 mice following caecal ligation and puncture

Study 1- Assessment of liver injury and renal function in male and female C57BL/6 mice that have underwent CLP surgery, when compared to sham operated animals treated with vehicle (sham), male C57BL/6 mice subjected to CLP surgery demonstrated no change in serum levels of urea ($P > 0.05$, Table 2.3.1) and creatinine ($P > 0.05$, Table 2.3.1) or AST ($P > 0.05$, Table 2.3.1) and ALT ($P > 0.05$, Table 2.3.1), 72 h post-surgery. However, due to the low n number in this group, a firm conclusion cannot be made and thus requires further confirmation. In contrast, when compared to sham operated animals treated with vehicle (sham), female C57BL/6 mice subjected to CLP surgery demonstrated significant increases in serum levels of urea ($P < 0.05$, Table 2.3.1), indicating possible renal dysfunction at 24 h post-surgery and AST and ALT, indicative of hepatic injury ($P < 0.05$, Table 2.3.1) at 24 and 48 h post-surgery.

Study 2- Assessment of liver and renal function in male and female CD-1 mice that underwent CLP surgery when compared to sham operated animals treated with vehicle (sham), male CD-1 mice subjected to CLP surgery demonstrated no significant increases in serum levels of urea ($P > 0.05$, Table 2.3.1), creatinine ($P > 0.05$, Table 2.3.1), or ALT ($P > 0.05$, Table 2.3.1), at 24, 48, 72, 96, or 120 h period post-surgery. However, a significant increase in serum level of AST ($P < 0.05$, Table 2.3.1) , indicating possible liver injury was observed at 24 and 48 h post-surgery.

Whereas, when compared to sham operated animals treated with vehicle (sham), female CD-1 mice subjected to CLP surgery demonstrated no significant increases in serum levels of urea ($P > 0.05$, Table 2.3.1) or creatinine ($P > 0.05$, Table 2.3.1) at 24, 48, 72, or 120 h post-surgery, but exhibited significant increases in serum levels of AST ($P < 0.05$, Table 2.3.1) and ALT ($P < 0.05$, Table 2.3.1) , indicative of hepatic injury ($P < 0.05$, Table 2.3.1) at 24 and 48 h post-surgery.

Study 3- Assessment of liver and renal function in male C57BL/6 and female CD-1 mice that underwent CLP surgery with subsequent alteration to fluid resuscitation and antibiotic therapy when compared to sham operated animals treated with vehicle (sham), male C57BL/6 mice subjected to CLP for 24 h and administered either no fluid resuscitation and antibiotic therapy (FRAT) or given only at 6 h or 18 h post-surgery demonstrated no change in serum levels of urea ($P > 0.05$, Table 2.3.1) or

creatinine ($P > 0.05$, Table 2.3.1) at 24 h post-surgery. Animals that were either administered no fluid resuscitation and antibiotic therapy or given only at 6 h post-surgery demonstrated significant increases in serum levels of AST ($P < 0.05$, Table 2.3.1) and ALT, indicative of hepatic injury ($P < 0.05$, Table 2.3.1) at 24 h post-surgery. In addition, animals that were given fluid resuscitation and antibiotic therapy as late as 18 h post-surgery demonstrated a significant increase in serum level of ALT, indicative of hepatic injury ($P < 0.05$, Table 2.3.1) at 24 h post-surgery.

Moreover, when compared to sham operated animals treated with vehicle (sham), female CD-1 mice subjected to CLP surgery and administered fluid resuscitation and antibiotic therapy only at 6 h post-surgery demonstrated no change in serum levels of urea ($P > 0.05$, Table 2.3.1), creatinine ($P > 0.05$, Table 2.3.1) or AST ($P > 0.05$, Table 2.3.1) but demonstrated an increased trend in serum levels of ALT at 24 h post-surgery which, could indicate possible hepatic injury. However due to the low n number in this group a firm conclusion cannot be made and thus requires further confirmation at 24 h post-surgery. In contrast, animals that were subjected to CLP surgery and administered fluid resuscitation and antibiotic therapy only at 6 h post-surgery demonstrated no significant change in serum levels of urea ($P > 0.05$, Table 2.3.1) and creatinine ($P > 0.05$, Table 2.3.1) at 72 h post-surgery, but demonstrated an increased trend in serum levels of ALT and AST at 72 h post-surgery which, could indicate possible hepatic injury.. However, due to the low n number in these groups, a firm conclusion cannot be made and thus requires further confirmation

Study 4- Assessment of renal and liver function in aging male C57BL/6 mice that underwent CLP surgery when compared to sham operated animals treated with vehicle (sham), 2 months-old male C57BL/6 mice subjected to CLP surgery demonstrated significant increases in serum level of urea ($P < 0.05$, Table 2.3.1), indicating possible renal dysfunction, and AST and ALT, indicative of hepatic injury ($P < 0.05$, Table 2.3.1) at 24 h post-surgery. In addition, 5 months-old male C57BL/6 mice subjected to CLP surgery demonstrated significant increases in serum levels of urea ($P < 0.05$, Table 2.3.1) indicating possible renal dysfunction, and AST and ALT, indicative of hepatic injury ($P < 0.05$, Table 2.3.1) at 24 h post-surgery. Furthermore, 8 months-old male C57BL/6 mice subjected to CLP surgery demonstrated significant increases in serum levels of urea ($P < 0.05$, Table 2.3.1) and creatinine, indicative of renal dysfunction ($P < 0.05$, Table 2.3.1),

and AST ($P < 0.05$, Table 2.3.1) and ALT, indicative of hepatic injury ($P < 0.05$, Table 2.3.1) at 24 h post-surgery.

Table 2.3.2. Serum levels of markers of organ injury/dysfunction in C57BL/6 and CD-1 male and female mice

Strain/ Sex	Group	Age (Months)	Needle Size	Average Urea (mmol/l)	Average Creatinine (µmol/l)	Average AST (iµ/l)	Average ALT (iµ/l)
Male C57BL/6	Sham (*n* = 5)	2	N/A	6.5 ± 0.4	27.3 ± 0.8	146.1 ± 22.4	52.1 ± 12.3
	CLP24 (*n* = 4)	2	20-G	ND	ND	ND	ND
	CLP48 (*n* = 3)	2	20-G	ND	ND	ND	ND
	CLP72 (*n* = 2)	2	20-G	10.4 ± 0.9	27.1 ± 2.0	387.6 ± 45.8	93.8 ± 11.5
Female C57BL/6	Sham (*n* = 4)	2	N/A	7.3 ± 0.58	30.28 ± 0.73	159.65 ± 18.51	40.40 ± 10.26
	CLP24 (*n* = 3)	2	20-G	18.7 ± 2.1§	30.1 ± 0.59	858.8 ± 77.7 §	359.2 ± 22.7 §
	CLP48 (*n* = 5)	2	20-G	10.0 ± 0.4	27.4 ± 1.5	828.5 ± 60.8 §	249.6 ± 25.6 §
Male CD-1	Sham (*n* = 4)	2	N/A	6.65 ± 0.36	27.78 ± 1.15	123.70 ± 29.30	110.15 ± 74.95
	CLP24 (*n* = 4)	2	20-G	9.6 ± 1.3	30.2 ± 1.8	681.0 ± 58.8 #	161.2 ± 25.7
	CLP48 (*n* = 3)	2	20-G	5.6 ± 1.2	25.6 ± 1.7	284.9 ± 11.0 #	107.7 ± 29.6
	CLP72 (*n* = 2)	2	20-G	4.7 ± 0.6	23.6 ± 0.7	189.2 ± 7.50	43.2 ± 12.6
	CLP96 (*n* = 4)	2	20-G	5.3 ± 0.1	25.8 ± 0.6	138.0 ± 10.0	23.1 ± 3.20
	CLP 120 (*n* = 3)	2	20-G	6.6 ± 1.3	27.8 ± 0.9	141.0 ± 14.5	24.4 ± 2.6
Female CD-1	Sham (*n* = 3)	2	N/A	4.93 ± 0.3	27.1 ± 1.1	128.2 ± 22.7	23.0 ± 3.6
	CLP24 (*n* = 4)	2	20-G	13.3 ± 4.6	30.2 ± 1.0	611.0 ± 56.7 $	235.5 ± 17.4 $
	CLP48 (*n* = 4)	2	20-G	6.0 ± 0.6	27.20 ± 1.1	405.4 ± 108.2 $	109.2 ± 40.0 $

FRAT = fluid resuscitation and antibiotic therapy; **N/A** = Not applicable;

ND = Not done; **G** = Gauge

Table 2.3.1. Serum levels of markers of organ injury/dysfunction in C57BL/6 and CD-1 male and female mice continued

Strain/ Sex	Group	Age (Months)	Needle Size	Average Urea (mmol/l)	Average Creatinine (µmol/l)	Average AST (iµ/l)	Average ALT (iµ/l)
	CLP72 (*n* = 4)	2	20-G	4.7 ± 0.4	25.8 ± 1.3	171.3 ± 29.1	31.0 ± 5.2
	CLP 120 (*n* = 4)	2	20-G	5.4 ± 0.1	27.2 ± 1.3	145.7 ± 17.5	20.8 ± 2.1
Male C57BL/ 6	CLP24 + No FRAT (*n* = 4)	2	20-G	23.9 ± 6.78	53.4 ± 15.3	572.9 ± 109.1 ★	403.1 ± 67.2 ★
	CLP24 + 6h FRAT (*n* = 14)	2	20-G	20.9 ± 3.5	53.0 ± 7.9	522.1 ± 49.9 ★	289.6 ± 30.4 ★
	CLP24 + 18h FRAT (*n* = 3)	2	20-G	15.5 ± 2.9	44.3 ± 3.5	466.6 ± 119.1	296.4 ± 95.3 ★
Female CD-1	CLP24 + 6h FRAT (*n* = 2)	2	20-G	4.4 ± 1.2	25.4 ± 1.8	370.5 ± 100.8	147.2 ± 34.6
	CLP72 + 6h FRAT (*n* = 2)	2	20-G	5.6 ± 0.1	29.9 ± 0.3	485.5 ± 132.5 9	74.2 ± 20.4
Male C57BL/ 6	CLP 2 months (*n* = 5)	2	18-G	19.8 ± 4.8 ★	40.9 ± 7.0	446.8 ± 71.5 ★	287.7 ± 64.9 ★
	CLP 5 months (*n* = 5)	5	18-G	25.6 ± 5.98 ★	38.1 ± 6.20	603.7 ± 110.0 ★	310.9 ± 53.8 ★
	CLP 8 months (*n* = 10)	8	18-G	36.8 ± 1.4 ★	76.3 ± 8.0 ★	673.5 ± 54.2 ★	356.6 ± 35.2 ★

FRAT = fluid resuscitation and antibiotic therapy; **N/A** = Not applicable;

ND = Not done; **G** = Gauge

Serum levels of (A) urea, (B) creatinine (C) aspartate aminotransferase (AST) and (D) alanine aminotransferase (ALT) in C57BL/6 and CD-1 male and female mice. Data are expressed as means ± SEM. for *n* number of observations. ★ $P < 0.05$ vs. Male C57BL/6 Sham; § $P < 0.05$ vs. Female C57BL/6; $ $P < 0.05$ vs. Female CD-1 Sham; # $P < 0.05$ vs. Male CD-1 Sham, (one-way analysis of variance with Dunnett's post-hoc test).

2.4. Discussion

The reality that promising new modalities that otherwise prove successful in treating sepsis in an experimental model fail to translate successfully into a clinical setting, highlights the importance for utilising animal models that more closely reproduce the pathophysiological changes associated with human sepsis. Animal models of sepsis can be classified into three strategies: i) administration of bacterial wall fragments such as lipopolysaccharide or PepG (see chapter I, section 1.1.2); ii) administration of life bacteria or viruses (or injection of faeces); and iii) alteration of the endogenous protective barrier of the animal, such as the model of CLP (Djager *et al*., 2011). Furthermore, many of the therapeutic failures can be attributed to initiating clinical trials on the basis of results obtained in animal models that do not take into consideration the multiple factors, which have a bearing on the clinical outcome of patients. These factors include i) animals housed in specific pathogen-free facilities, ii) usage of inbred strains, iii) animals being of the same age, weight and gender. The reality is that patients are far more heterogenous with respect to gender, age, nutritional status, and supportive care than any animals used in preclinical studies (Djager *et al*., 2011).

I sought to refine an existing model of CLP (Wichterman *et al*., 1980) and develop a more clinically relevant model of CLP-induced cardiac dysfunction, by altering factors such as strain, sex, age, insult (bacterial load - needle size) and alterations in fluid resuscitation and antibiotic therapy, factors, which are thought to play a significant role in the development of organ dysfunction in animal models of CLP. In my study changes in gender as well as alterations in fluid resuscitation and antibiotic therapy did not appear to have an effect on the susceptibility of young inbred C57BL/6 and outbred CD-1 mice to develop cardiac dysfunction. In contrast, having seen the difficulty in obtaining reproducible cardiac dysfunction in young mice, I have set-up a modified model of sepsis-induced cardiac dysfunction in inbred C57BL/6 mice, based upon the standard polymicrobial CLP model with two distinctive features: i) use of aged mice, and ii) the use of appropriate fluid resuscitation and antibiotic therapy.

Various preclinical and clinical studies have demonstrated a gender dimorphism of the immune and organ responsiveness in susceptibility to and morbidity from shock, trauma, and sepsis (Angele *et al*., 2000). Females exhibit a form of protection in terms of their cardiovascular (Mizushima *et al*., 2000) and immunologic (Angele *et al*., 1997) responses during infectious challenge, while male patients demonstrate a higher incidence

of severe sepsis (Aube *et al*., 1992; Wichmann *et al*., 2000) and of infections in general (McGowan *et al*., 1975; Holmes *et al*., 1998; Offner *et al* 1999). The difference in the gender-mediated response to injury has been predominantly attributed to the differing hormonal milieu of each sex (De Maio *et al*., 2005). Various investigators that have demonstrated i) during the proestrus phase, oestrogen has effects on immune function and confers protection in sepsis models (Diodato *et al*., 2001; Merkel *et al*., 2001; Knoferl *et al*., 2002; Kher *et al*., 2005), ii) a decrease in testosterone levels by either castration or pharmacological blockade has shown to be beneficial following haemorrhagic shock (Remmers *et al*., 1997; Remmers *et al*., 1998), and iii) females in the proestrus phase are able to maintain splenic immune functions and, thus, tolerate sepsis better compared to their male littermates (Zellweger *et al*., 1997). I report here that young (2 month old) female C57BL/6 and CD-1 mice failed to develop significant cardiac dysfunction 24 h subsequent to undergoing CLP surgery. This finding was associated with an overall resistance to develop significant renal dysfunction and liver injury, two organs, which are known to fail early on in the septic syndrome (Abraham & Singer, 2007). I failed to see a gender difference in the response to CLP, as also male C57BL/6 and CD-1 mice demonstrated a similar trend, possibly as a result of the CLP procedure not being severe enough to result in organ dysfunction.

In addition, the implications of a genetic variance for the immune response to sepsis has been an area of great research, with previous investigators demonstrating that the response to endotoxaemia (De Maio *et al*., 1998; O'Malley *et al*., 1998) and polymicrobial sepsis (Stewart *et al.*, 2002) amongst inbred mice is strain-dependent, a factor leading to increased susceptibility amongst the C57BL/6 strain. Of note, the CLP protocol implemented by Stewart and colleagues, comprises of a single puncture technique with a 25 G needle size. Additionally, animals were only given 1 ml of saline post-operatively with no mention of administration of fluid resuscitation or antibiotics. It could be that this group wished to assess the mortality between the two strains they investigated using a model of severe sepsis but because the purpose of the study was to determine the influence of genetic variation in the response to sepsis, one would think to use a model that was more representative of the clinical setting. Therefore, I think the lack of fluid resuscitation and antibiotic administration in this case may have been a huge contributor to the increased mortality they observed in their C57BL/6 strain of mice. I failed to see a significant effect of the strain difference in mice on the susceptibility to succumb to cardiac dysfunction between the inbred and outbred mice. A possible

explanation for not seeing an increase in C57BL/6 susceptibility to organ injury/dysfunction which has been shown by other studies could due to the difference in model severity i.e. needle size and whether or not fluid resuscitation and antibiotic therapy was initiated as part of the protocol.

Currently, the therapeutic approach of patients with sepsis is centred on early administration goal-directed haemodynamic resuscitation and appropriate antibiotics (Hollenberg *et al*., 2004; Kumar *et al*., 2006). Clinically intravenous antibiotic therapy should be initiated immediately following obtaining positive blood culture. A retrospective cohort study of 14 intensive care units (ICU) found that each hour delay in antimicrobial administration correlates with decreased survival (Kumar *et al*., 2006). Other studies show that failure to initiate adequate antimicrobial therapy correlates with increased morbidity and mortality in septic patients admitted to the ICU (Ibrahim *et al.*, 2000; Garnacho-Montero *et al.*, 2003; Harbarth *et al*., 2003). Likewise, adequate fluid resuscitation serves as one of the keystones in the management of shock, with the purpose of counteracting the effects of the sepsis-induced hypovolaemia, by preserving intravascular fluid volume, restore effective tissue perfusion and re-establish and maintain a balance between tissue oxygen demand and supply (Vincent & Gerlach, 2004). Although patients with septic shock can be successfully resuscitated with crystalloids (i.e. Ringer's solution) and colloids (i.e. Hydroxyethyl starch), the choice of fluids in this setting remains controversial. Titration with either colloids or crystalloids to the same level of filling pressure restores tissue perfusion to the same degree (Rackow *et al.*, 1983). However, colloids are evidently the preferred choice in various countries despite the possible risks and higher costs compared to crystalloids (Finfer *et al.*, 2010). A choice seen to be due data suggesting that they improve cardiac performance more effectively in patients with hypovolaemia (Rackow *et al*., 1983; Trof *et al*, 2010) and the lower resuscitation volumes required (Lamke *et al*., 1976; Rackow *et al*., 1983), although the latter is put into question following a more recent study (Bayer *et al*., 2012).

This being said, the utilisation of sufficient antibiotic therapy and fluid resuscitation is still variable in animal models of sepsis. The importance of these supportive treatments in the severity of the sepsis response has been illustrated by using different resuscitation strategies and by the use of antibiotic in CLP. The importance of antibiotic in animal studies is highlighted in the study of Romero and colleagues, whereby they observed increased resistance of IFN-γ knockout mice to CLP-induced mortality (Romero *et al*., 2010). This was in contrast to the study of Echtenacher and colleagues in

which no antibiotic treatment was applied (Echtenacher *et al*., 2001). Furthermore, a study by Vianna and colleagues in a CLP model, demonstrated that animals treated with imipenem exhibited higher survival rates with significant infection control, effects explained by i) reduced levels of plasma TNF-α and plasma and peritoneal fluid levels of IL-6 and ii) higher endotoxin plasma concentrations, possibly as a result from rapid killing of bacteria in the peritoneum (Vianna *et al*., 2004). There was no difference in mortality nor in the susceptibility to develop organ dysfunction between the mice that had the normal fluid resuscitation and antibiotic therapy (Imipenem/Cilastatin with those that had an altered fluid resuscitation and antibiotic therapy. Although the purpose of altering the fluid resuscitation antibiotic therapy either by giving this late or not at all, was to try and induce significant cardiac dysfunction, it does not serve as a clinically relevant model for this particular study. Patients presenting or suspected to have sepsis are resuscitated with the preferred colloid solution and are put on a course of antibiotic therapy straight away to fight off the course of infection. Therefore, since the aim of setting up a CLP model of cardiac dysfunction is to be able to test a potential translatable drug (Chapter 3), it was important to mimic the clinical situation as much as possible. Thus a regime comprising of antibiotic therapy commencing at 6 h post-surgery to simulate a short window of undiagnosed sepsis, and continued 12 h after that, was applied in my final 24 h aged model which, successfully developed significant organ dysfunction. In retrospect, having used a crystalloid as the preferred choice of resuscitation fluid in this study, it would be interesting to see if there is any difference in the model response to the CLP insult when using a colloid such as hydroxyethyl starch, since a recent study showed improved short-term survival and acid-base balance when using a colloid compared with a crystalloid, in a model of endotoxin-induced septic shock (Kellum, 2002).

Sepsis is predominantly a disease affecting the elderly with recent epidemiologic studies demonstrating mean ages of approximately 65 years in United States and European intensive care units (Angus *et al*., 2001; Padkin *et al*., 2003). An observational study analysing approximately 500 geographically separated acute care hospitals in the United States during 1979 to 2002, showed that the incidence of sepsis is disproportionately increased in elderly adults, with age being an independent predictor of mortality (Martin *et al.,* 2006). Additionally, there is very good evidence from a multitude of studies documenting a relationship between age and increased mortality in CLP (Turnbull *et al*., 2009). Compared to young mice, aged mice subjected to CLP demonstrate increased systemic levels of inflammatory cytokines (Turnbull *et al*., 2003;

Saito *et al*., 2003) in addition to increased splenic apoptosis (Turnbull *et al*., 2004), and both of these two factors are associated with increased mortality in sepsis in humans (Hotchkiss *et al*., 1999; Bozza *et al*., 2007) and animal studies (Remick *et al*., 2002; Chang *et al*., 2007). I demonstrate that unlike young mice, 8-months old C57BL/6 male mice developed multi-organ failure (despite adequate fluid resuscitation and antibiotics therapy) including kidney dysfunction, hepatic injury, and most notably cardiac dysfunction at 24 h. The increased susceptibility of 8-months old mice to develop both renal dysfunction and hepatic injury is consistent with a prior study whereby Miyagi and colleagues demonstrated that inbred C57BL/6 only develop acute kidney injury at an age of 42-44 weeks (Miyagi *et al*., 2003).

Limitations

As with all animal studies, this study has certain limitations. Performing experiments in a temperature-controlled room on a heat mat does not necessarily equate to maintaining adequate body temperature. It is known that administration of anaesthetics leads to significant loss of body temperature (Gardner *et al*., 1995; Sessler & Akca, 2002) and that due to the nature of the surgical procedure in CLP, post-operative hypothermia becomes a factor that modulates outcome (Nemzek *et al*., 2008), as studies demonstrate that correction of perioperative hypothermia has been shown to decrease the incidence of wound infections (Kurz *et al.,* 1996; Xiao & Remick, 2005). Therefore, more stringent measurement and thus control of individual animal body temperature should be adhered to for future experiments. In addition, some groups in this study were made up of low *n* numbers, a result of not only financial constraints but also insufficient experimental planning. Therefore, for these particular groups any organ-specific effects seen should be confirmed by doing more *n* numbers. It is not expected that cardiac parameters for naïve animals would be any different from sham animals (Takuma *et al*., 2001; Roth *et al*., 2002). Since the shams in this study exhibit normal cardiac parameters which are expected for such animals under the influence of anaesthesia, one could assume that they are not exerting any effect on the parameters measured. Nevertheless, the inclusion of naïve animals serves as part of good experimental practice and will exclude the possibility of shams having any effect on the parameters measured. Furthermore, inclusion of age-specific sham controls should be made not only to ensure that there is no age-dependent

effects on any parameters measured for the sham groups, but also to allow the correct comparison of age-specific CLP treated animals to their respective shams.

Conclusion

I demonstrate that young inbred and outbred mice show resistance to develop cardiac dysfunction following CLP. Having utilised 8-months old mice, I managed to establish a model of renal dysfunction, hepatic injury, and most notably consistent cardiac dysfunction, whilst employing a clinically relevant fluid resuscitation and therapy.

Chapter 3

Erythropoietin Attenuates Cardiac Dysfunction in Experimental Sepsis via Activation of the β-Common Receptor

3.1. Introduction

The haematopoietic growth factor erythropoietin (EPO) has long been known to function in maintaining optimal tissue oxygenation through the regulation of erythrocytes, via a negative feedback control system, which enables cross-talk between the kidney and the bone marrow (Sharples & Yaqoob, 2006). Recombinant human EPO (rhEPO) is widely recognised clinically for the management of anaemia associated with end-stage renal disease (Eschbach *et al*., 1987) and non-myeloid malignancies (Gabrilove *et al*., 2001). However, during the past two decades, our understanding of the actions of EPO has shifted from a belief that the hormone acts exclusively on haematopoietic progenitor cells to the knowledge that this agent possesses much broader salutary effects, like those seen in preclinical models of, haemorrhagic shock (Abdelrahman *et al*., 2004), and endotoxic shock (Aoshiba *et al*., 2009).

The beneficial non-erythropoietic effects of EPO have been reported to be 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), present also in the myocardium (Brines *et al*., 2004). The βcR is also the signal-transducing component common to IL-3, IL-5, and granulocyte-macrophage colony stimulating factor receptors (D'Andrea & Gonda, 2000). In addition, the 'tissue-protective receptor' is thought to specifically bind to EPO with a lower affinity than the classical EPOR (Masuda *et al*., 1993).

The discovery of a functional EPO receptor in the cardiovascular system, particularly in endothelial cells (Anagnostou *et al*., 1994) and cardiomyocyes (Wright *et al*., 2004), as well as the finding of a cellular co-localization of EPO, βcR and EPOR in cardiomyocytes (Brines *et al.*, 2004) points towards a role of EPO in modulating cardiac function. Evidence from animal models support the notion that EPO (Cai *et al.*, 2003; Calvillo *et al*., 2003) as well as non-haematopoietic tissue-protective EPO-analogues such as carbamylated EPO (Moon *et al*., 2006; Ueba *et al*., 2010) exert cardioprotective effects in a variety of disease models. These effects are primarily through the inhibition of myocardial apoptosis (Calvillo *et al.*, 2003), the reduction in inflammation (Rui *et al*., 2005), and the induction of angiogenesis (Carlini *et al*., 1995), to name but a few.

Therefore, I sought to investigate the effects of EPO and, specifically, the role of the βcR in the cardiac dysfunction associated with endotoxaemia (caused by LPS) and

polymicrobial sepsis (caused by CLP). Specifically, I have investigated i) the effect EPO has on the impairment of systolic contractility (measured *in vivo* by echocardiography or *ex vivo* in the isolated Langendorff-perfused heart) associated with endotoxaemia and polymicrobial sepsis using WT C57BL/6 mice; ii) whether the effect of EPO is mediated through the proposed tissue protective receptor, with the use of βcR KO mice; iii) the mechanisms underlying the observed beneficial effects of EPO including the phosphorylation of Akt on Ser^{473}, phosphorylation of GSK-3β on Ser^{9}, phosphorylation of eNOS on Ser^{1177}, activation of NF-κB (measured as nuclear translocation of p65) and expression of IL-1β. Because sepsis most frequently occurs in aging patients (Girard *et al*., 2005), I subsequently investigated the effects of EPO and the role of the βcR in aging animals with sepsis.

3.2. 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 Stationary 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) male C57BL/6 mice (Harlan Laboratories, Wyton, UK) and 101 βcR KO male mice (B6.129S1-Csf2rbtm1Cgb/J) on a C57BL/6J genetic background (see below for further details) weighing 24.65 ± 1.29 g (2 months old) or 32.30 ± 1.34 g (8 months old), receiving a standard diet and water *ad libitum*.

Animals were either administered vehicle (saline) or EPO (1000 IU kg^{-1}). This dose of EPO was chosen as it was previously shown to confer tissue protection in a model of endotoxaemia (Aoshiba *et al*, 2009) which, I wanted to use as a starting point in this study. Although this dose exceeds that given routinely (100 – 200 IU kg^{-1} $week^{-1}$; Aoshiba *et al*., 2009) for patients with anaemia, as well as the circulating plasma concentrations of EPO (6-32 IU kg^{-1}; Jelkmann, 2011) various studies have demonstrated that a larger dose is needed to protect against animal organ injury and dysfunction in endotoxaemia/sepsis (Abdelrahman *et al*., 2004; Kao *et al*., 2007; Mitra *et al.*, 2007)

Mice were anaesthetised with a ketamine (100 mg ml^{-1}) and xylazine (20 mg ml^{-1}) mixture (2:1; 1.5 ml kg^{-1}, i.p.) before sacrifice. At 16-18 h after the administration of LPS (9 mg kg^{-1} i.p.) or 24 h after CLP surgery, cardiac function was assessed by echocardiography and/or the isolated Langendorff-perfused heart. Additionally, heart samples were taken and stored at -80 ^{0}C for further analysis.

βcR KO mice

βcR KO mice colonies were kindly donated from M. Brines and A. Cerami (Kenneth S. Warren Institute, Kitchawan, New York) and these were bred and maintained at Queen Mary University of London, Biological Services Unit. These mice are grossly normal phenotypically and are fertile (Brines *et al*., 2004), exhibit a normal baseline haematopoiesis except for a low basal circulating eosinophil level (Scott *et al*., 1998). They have been shown to respond normally to infectious insult (Nishinakamura *et al*.,

1996) however, ultimately develop a progressive pulmonary fibrosis with advancing age (Brines *et al*., 2004). Breeding was achieved with Trio's (one male and two females) per individually ventilated cage which, were equipped with their nesting material, environmental toys and tunnel, to enable nest making for the mice. Litters were weaned and transferred into separate male/ female cages after approximately 21 days. Although at this age mice were genotyped to confirm identification, confirmation of βcR -/- was accomplished for randomly picked mice from different cages by PCR genotyping as mentioned below).

Isolation of cDNA

Approximately 0.2 cm of ear clip was taken from randomly selected βcR KO mice and placed into a 0.5 ml Eppendorf tube and stored at -20 ^{0}C until ready to digest. The ear clips were digested in a 50 µl (to 200 µg ml^{-1} final concentration) mixture of lysis buffer (100 mM Tris pH8, 500 mM KCL, 0.1 mg ml^{-1} Gelatin, 0.45 % NP-40, 0.45 % Tween 20) and protease K (500 µg ml^{-1}). The mixture was first incubated at 55 ^{0}C in a water bath overnight and then spun briefly the next day. Following incubation at 94 ^{0}C for 10 min, the mixture was spun at 3, 000 rpm for 5 min in a bench centrifuge fitted with a plate carrier, in order to isolate the cDNA.

PCR analysis

A standard polymerase chain reaction was carried out. Primers (Beta 823, GTGTAGACACTGGCCCCCG; Beta 891, GAACCTTCAATGCTTCTTTGATGGGAT; Beta Neo F, ATATTGGTGAAGAGCTTGGCGGC) were initially reconstituted in dH_2O to produce stock 100 µM solutions. These were further diluted in dH_2O to achieve working concentrations of 10 µM. For each ear clip the following components were added to a sterile 0.5-ml microcentrifuge tube sitting on ice: 10 uL 10X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl) [final concentration was 1x], 2 uL10 mM dNTP mixture final concentration was 0.2 mM], 3 uL 50 mM MgCl2 [final concentration was1.5 mM], 5 µL each Primer (10 µM each) [final concentration was 0.5 µM], 2 µL Template DNA, 0.5 µL *Taq* DNA Polymerase (5 U/µL, New England Biolabs, Hertfordshire, UK), Autoclaved distilled water to 100 µL. For multiple ear clips a master mix was prepared. The tube was loaded into a PCR thermocycler (G-storm, East Sussex, UK). The

amplification reactions started at 94 °C for 5 min, cycled at 35 times at 94 °C for 30 sec, 60 °C for 50 sec, 72 °C for 3 min, a 7 min hold at 72 °C followed by a 4 °C hold.

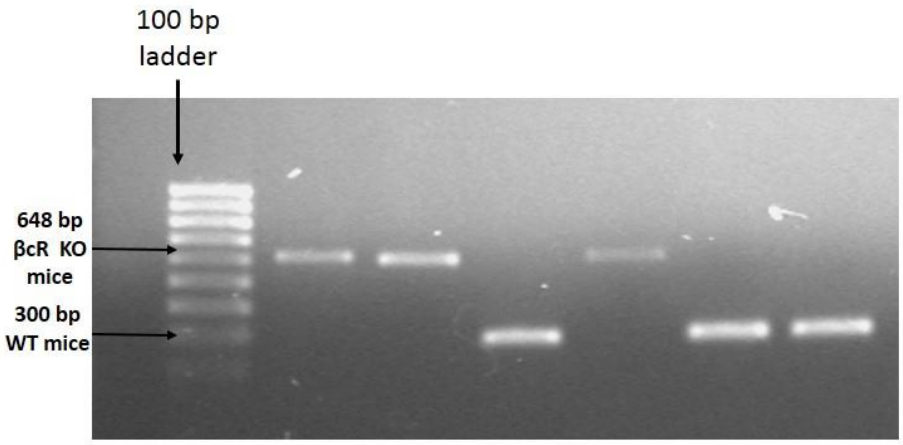


Figure 3.2.3. βcR KO mice genotyping PCR product. The murine mutant βcR gene (648 bp) was amplified from ear clip samples of βcR KO mice using three primers for the βcR gene (Beta 823, Beta 891, Beta Neo F). Twenty microliters of the PCR product was visualised by electrophoresis on a 1 % agarose gel (containing ethidium bromide and 1 x Tris borate) alongside a 100 bp DNA ladder.

Caecal Ligation and Puncture

Methodology is as described in chapter 2. Based on previous evidence and preliminary data (Chapter 2) an 18-G needle was used with the double puncture technique in order to generate cardiac dysfunction during the early phase of sepsis (24 h) in aging mice.

Experimental Design

Animals were allocated into the following studies, receiving the relevant treatment:

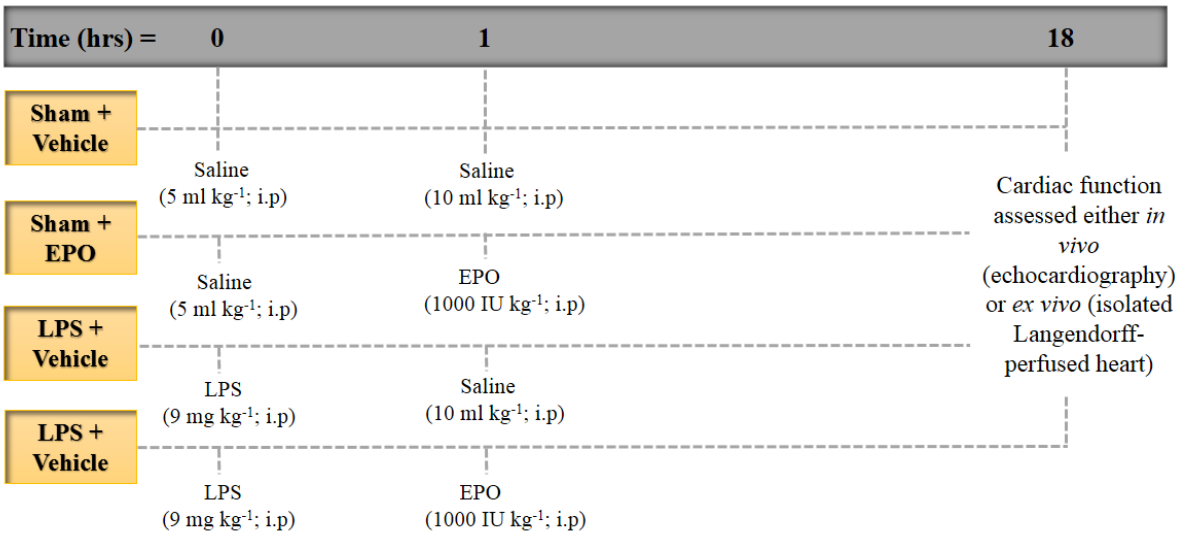
Study 1 - Cardiac function was assessed in 2 months old mice randomized to undergo either i) sham operation and treated with either vehicle (Sham + Vehicle) or EPO (Sham + EPO) or ii) subjected to endotoxaemia for 16-18 h and treated with either vehicle (LPS + Vehicle) or EPO (LPS + EPO).

Study 2 - Cardiac function was assessed in 8 months old mice randomised to undergo either i) sham operation and treated with either vehicle (Sham + Vehicle) or EPO (Sham

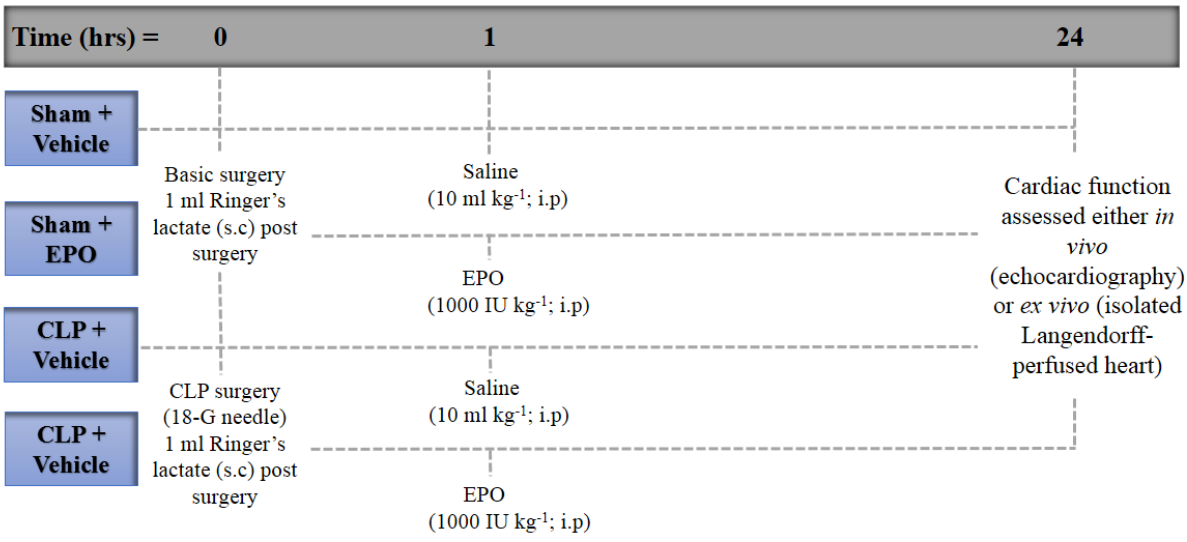
+ EPO) or ii) CLP for 24 h and treated with either vehicle (CLP + Vehicle) or EPO (CLP + EPO). The detailed protocols of the 2 study groups are shown in Figure 3.2.1

Figure 3.2.1 Schematics showing the experimental protocols for mice subjected to either (A) endotoxaemia or (B) CLP.

(A)



(B)



Assessment of Cardiac Function *in vivo* (Echocardiography)

Methodology is as described in chapter 2. Cardiac function was assessed 18 h after administration of LPS or 24 h after CLP surgery. Left ventricular end-diastolic volume

(LVEDV) and left ventricular internal dimension-diastole (LVIDD) were calculated from M-mode in the parasternal short axis view at the level of the papillary muscles.

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 h after the administration of LPS, mice were anaesthetised and heparinised (heparin sodium, 1000 IU/100g, 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, $MgSO_4$ 1.19, $NaHCO_3$ 25, $CaCl_2$ 1.25, KH_2PO_4 1.18, sodium pyruvate 5, and glucose 10; equilibrated with 95 % O_2/ 5 % CO_2 (pH 7.4). Aortic cannulation was performed (within 1 min of excision), hearts were perfused immediately with filtered KHB, gassed continuously with 95 % O_2/ 5 % CO_2 and maintained at 37 ^oC. Hearts were retrogradely perfused in a non-recirculating Langendorff mode at a constant flow. 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 left ventricle (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 inserted into the wall of the right atrium and then allowed to stabilise for at least 10 min before any experimental protocols were carried out. Alterations in isovolumic LV developed pressure (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

Heart samples were homogenized at 10 % (w v^{-1}) in a Potter Elvehjem homogenizer (Wheaton, Millville, NJ, USA) using a homogenization buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 % NP-40, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethyl sulphonyl fluoride (PMSF), 5 µg ml^{-1} aprotinin, and 2.5 µg ml^{-1} leupeptin. Homogenates were centrifuged at 4, 000 RPM (1323 g) for 5 min at 4 ^0C, after which the supernatant was centrifuged at 14, 000 RPM (16250 g) for 40 min to obtain the cytosololic fraction. The pelleted nuclei was resuspended in extraction buffer containing 20mM HEPES, pH

7.9, 1.5 mM $MgCl_2$, 0.2mM EDTA, 1mM EGTA, 20 % glycerol, 420 mM NaCl, 1mM DTT, 0.5 mM PMSF, 5 µg ml^{-1} aprotinin, and 2.5 µg ml^{-1} leupeptin. Pellet was incubated in ice for 30 min (with occasional vortexing) and was centrifuged at 14, 000 for 20 min at 4 ^{0}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 kit following the manufacturer's directions (Thermo Fisher Scientific, Rockford, IL). Briefly, 30 µl of sample was added to 27 µl of dH_2O and 570 µl of a mixture (1:50 of Cu^{2+}; BCA reagent). The plate was read at 570 nm yielding a graph to enable protein contents of the sample to be determined.

Fifty µg of cytosolic or nuclear protein was loaded. Proteins were separated by 8 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinyldenedifluoride membrane, which was then incubated with a solution of albumin 5 % in PBS for 1 h. Membranes were incubated with primary antibody (rabbit anti-total GSK-3β, dilution 1:200; goat anti-pGSK-3β Ser^9 dilution 1:200, rabbit anti-total Akt, dilution 1:1000, mouse anti-pAkt Ser^{473}, dilution 1:1000, goat anti-peNOS Ser ^{1177}, dilution 1:200, rabbit anti-eNOS, dilution 1:200, and rabbit anti-NF-κB, dilution 1:1000) over night at 4 ^{0}C. Blots were incubated with secondary antibody conjugated with horseradish peroxidase for 30 min in at room temperature and developed with the enhanced chemiluminescence detection system. The immunoreactive bands were visualized by autoradiography and the density of the bands was evaluated densitometrically using the Quantity One 1-D Analysis software (Bio-Rad Laboratories Ltd, Hertfordshire, UK). The membranes were stripped and incubated with β-actin monoclonal antibody for 30 min and subsequently with anti-mouse antibody for 30 min, at room temperature, in order to assess gel-loading homogeneity. Relative band intensity was assessed and normalized against parallel β-actin expression. In the sham group, the immunoreactive bands of the gel were respectively measured and normalised 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 an average value of 1 is relative to the first immunoreactive band. Each group was then adjusted against corresponding sham-control data to establish relative protein expression when compared to sham-control animals.

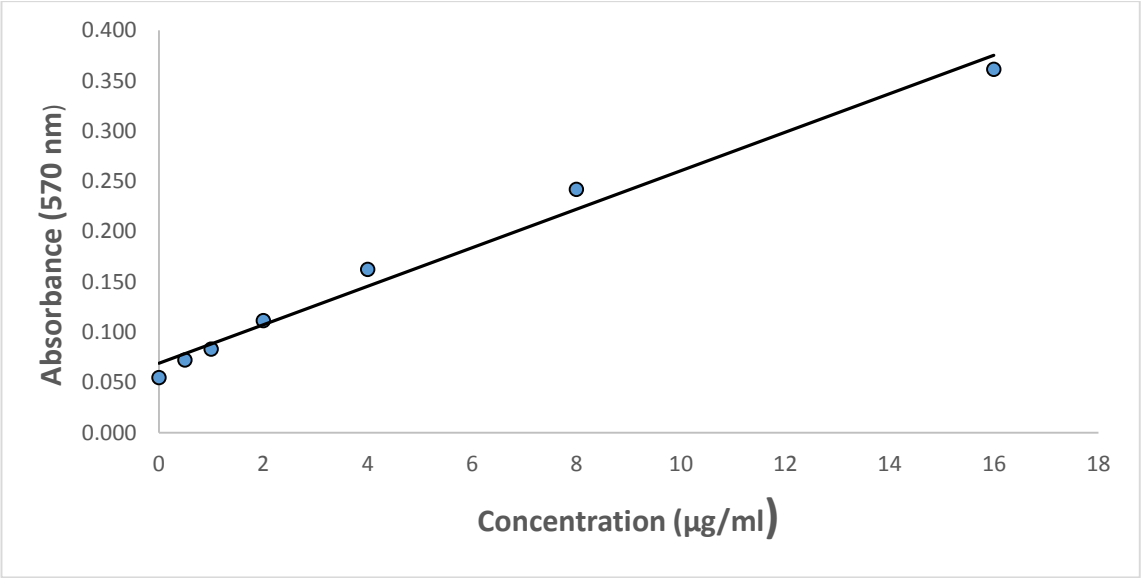


Figure 3.2.2. A representative standard protein curve for western blot analysis

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, USA) which, is specific to recognise natural and recombinant IL1β with no significant cross-reactivity/interference between different species. This assay exhibits the sensitivity to detect a minimum detectable dose of mouse IL1β in the range from 0.46 – 4.80 pg/ml, with a mean of 2.31 pg/ml.

Heart samples were homogenized at 10 % (w v^{-1}) in a Potter Elvehjem homogenizer (Wheaton, Millville, NJ, USA) using a homogenization buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 % NP-40, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethyl sulphonyl fluoride (PMSF), 5 µg ml^{-1} aprotinin, and 2.5 µg ml^{-1} leupeptin. Homogenates were centrifuged at 4, 000 RPM (1323 g) for 5 min at 4 ^{0}C to remove cell debris after which aliquots of the cell lysate was removed in preparation for the ELISA analysis. 50 µl of Assay Diluent RD1N was added to each well of the microplate. Once 50 µl of Standard, Control or Sample were added per well, the plate was covered with adhesive strip and left to incubate for 2 h at room temperature. Each well was washed with Wash Buffer (400 µl) and aspirated, a process that was repeated 4 times. Once 100 µl of mouse IL1β Conjugate was added to each well, the plate was covered with an adhesive strip and

left to incubate for 2 hrs at room temperature. The plate was aspirated/washed again as previously described, followed by the addition of 100 µl of Substrate Solution to each well. Subsequently the plate was incubated for 30 min at room temperature, away from light. Following the addition of 100 µl of Stop Solution was added to the plate and thoroughly mixed, the optical density of each well was determined using a microplate reader set at 570 nm.

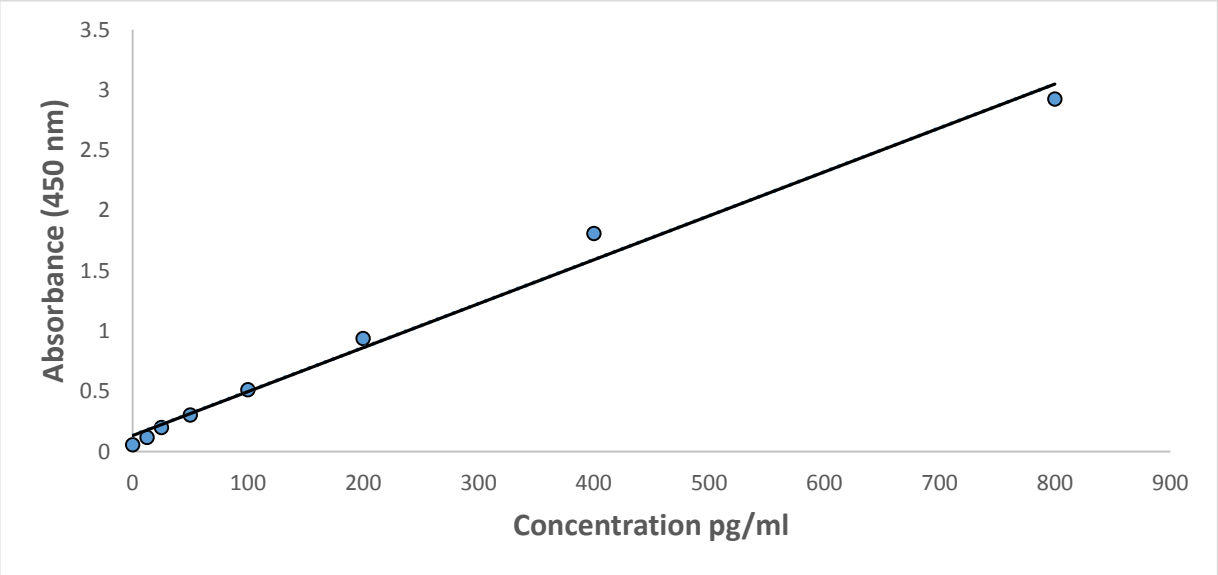


Figure 3.2.3. A representative standard curve for mouse IL-1β ELISA assay

Materials

All solutions were prepared using non-pyrogenic saline (0.9 % w v^{-1} NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK). *Escherichia coli* LPS (serotype 0111:B4) was purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Recombinant human EPO (epoetin beta) was manufactured by Roche Diagnostics (Sussex, UK). The BCA protein Assay kit, SuperBlock blocking buffer and ECL detection reagent were from Thermo Scientific UK Ltd (Loughborough, UK). Antibodies against rabbit anti-total GSK-3β, rabbit anti-total Akt, mouse anti-pAkt Ser^{473}, rabbit anti-NF-κβ p65 were purchased from New England Biolabs (Ipswich, MA, US).

Statistical Analyses

All values described in the text and figures are presented as mean ± SEM of n observations, where n represents the number of animals studied. Statistical analysis was performed using GraphPad Prism 5.0d (GraphPad Software, San Diego, California, USA). 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.

3.3. Results

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

To investigate the effect EPO has on the LPS-induced cardiac dysfunction, LV function was assessed using echocardiography in WT mice, 18 h after administration of vehicle or LPS. There were no differences in LV dimensions (LVIDD and LVEDV) between the WT groups (Table 3.3.1). Figure 3.3.1A shows representative M-mode echocardiograms of Sham + Vehicle, Sham + EPO, LPS + Vehicle, and LPS + EPO in WT mice. When compared to Sham + Vehicle mice, sham mice treated with EPO demonstrated no significant alterations in percentage ejection fraction, fractional shortening, and fractional area change ($P > 0.05$) (Figures 3.3.1B-D). When compared to sham mice, mice subjected to 18 h of endotoxaemia demonstrated a significant reduction in percentage ejection fraction, fractional shortening, and fractional area change ($P < 0.05$) (Figures 3.3.1B-D), indicating impairment in systolic contractility *in vivo*. Administration of EPO significantly attenuated the impairment in systolic contractility associated with endotoxaemia ($P < 0.05$) (Figures 3.3.1B-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 LVDP were assessed in WT mice 16-18 h 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. When compared to 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 ($P < 0.05$) (Figures 3.3.1E-F). Administration of EPO to endotoxaemic WT mice significantly attenuated the impairment in systolic contractility at this volume load ($P < 0.05$) (Figures 3.3.1E-F, one-way ANOVA followed by Bonferroni post-hoc test).

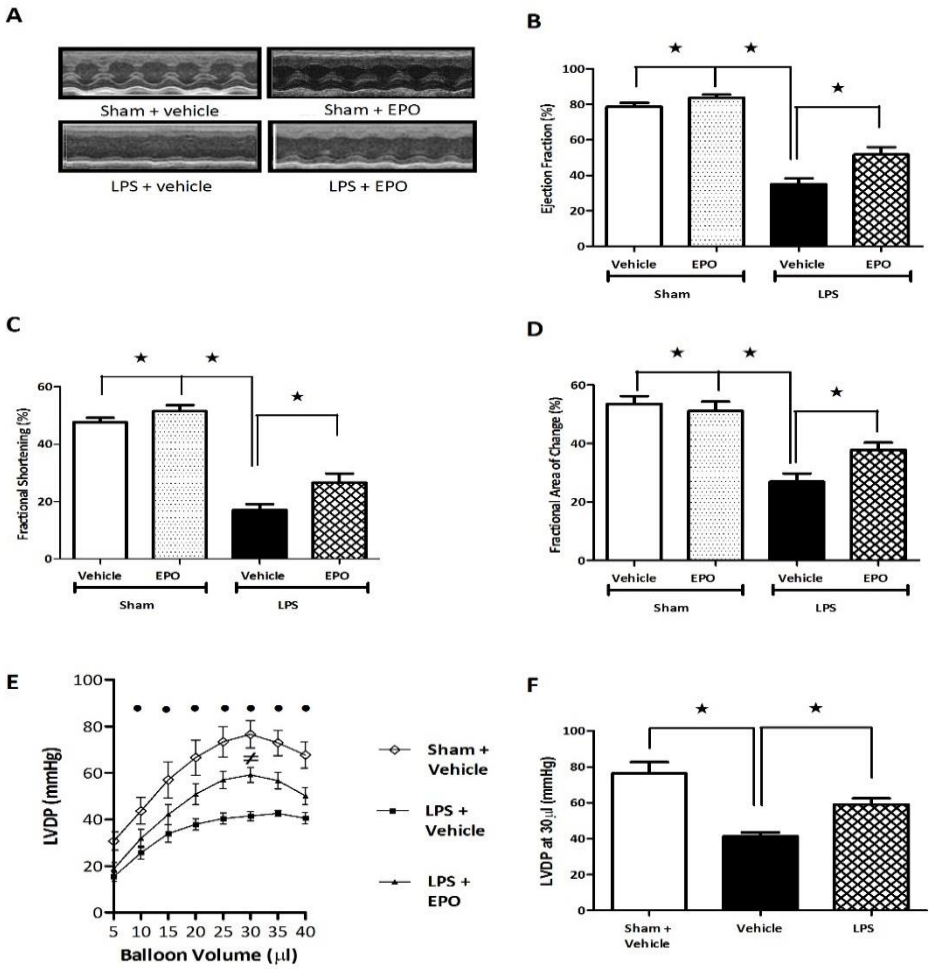


Figure 3.3.1. Effect of EPO on the cardiac dysfunction in endotoxaemic WT mice. Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) 18 h subsequent to sham-operation or LPS administration. Volume-pressure curves were generated from isolated Langendorff-perfused hearts to assess alterations in isovolumic left ventricular developed pressure in response to 5 µl incremental increases in balloon volume (E), and in response to 30 µl (maximum response) volume loading (F), 16-18 h subsequent to sham-operation or LPS administration. Mice received either LPS (9 mg kg^{-1} i.p.) or vehicle (5 ml kg^{-1} 0.9% saline i.p.). At one hour after induction of endotoxaemia mice were treated either with Erythropoietin (EPO) (1000 IU kg^{-1} s.c.) or vehicle (10 ml kg^{-1} 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 B-D + F, one-way ANOVA followed by Bonferroni post-hoc test; Fig E, two-way ANOVA followed by Bonferroni post-hoc test). (Figure taken from Master's thesis 2010)

Effect of EPO on cardiac dysfunction in endotoxaemic 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, I evaluated the cardioprotective effects of EPO in βcR KO mice. There were no differences in LV dimensions between the KO groups with the exception of a significant increase in LVIDD between Sham + Vehicle and endotoxtaemic βcR KO mice, which was not altered with the administration of EPO (Table 3.3.1). Figure 3.3.2A shows representative M-mode echocardiograms of Sham + Vehicle, Sham + EPO, LPS + Vehicle, and LPS + EPO in βcR KO mice. When compared to Sham + Vehicle mice, sham mice treated with EPO demonstrated no significant alterations in ejection fraction, fractional shortening, and fractional area change ($P > 0.05$) (Figures 3.3.2B-D). When compared to sham βcR KO mice, endotoxaemia in βcR KO mice resulted in a significant reduction in percentage ejection fraction, fractional shortening, and fractional area change ($P < 0.05$) (Figures 3.3.2B-D), indicating impairment in systolic contractility. Administration of EPO to endotoxaemic βcR KO mice did not alter this impairment in systolic contractility ($P > 0.05$) (Figures 3.3.2B-D).

I sought to confirm these effects in an isolated, perfused heart *ex vivo* (in the absence of any influences of cardiac pre- or afterload). When compared to sham mice, βcR KO 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 ($P < 0.05$) (Figures 3.3.2E-F). Administration of EPO to endotoxaemic βcR KO mice caused no significant change in the impairment in systolic contractility associated with endotoxaemia ($P > 0.05$) (Figures 3.3.2E-F).

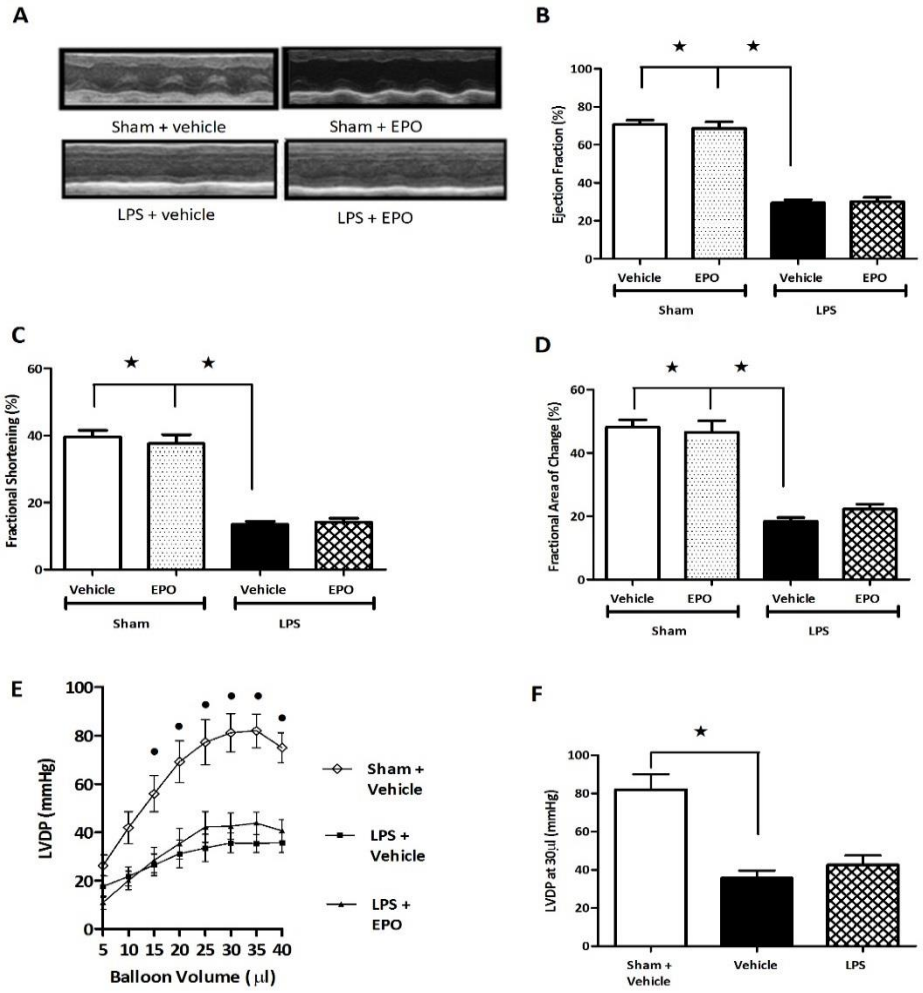


Figure 3.3.2. Effect of EPO on the cardiac dysfunction in endotoxaemic βcR KO mice. Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) 18 h subsequent to sham-operation or LPS administration. Volume pressure curves were generated from isolated Langendorff-perfused hearts to assess alterations in left ventricular developed pressure in response to 5 µl incremental increases in balloon volume (E), and in response to 30 µl (maximum response) volume loading (F), 16-18 h subsequent to sham-operation or LPS administration. Mice received either LPS (9 mg/kg i.p.) or vehicle (5 ml kg^{-1} 0.9% saline i.p.). At 1 hour after induction of endotoxaemia, mice were treated either with Erythropoietin (EPO) (1000 IU kg^{-1} s.c.) or vehicle (10 ml kg^{-1} 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 (Fig B-D + F, one-way ANOVA followed by Bonferroni post-hoc test; Fig E, two-way ANOVA followed by Bonferroni post-hoc test). (Figure taken from Master's thesis 2010)

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

In order to gain a better insight into the potential mechanism(s) underlying the observed cardioprotective effects of EPO, I 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 to sham mice, WT and βcR KO mice subjected to LPS demonstrated no change in the phosphorylation of Akt on Ser^{473} ($P > 0.05$; 60 kDa) (Figure 3.3.3A), GSK-3β on Ser^{9} ($P > 0.05$; 46 kDa) (Figure 3.3.3B), and eNOS on Ser^{1177} ($P > 0.05$; 140 kDa) (Figure 3.3.3C). Administration of EPO to endotoxaemic WT mice, however, resulted in a significant increase in the phosphorylation of Akt on Ser^{473} ($P < 0.05$) (Figure 3.3.3A), GSK-3β on Ser^{9} ($P < 0.05$) (Figure 3.3.3B), and eNOS on Ser^{1177} ($P < 0.05$) (Figure 3.3.3C). In contrast, administration of EPO to endotoxaemic βcR KO mice caused no change in the phosphorylation of Akt on Ser^{473} ($P > 0.05$) (Figure 3.3.3A), GSK-3β on Ser^{9} ($P > 0.05$) (Figure 3.3.3B), or eNOS on Ser^{1177} ($P > 0.05$) (Figure 3.3.3C).

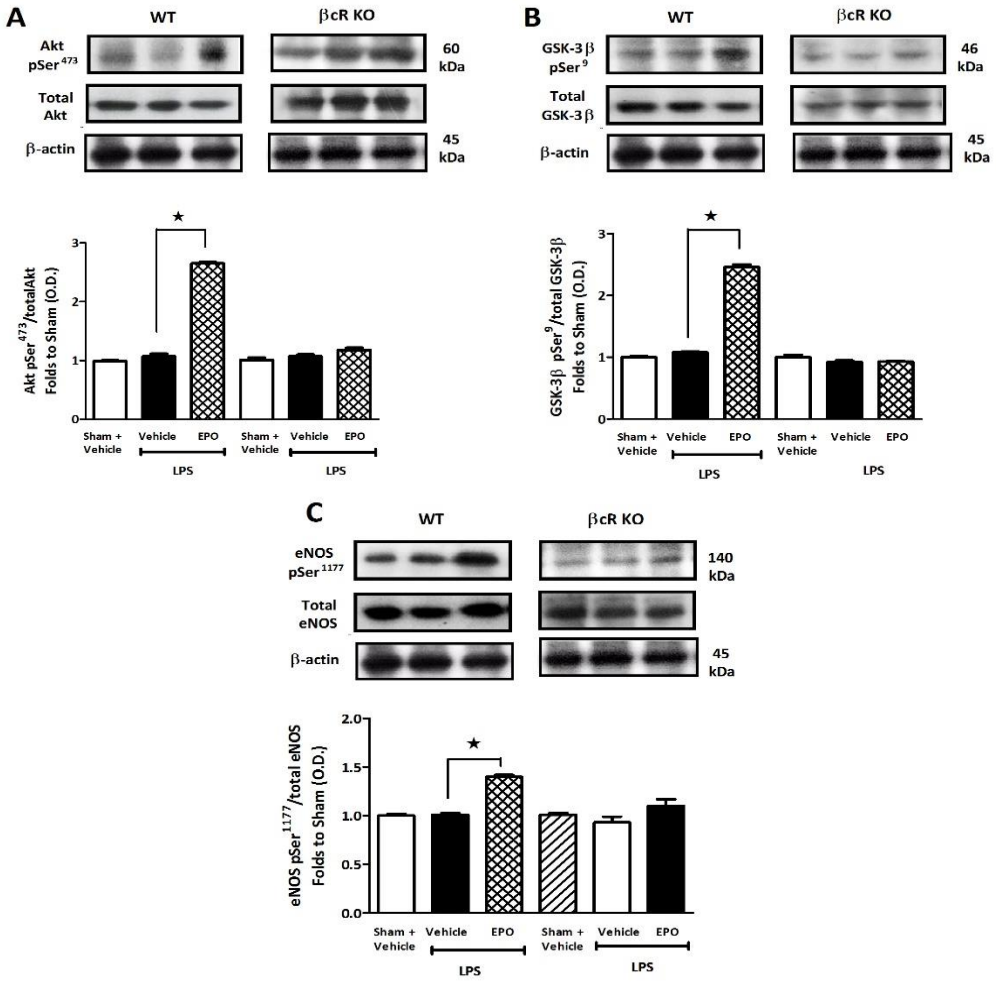


Figure 3.3.3. Effect of EPO on signalling pathways in the hearts of WT and βcR KO mice with endotoxaemia. Mice received either LPS (9 mg kg^{-1} i.p.) or vehicle (5 ml kg^{-1} 0.9% saline i.p.). One hour after induction of endotoxaemia mice were treated either with Erythropoietin (EPO) (1000 IU kg^{-1} s.c.) or vehicle (10 ml kg^{-1} 0.9% saline s.c.). Densitometric analysis (Quantity One 1-D Analysis Software) of the bands is expressed as relative optical density (O.D.) of (A) Akt phosphorylation at Ser^{473} ($pSer^{473}$), corrected for the corresponding total Akt content and normalized using the related sham band, (B) GSK-3β phosphorylation at Ser^{9} ($pSer^{9}$), corrected for the corresponding total GSK-3β content and normalized using the related sham band, (C) eNOS phosphorylation at Ser^{1177} ($pSer^{1177}$), corrected for the corresponding total eNOS content and normalized using the related sham band. Each immunoblot is from a single experiment and is representative of four separate experiments. Data are expressed as means ± S.E.M. for *n* number of observations.★ $P < 0.05$ versus LPS + Vehicle, one-way ANOVA followed by Bonferroni post-hoc test. Blots generated by M. Collino, University of Turin.

Effect of EPO on the nuclear translocation of the p65 NF-κB subunit in the hearts of endotoxaemic WT and βcR KO mice

When compared to hearts from sham mice, the hearts of endotoxaemic WT and βcR KO mice exhibited significant increases in the nuclear translocation of the p65 subunit ($P < 0.05$) (Figure 3.3.4), indicating activation of NF-κB (65 kDa). Administration of EPO to endotoxaemic WT mice significantly attenuated the increase in nuclear translocation of p65 and, hence, inhibition of activation of NF-κB in the heart ($P < 0.05$) (Figure 3.3.4). In contrast, administration of EPO to endotoxaemic βcR KO mice did not attenuate the nuclear translocation of p65 caused by LPS ($P > 0.05$) (Figure 3.3.4).

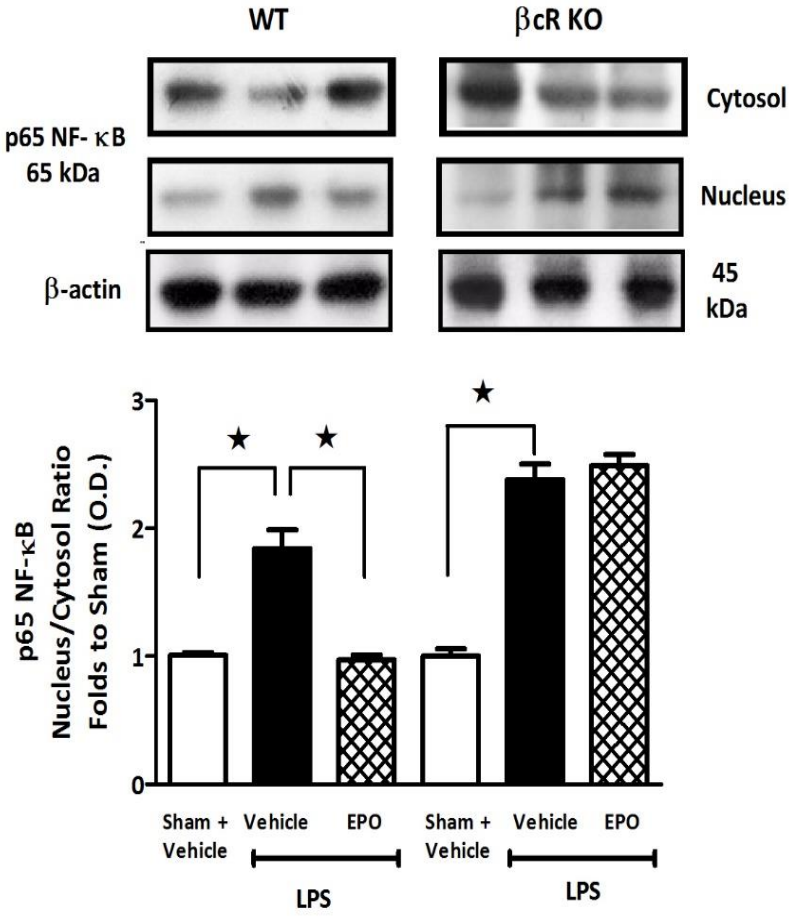


Figure 3.3.4. Effect of EPO on signalling pathways in the hearts of WT and βcR KO mice with endotoxaemia. Mice received either LPS (9 mg kg^{-1} i.p.) or vehicle (5 ml kg^{-1} 0.9% saline i.p.). One hour after induction of endotoxaemia mice were treated either with EPO (1000 IU kg^{-1} s.c.) or vehicle (10 ml kg^{-1} 0.9% saline s.c.). Densitometric analysis (Quantity One 1-D Analysis Software) of the bands is expressed as relative optical density (O.D.) of NF-κB p65 subunit levels in both cytosolic and nuclear fractions and expressed as a nucleus/cytosol ratio. Each immunoblot is from a single experiment and is representative of four separate experiments. Data are expressed as means ± S.E.M. for *n* number of observations.★ $P < 0.05$ versus LPS + Vehicle, one-way ANOVA followed by Bonferroni post-hoc test. Blots generated by M. Collino, University of Turin.

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

IL-1β expressed by various cells namely mononuclear leukocytes is a pro-inflammatory cytokine, known to many of the immunopathological features of the response during sepsis (Cannon *et al*., 1990). This role in sepsis has been well documented in numerous reports in experimental models (Hesse *et al*., 1988) and human studies (Hesse *et al*., 1988; Cannon *et al*., 1990). Circulating concentrations of IL-1β as well as other pro-inflammatory cytokines is not only associated to morbidity and mortality in patients with sepsis (Casey *et al*., 1993) but has also been linked to acting as a cardio depressant factor in sepsis (Hoffman *et al*., 1999; Cain *et al*., 1999; Fernandes Jr & Cesar de Assunca, 2012).

When compared to hearts from sham mice, the hearts of endotoxaemic WT and βcR KO mice demonstrated a significant increase in IL-1β expression ($P < 0.05$) (Figure 3.3.5). Administration of EPO to endotoxaemic WT mice significantly attenuated IL-1β expression ($P < 0.05$). In contrast, administration of EPO to endotoxaemic βcR KO mice caused no alteration in IL-1β expression ($P > 0.05$) (Figure 3.3.5). To note, it was surprising to find that the baseline IL-1β expression was significantly higher in the Sham Vehicle and LPS Vehicle groups of the βcR KO mice compared to their corresponding groups in the WT animals.

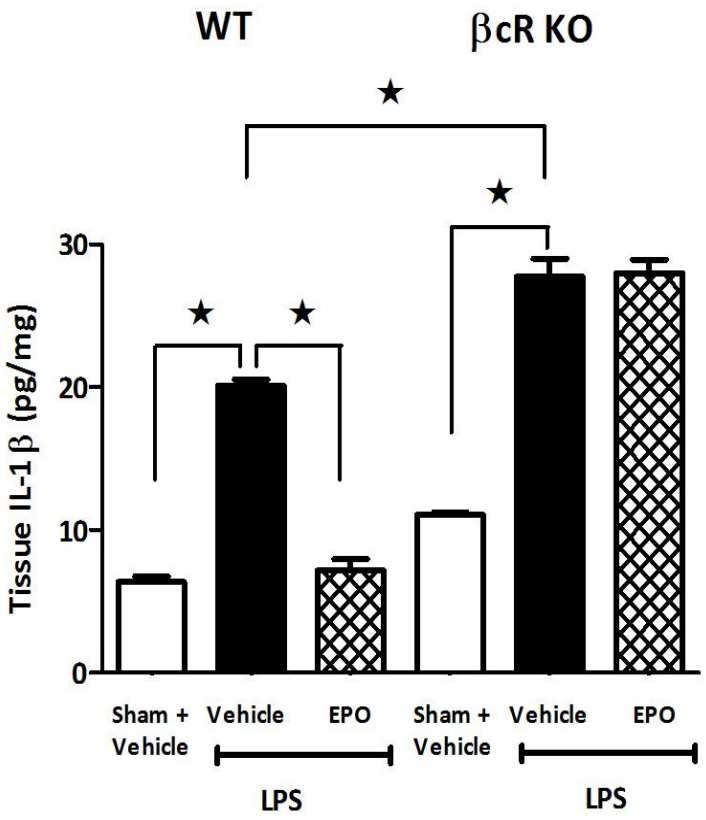


Figure 3.3.5. Effect of EPO on IL-1β expression in the hearts of WT and βcR KO mice with endotoxaemia. Concentration of IL-1β was quantitatively measured in mouse heart tissues by using a mouse IL-1β/IL-1F2 Immunoassay kit. WT or βcR KO mice received either LPS (9 mg kg^{-1} i.p. n = 3) or vehicle (5 ml kg^{-1} 0.9% saline i.p. n = 3). One hour after induction of endotoxaemia mice were treated either with Erythropoietin (EPO) (1000 IU kg^{-1} s.c. n = 3) or vehicle (10 ml kg^{-1} 0.9% saline s.c.). Data are expressed as means ± S.E.M. for n number of observations. ★ $P < 0.05$ (one-way ANOVA followed by Bonferroni post-hoc test). ELISA experiment was performed by M.Collino, University of Turin, Italy.

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

The murine model of CLP with fluid resuscitation and antibiotics is regarded as a clinically relevant model of abdominal polymicrobial human sepsis. There were no differences in LV dimensions (Table 3.3.1) between the WT groups. Figure 3.3.6A shows representative M-mode echocardiograms of Sham + Vehicle, Sham + EPO, CLP + Vehicle, and CLP + EPO in WT mice. When compared to Sham + Vehicle mice, sham mice treated with EPO demonstrated no significant alterations in EF, FS, and FAC ($P > 0.05$) (Figures 3.3.6B-D). When compared to sham mice, WT mice subjected to CLP demonstrated a significant reduction in percentage EF, FS, and FAC ($P < 0.05$) (Figures 3.3.6B-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 ($P < 0.05$) (Figures 3.3.6B-D).

There were no differences in LV dimensions between the KO groups (Table 3.3.1). Figure 3.3.7A shows representative M-mode echocardiograms of Sham + Vehicle, Sham + EPO, CLP + Vehicle, and CLP + EPO in βcR KO mice. When compared to Sham + Vehicle mice, sham mice treated with EPO demonstrated no significant alterations in EF, FS, and FAC ($P > 0.05$) (Figure 3.3.7B-D). When compared to sham mice, βcR KO mice subjected to CLP demonstrated a significant reduction in percentage EF, FS, and FAC ($P < 0.05$) (Figures 3.3.7B-D), indicating the development of impaired systolic contractility *in vivo*. Treatment of βcR KO mice with EPO did not alter this cardiac dysfunction ($P > 0.05$) (Figures 3.3.7B-D).

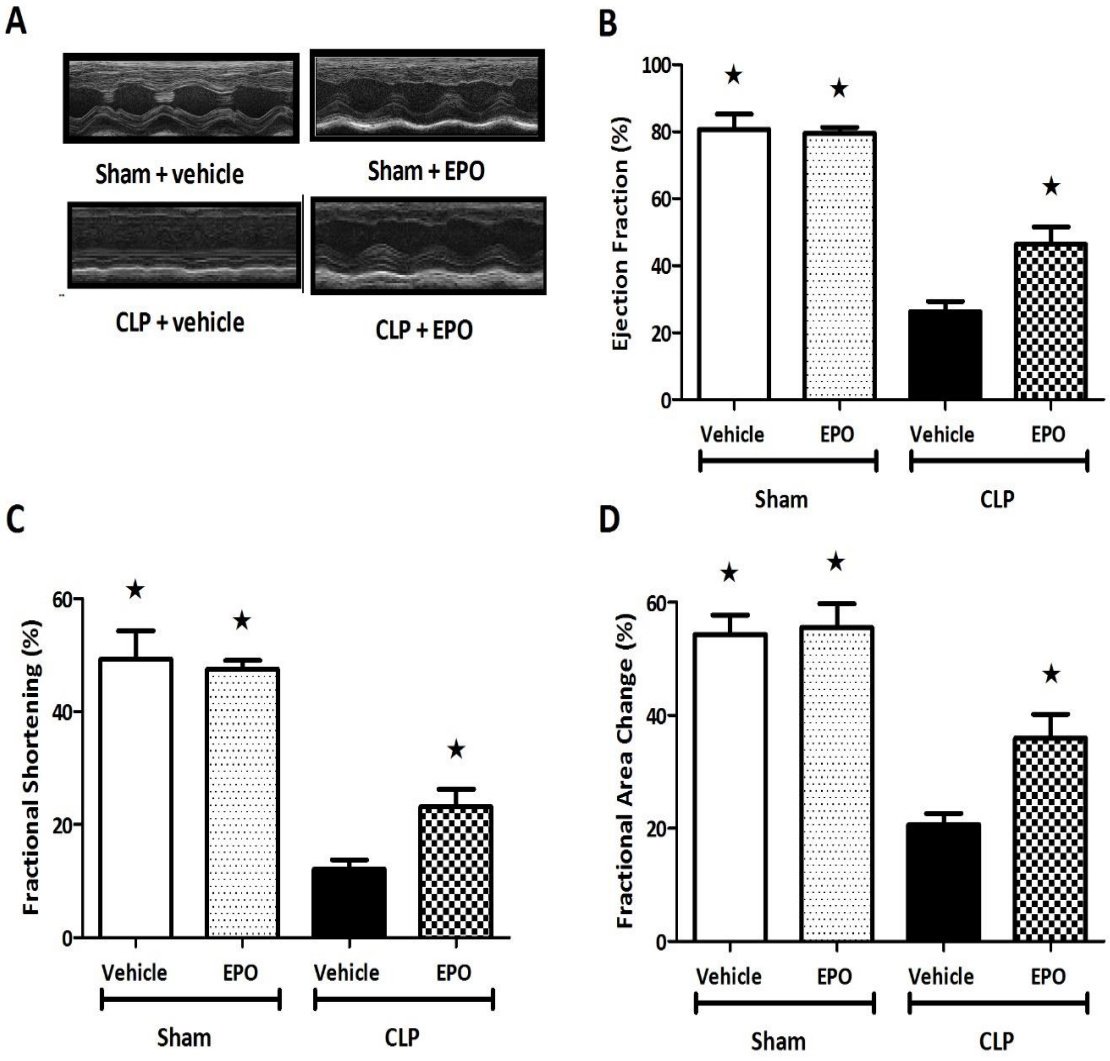


Figure 3.3.6. Effect of EPO on the cardiac dysfunction in WT mice that underwent CLP. Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D), in wild-type mice 24 h subsequent to CLP surgery. One hour after induction of CLP, mice were treated either with EPO (1000 IU kg^{-1} s.c.) or vehicle (10 ml kg^{-1} 0.9% saline s.c.). B-D: Sham + Vehicle (n = 3); Sham + EPO (n = 3); CLP + Vehicle (n = 9); CLP + EPO (n = 10). ★ $P < 0.05$ versus CLP + Vehicle.

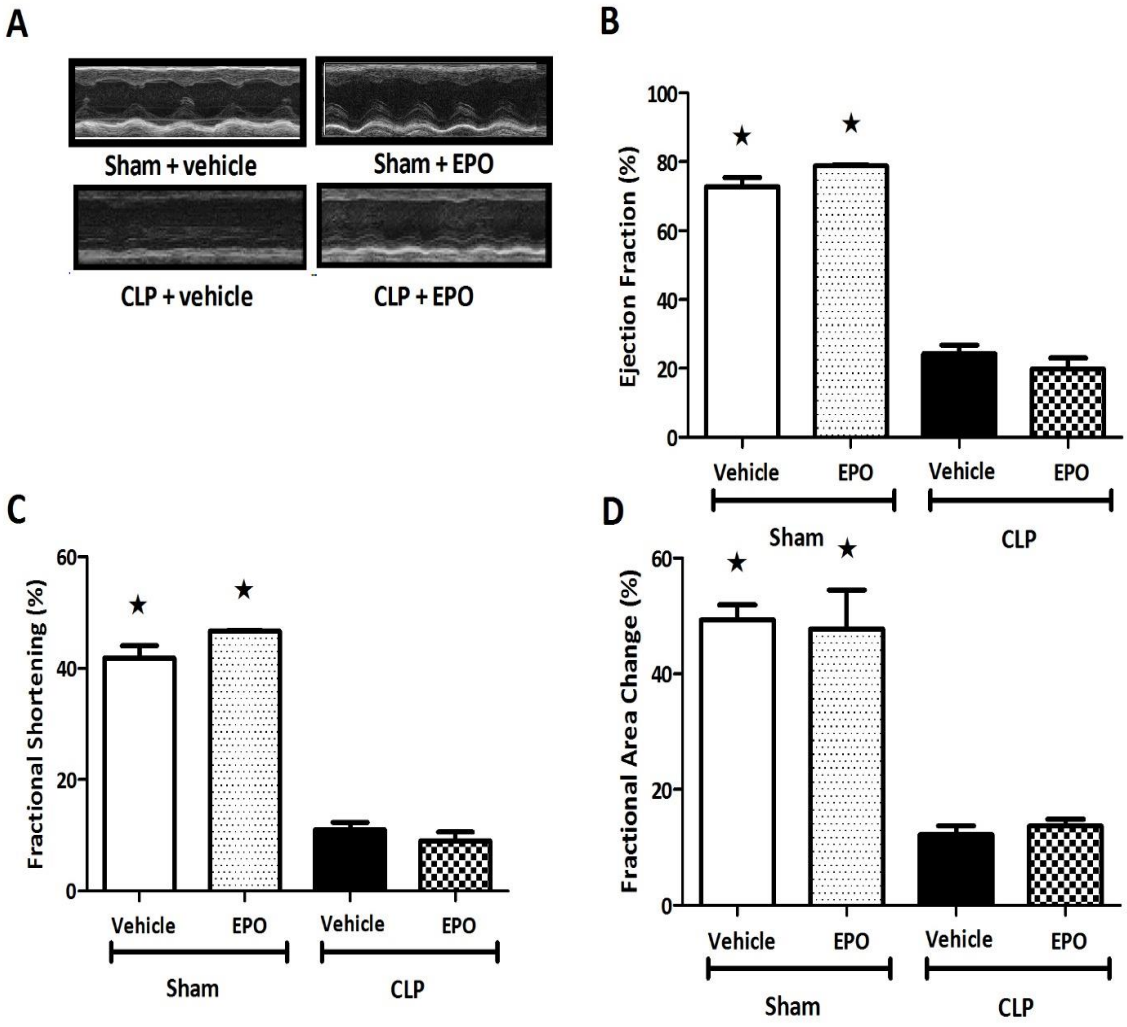


Figure 3.3.7. Effect of EPO on the cardiac dysfunction in βcR KO mice that underwent CLP. Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D), 24 h subsequent to CLP surgery. One hour after induction of CLP mice were treated either with EPO (1000 IU kg^{-1} s.c.) or vehicle (10 ml kg^{-1} 0.9% saline s.c.). B-D: Sham + Vehicle (n = 5); Sham + EPO (n = 2); CLP + Vehicle (n = 7); CLP + EPO (n = 7). ★ $P < 0.05$ versus CLP + Vehicle.

Table 3.3.1: Effect of EPO on echocardiographic parameters (diastolic function) in WT or βcR KO mice subjected to endotoxaemia for 16-18 h or CLP for 24 h

Treatment	Group	Genotype	LVIDD (mm)	LVEDV (µl)	*n*
Endotoxaemia in 2 months old mice	Sham + Vehicle	WT	3.4 ± 0.06	47 ± 2.13	10
	Sham + EPO	WT	3.1 ± 0.14	39 ± 4.34	6
	LPS + Vehicle	WT	3.6 ± 0.07	54 ± 2.60	15
	LPS + EPO	WT	3.6 ± 0.08	53 ± 2.61	15
	Sham + Vehicle	KO	★3.1 ± 0.09	39 ± 2.91	9
	Sham + EPO	KO	3.5 ± 0.15	52 ± 5.36	3
	LPS + Vehicle	KO	3.5 ± 0.08	53 ± 2.96	14
	LPS + EPO	KO	3.5 ± 0.07	53 ± 2.65	25
Polymicrobial Sepsis in aged mice	Sham + Vehicle	WT	3.5 ± 0.30	51 ± 9.44	3
	Sham + EPO	WT	3.5 ± 0.12	51 ± 3.98	3
	CLP + Vehicle	WT	3.9 ± 0.09	68 ± 3.55	9
	CLP + EPO	WT	3.8 ± 0.13	62 ± 5.03	10
	Sham + Vehicle	KO	4.2 ± 0.11	80 ± 4.83	5
	Sham + EPO	KO	3.5 ± 0.00	52 ± 0.76	2
	CLP + Vehicle	KO	3.8 ± 0.12	64 ± 4.24	7
	CLP + EPO	KO	4.1 ± 0.14	74 ± 6.05	7

WT or βcR KO mice received either LPS (9 mg kg^{-1} i.p.) or vehicle (5 ml kg^{-1} 0.9% saline i.p.) or underwent CLP surgery. One hour after induction of endotoxaemia or CLP surgery, mice were treated either with Erythropoietin (EPO) (1000 IU kg^{-1} s.c.) or vehicle (10 ml kg^{-1} 0.9% saline s.c.). All echocardiographic images were assessed for changes in left ventricular internal dimension-diastole (LVIDD) and left ventricular end-diastolic volume (LVEDV). ★ $P < 0.05$ versus LPS + Vehicle (KO).

3.4. DISCUSSION

Sepsis-associated myocardial dysfunction occurs in almost 40 % – 50 % of patients and is a major contributor to morbidity and mortality. There continues to be an unmet clinical need for the discovery of specific therapeutic interventions for this condition. I report here, for the first time, that EPO attenuates the impaired systolic contractility caused by endotoxaemia in young mice or polymicrobial sepsis in aged mice, an effect not seen in mice where the βcR is knocked out. The 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 indicate that the effects of EPO relate to a specific improvement in cardiac contractility, independent of changes in 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 the βcR is essential for the cardioprotective effects of EPO in sepsis, I subsequently investigated the molecular pathways that are activated by EPO in a βcR-dependent fashion. In summary, EPO activated Akt and eNOS and inhibited GSK-3β (an effect secondary to activation of Akt), NF-κB and expression of IL-1β (Figure 3.3.8). Most notably, all of these beneficial effects of EPO were lost in βcR KO mice.

Akt is a member of the PI3K signal transduction enzyme family, which 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 this study, EPO caused a significant increase in phosphorylation of Akt on Ser^{473} (resulting in activation of this kinase), an effect, which was lost in βcR KO mice. The hypothesis that activation of Akt importantly contributes to the improvement in cardiac contractility afforded by EPO is supported by the following findings: i) transgenic mice with cardiac-specific overexpression of Akt exhibit a significant increase in cardiac contractility compared with WT mice (Condorelli *et al*., 2002); ii) protection by EPO against doxorubicin-induced cardiotoxicity was mediated by PI3K activation (Kim *et al*., 2008); iii) the reduction of infarct size afforded by EPO in a murine model of myocardial infarction were associated with activation of Akt (Calvillo *et al*., 2003); and iii) both the activation of Akt and the cardioprotective effects of EPO were lost when animals were pre-treated with an inhibitor of the PI3K/Akt pathway (Cai & Semenza, 2004). Interestingly, Recknagel and

colleagues demonstrated that PI3K signalling also plays a crucial role in the development of liver dysfunction in septic rats (Recknagel *et al.*, 2012). I report here that the increase in Ser^{473} 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 prevention of apoptosis in the heart and that activation by EPO elicits its cytoprotective effects through an Akt-dependent pathway, in part by antagonizing the effects of TNF-α (Ueba *et al.*, 2010). Taken together, all of the above results support the view that activation of Akt, secondary to activation of the 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 Ser^9. 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 Ser^9. Activation of Akt inactivates GSK-3β by causing Ser^9 phosphorylation (Cross *et al.*, 1995). I report here that EPO caused a significant increase in phosphorylation of GSK-3β on Ser^9 (resulting in inhibition of this kinase), an effect, which was lost in βcR KO mice. The hypothesis that inhibition of GSK-3β could contribute to the improvement in cardiac contractility afforded by EPO is supported by the following findings: i) 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 endotoxaemia (Martin *et al.*, 2005); ii) the cardioprotective effects of EPO are secondary to inhibition of GSK-3β as measured by Ser^9 phosphorylation (Nishihara *et al.*, 2006); iii) 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 iii) 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 resulting from activation activation of the βcR by EPO could contribute 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 $Serine^{1177}$ in endothelial cells (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999) and cardiomyocytes (Burger *et al.*, 2006). There is evidence that EPO induces

NO production through eNOS phosphorylation (Su *et al*., 2011; Souza *et al*., 2012a; Kao *et al*., 2011) and its cardioprotective effects appear, in part, to be mediated by the up-regulation of eNOS, *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 or JAK 2 in endothelial cells (Su *et al*., 2011). Indeed, Sautina and colleagues demonstrated the requirement of the βcR subunit in the EPO induction of NO (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. I report here that the increase in Ser^{1177} phosphorylation of eNOS by EPO in the heart is lost in βcR KO mice. In conditions associated with sepsis, activation of eNOS is beneficial as enhanced formation of NO cause's 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 may contribute to the beneficial effects of EPO reported here.

Downstream of GSK-3β, several studies have now reported an association between GSK-3β and NF-κB activity *in vitro* (Hoeflich *et al*., 2000; Schwabe & Brenner, 2002), and *in vivo* (Dugo *et al*., 2005; Dugo *et al*., 2006). NF-κB is a transcriptional factor that plays an important role in regulating the transcription of a number of genes, especially those involved in producing mediators involved in local and systemic inflammation, such as cytokines, chemokines, cell adhesion molecules, apoptotic factors, and other mediators (Senftleben & Karin, 2002). Treatment of TNF-α-stimulated hepatocytes with a specific GSK-3β inhibitor resulted in a decrease of the NF-κB-dependent gene transcription (Schwabe & Brenner, 2002). This study also indicated four potential phosphorylation sites for GSK-3β on the NF-κB subunit, p65. Most notably, pre-treatment with a number of chemically distinct inhibitors of GSK-3β attenuates organ injury and dysfunction caused by endotoxaemia (Dugo *et al*., 2005; Martin *et al*., 2005). This protective effect was associated with inhibition of the activation of NF-κB and NF-κB-dependent pro-inflammatory genes (e,g, IL-1β and TNF- α) , along with a reduced phosphorylation of Ser^{536} on the NF-κB p65 subunit. In addition, GSK-3β may also inhibit the activation of NF-κB by phosphorylating and degrading IκBα, which is required to prevent NF-κB translocation (Takada *et al*., 2004). In this study, EPO attenuated the activation of NF-κB caused by endotoxaemia in the heart, an effect, which was lost in βcR KO mice. NF-κB 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 TNF-α, IL-1β, and IL-6 (Jia *et al*., 2009). I 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 endotoxaemic βcR KO mice.

Clinical relevance

Myocardial function is depressed in sepsis and has a significant impact on patients' outcome (Rudiger *et al*., 2013). Using a long-term rat model of faecal peritonitis, Rudiger and colleagues demonstrated most recently that significant differences in stroke volume and heart rate assessed 6 h 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 was significantly lower in the survivors. Improved outcome of "dilators" vs. "non-dilators" was also shown in resuscitated murine CLP-induced septic shock (Zanotti Cavazzoni *et al*., 2010). However, other authors demonstrated that mortality was higher (47 % vs. 16 %) in patients with a subnormal fractional area contraction upon initial echocardiography (Charpentier *et al*., 2004). Moreover, Kumar and colleagues 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 EF coincided with unchanged end-diastolic diameter and volume. Hence, diastolic relaxation was not affected by the treatment. This observation is in contrast to a previous study in murine CLP-induced septic shock (Barth *et al*., 2006); genetic deletion and pharmacological blockade of iNOS increased systolic contractility and improved myocardial catecholamine responsiveness at the expense of impaired diastolic relaxation, i.e. "stiffening of the ventricle".

Limitation

In retrospect, a limitation of this study is the utilization of two different models in two different age groups; a model of endotoxaemia in young mice and a model of polymicrobial sepsis in aged mice. The main focus of the study was to be able to reproduce the findings of EPO in the endotoxaemic model, in a more clinically relevant model of polymicrobial sepsis. Although, I 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, however, that an investigation into the effects of age on the pathophysiology of sepsis was not the main focus of this study, but rather a by-product of the development of a reproducible model of CLP-induced cardiac dysfunction. In addition, although I clearly demonstrate here by western blot analysis that EPO exerts its cardioprotective effects in both models of endotoxaemia and polymicrobial sepsis through activation of survival pathways, one could argue that it is not certain that these effects are associated with the pathophysiology of the cardiac dysfunction in these models. Therefore, confirmation of the involvement of these pathways in the protective effects of EPO could be confirmed by the use of pharmacological inhibitors specific to components of these pathways (PI3K, LY294002; GSK-3β, SB216763; eNOS, N (5)-(1-Iminoethyl)-L-ornithine HCL), to see if there is a reversal of EPO effects. Furthermore, due to time and financial constraints it was not possible to generate western blot for Sham-EPO animals which are important as it would not only confirm that the effects of EPO (activation of survival pathways) are solely related to the model but also since the dose used here is much more than that generally administered to anaemic patients, its best to be sure that there are no toxic effects by EPO.

Conclusion

My results show for the first time that the administration of EPO reduces the impaired systolic contractility associated with sepsis. In endotoxaemia the observed beneficial effects of EPO are associated with i) activation of Akt, ii) inhibition of GSK-3β, iii) activation of eNOS, iv) inhibition of NF-κB, and v) inhibition of the expression of IL-1β, in a model of endotoxaemia (Figure 3.3.8). 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.

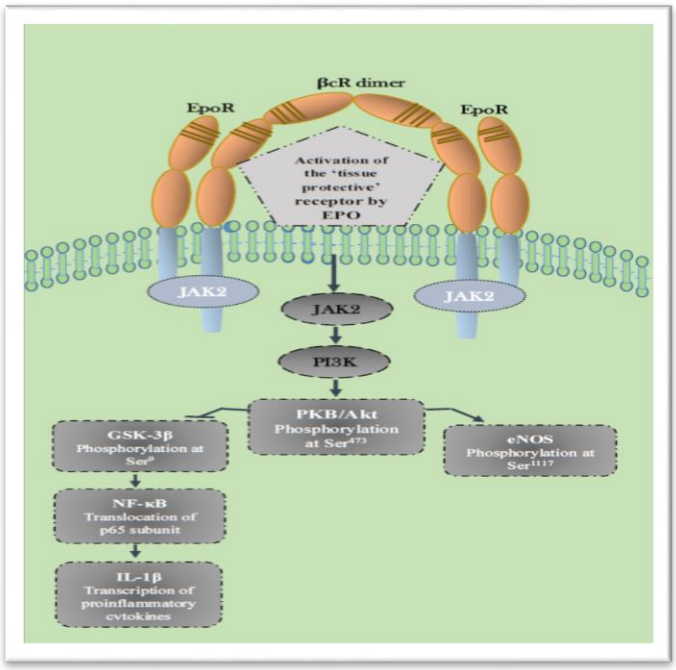


Figure 3.3.8. Schematic overview demonstrating the intracellular signalling pathways implicated in EPO's cardioprotective effects in endotoxaemic WT mice. Binding of EPO to the proposed model of the tissue-protective receptor (EpoR-βcR) is associated with i) activation of PI3K-Akt signalling pathway, which results in the inhibition of the activation of glycogen synthase kinase-3β, which subsequently suppresses NF-κB activity resulting in a blunted expression of the NF-κB-driven gene transcription of pro-inflammatory mediators such as interleukin-1β, and ii) activation of the PI3K-Akt signalling pathway results in the increased activation of eNOS and thus the enhanced formation of NO.

Chapter 4

The Anti-malarial Drug Artesunate Attenuates Cardiac Injury in a Rodent Model of Myocardial Infarction

4.1. Introduction

Artesunate (ARS) is a semi-synthetic derivative of artemisinin, a sesquiterpenetrioxane lactone, isolated from the plant *Artemesia annua* (Meshnick *et al.*, 1996). In the past decade, artemisinin and its derivatives have emerged as the most effective drugs for the treatment of severe malaria (Haynes *et al*., 2001; Rosenthal, 2008). ARS, the only artemisinin analogue that can be administered intravenously, results in rapid parasite and fever clearance in falciparum malaria (Batty *et al*., 1998). ARS has a very short half-life of approximately 10 min and is rapidly metabolised to dihydroartemisinin (DHA), which has a half-life of approximately 1 h and regarded as the active metabolite of ARS (Krishna *et al*., 2004).

Today we know that ARS has a multitude of pharmacological effects that include anti-angiogenic effects (Wartenberg *et al*., 2003; Huan-huan *et al*., 2004), anti-viral effects (Efferth *et al*., 2008), as well as induction of cell apoptosis (Huan-huan *et al*., 2004) and oxidative stress (Efferth *et al*., 2008; Ho *et al*., 2012). Furthermore, there is increasing evidence to support the notion that artemisinin and its derivatives also possess anti-inflammatory effects (Aldieri *et al*., 2003; Li *et al*., 2006; Xu *et al*., 2007; Li *et al*., 2008; Cheng *et al*., 2011; Souza *et al*., 2012b; Yang *et al*., 2012). Indeed, ARS has been reported to i) reduce the production of cytokines from TNF-α-stimulated human rheumatoid arthritis fibroblast-like synoviocytes (Xu *et al*., 2007), ii) inhibit lipopolysaccharide-induced production of TNF-α, IL-6, and to reduce the expression of TLR-4, and TLR-9 in macrophages (Li *et al*., 2008).

The exact mechanism of action that mediates these anti-inflammatory effects by ARS is not completely understood. However, it could correlate with several other sesquiterpene lactones which, are known to inhibit the activation of NF-κB by either alkylating it (Garcia-Pineres *et al*., 2001) or preventing the degradation of its inhibitory protein IκB (Wong & Menendez., 1999; Kim *et al*., 2001). Indeed, various investigators have shown ARS (Li *et al*., 2008) and other artemisinin derivatives (Li *et al*., 2006) to exert anti-inflammatory effects via inhibition of the nuclear translocation of NF-κB. Additionally, ARS has been reported to possess inhibitory activity against the PI3K/Akt signalling pathway (Xu *et al.,* 2007; Cheng *et al*., 2011).

As the PI3K/Akt signalling pathway plays a critical role in the pathogenesis of myocardial injury by which, its activation confers protection against I/R injury, I investigated the effect of ARS in a model of transient I/R in the rat and explored the

mechanism of action of ARS. The rational to use rats instead of mice is due to the existence of an already available model of rat myocardial I/R, since setting up a mouse model of myocardial I/R is more tricky and time consuming, a luxury I did not have. Specifically, I have investigated (i) the effect of ARS on the infarct size 2 h post reperfusion; (ii) the mechanisms underlying the observed effects of ARS including the involvement of the RISK pathway; activation of PI3K signalling pathway (the subsequent phosphorylation Akt on Ser^{473}) and ERK 1/2 signalling pathway, and the SAFE pathway (phosphorylation of STAT3 on Tyr^{705}). In addition, the involvement of downstream targets of the above pro-survival pathways were investigated; phosphorylation of GSK-3β on Ser^{9}, phosphorylation of endothelial NO synthase (eNOS) on Ser^{1177} and activation of nuclear factor NF-κB (measured as nuclear translocation of p65).

4.2. Methods

This study was carried out on 169 male Wistar rats (Charles River, UK) weighing 240-340 g receiving a standard diet and water ad libitum. The investigation was performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, published by HMSO, London.

Surgical Instrumentation and Haemodynamic Measurements

Rats were anaesthetised with thiopentone sodium (Intraval 120 mg kg^{-1} i.p.) and anaesthesia was maintained by supplementary doses of thiopentone sodium as required. The trachea was cannulated and the animals were allowed to artificially respire using a Harvard ventilator (inspiratory oxygen concentration: 30 %; 70 strokes/min tidal volume: 8-10 ml kg^{-1}). It has been reported that a change of 1 ^{0}C in temperature affects the infarct size (Chien *et al*., 1994). Thus, body temperature was maintained at 37 ± 1 ^{0}C with the aid of a rectal probe thermometer attached to a homeothermic blanket unit (Harvard Apparatus Ltd., Edenbridge, Kent. U.K.).

The right carotid artery was cannulated with a polyethylene catheter and connected to a pressure transducer (Senso-Nor 844, Senso-Nor, Horten, Norway), to monitor mean arterial pressure (MAP) and heart rate (HR), which were displayed on a data acquisition system (MacLab 8e, ADI Instruments, Hastings, U.K.), installed on an IBM compatible computer. Pressure rate index (PRI), a relative indicator of myocardial oxygen consumption (Baller *et al*., 1981), was calculated as a product of MAP and HR, and expressed in mmHg min^{-1} 10^3. Subsequently, the right jugular vein was cannulated for the administration of drugs and saline.

Myocardial Ischaemia and Reperfusion

Following anaesthesia and instrumentation of the rat as described above, a parasternal thoracotomy was performed, using an electrosurgery device to cauterise the intercostal arteries before cutting through three ribs. The chest was retracted and the pericardium dissected from the heart. The left anterior descending (LAD) coronary artery was isolated, and a snare occluder was placed around the LAD.

Subsequently, upon completion of the surgical procedure, the retractor was removed, and animals were allowed to stabilize for 15 min, after which, the occluder was tightened, at time 0. After 25 min of LAD-occlusion, the occluder was released to allow

reperfusion of the previously ischaemic myocardium for 2 h. Hemodynamic parameters were continuously monitored. Baseline readings were taken prior to treatment, and, myocardial I/R injury. Saline was administered immediately after reperfusion and throughout reperfusion at a rate of 2 ml kg^{-1} h^{-1}.

Quantification of myocardial tissue injury

At the end of the 2 h reperfusion period, the LAD was re-occluded and 1 ml of Evans Blue dye (2 % w v^{-1}) was administered via the jugular vein. The dose of Evans Blue dye used in this study is within the range shown to exclusively bind to plasma proteins (Lindner & Heinle, 1982). This dye stains perfusable tissue, therefore the non-perfused (occluded) vascular tissue remains un-coloured. Each animal was killed with an overdose of anaesthetic, the heart excised, and excess dye washed off. The heart was then sectioned into 3 slices of 3-4 mm, the right ventricular wall was removed, and the area at risk (AAR) – essentially the non-perfused and, hence, non-stained myocardium was separated from the non-ischemic (blue) tissue. The ischaemic and non-ischaemic tissue was weighed, and the AAR expressed as a percentage of the left ventricle. The tissue from the AAR was cut into small pieces and incubated with p-nitroblue tetrazolium (NBT, 0.5 mg ml^{-1}) for 30 min at 37 ^{0}C. NBT is a reducing agent that reacts with formazon. In the presence of intact dehydrogenase enzyme enzymes, found in normally perfused myocardium tissue, NBT forms a dark blue formazon, whilst infarcted tissue (non-viable), lacking dehydrogenase activity will fail to stain (Nachlas & Shnitka, 1963). The stained tissue was separated from the infarcted tissue, weighed, and the infarct size expressed as a percentage of the AAR.

Experimental Design

Initially, the aim was to establish the optimal dose of and vehicle for ARS to be used for this study. As there was no data regarding the use of ARS in an *in vivo* model of myocardial I/R three vehicles were used to determine which is best to completely dissolve the ARS and not have adverse effects in this model. Additionally, differential doses of ARS was used to determine which dose would result in the maximum reduction in infarct size.

Animals were randomized into 23 Groups and were either subjected to i) surgical procedure alone with no LAD occlusion and reperfusion and treated with vehicle

(dimethylsulphoxide [DMSO] 10 % v v^{-1}) (sham vehicle), or ii) surgical procedure with LAD occlusion and reperfusion and treated with either vehicle [DMSO, sodium bicarbonate ($NaHCO_3$), or cyclodextrine (CD 30 % v v^{-1})] ('control' animals) or ARS ('treated' animals) at the start of reperfusion, with/without pre-treatment with LY294002 (LY) or U0126 10 mins before occlusion of the LAD. For experiments that served as positive controls, an additional group of animals were subjected to surgical procedure followed by either 2 cycles of ischaemic preconditioning (IPC – 1 cycle: 5 min ischaemia and 5 min reperfusion) or pre-treated 10 mins before initiation of IPC with LY or U0126, followed by LAD occlusion and reperfusion and treated with vehicle. The involvement of the PI3K/Akt pathway (Xu *et al*., 2007) and ERK (Lee, *et al*., 2012) in the action of ART have been established. Therefore, in order to determine whether these pathways are involved in this model of myocardial I/R LY and UO126, highly selective inhibitors of PI3K and ERK, respectively were used. The surgical protocols and experimental groups of the 23 study groups are shown in Figure 4.2.1 and Table 4.2.1 respectively.

Figure 4.2.1. Surgical protocols of animals subjected to LAD occlusion and reperfusion

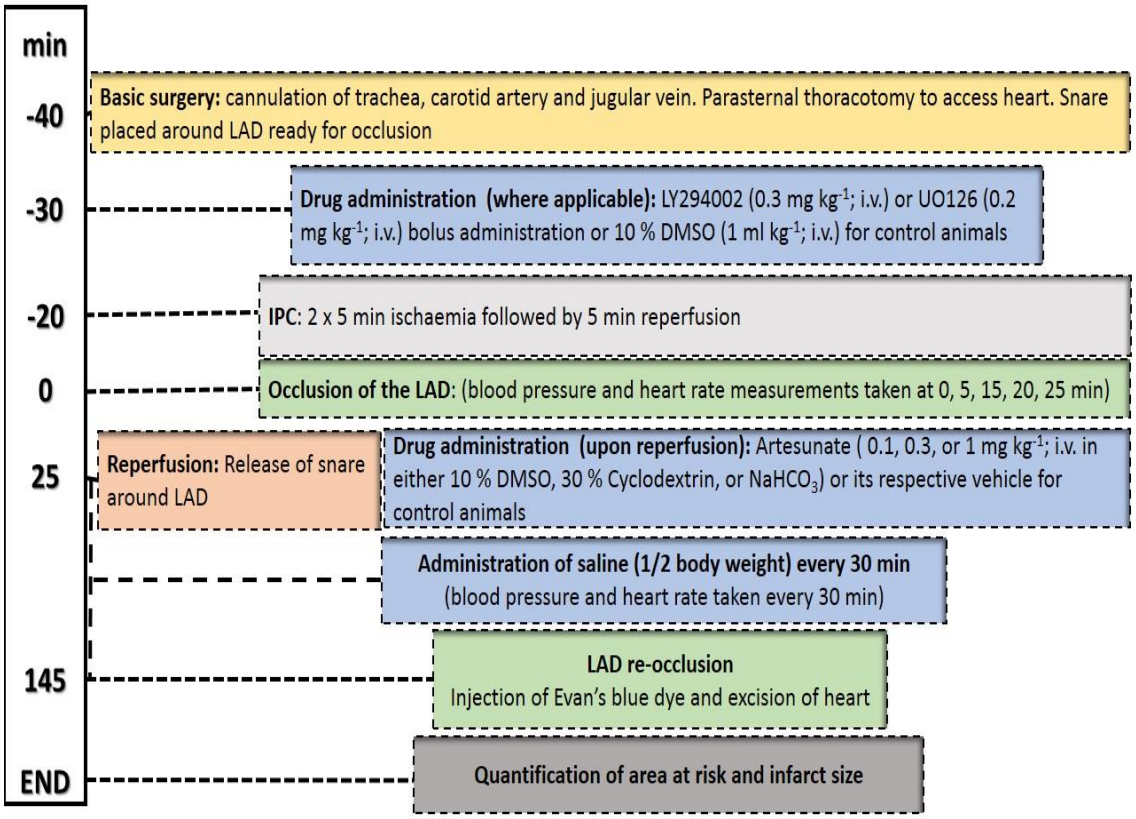


Table 4.2.1. Experimental groups of animals subjected to LAD occlusion and reperfusion

Group	*n*	Ischaemia (min)	Reperfusion (h)
Sham vehicle (1 ml kg^{-1}; 10 % DMSO)	6	N/A	N/A
MI Vehicle (1 ml kg^{-1}; 10 % DMSO)	14	25	2
MI Vehicle (1 ml kg^{-1}; 30 % Cyclodextrin)	7	25	2
MI Vehicle (1 ml kg^{-1}; $NaHCO_3$)	5	25	2
MI + ARS (0.1 mg kg^{-1}; 10 % DMSO)	3	25	2
MI + ARS (0.3 mg kg^{-1}; 10 % DMSO)	7	25	2
MI + ARS (1 mg kg^{-1}; 10 % DMSO)	7	25	2
MI + ARS (0.3 mg kg^{-1}; 30 % Cyclodextrin)	7	25	2
MI + ARS (1 mg kg^{-1}; 30 % Cyclodextrin)	7	25	2
MI + ARS (0.1 mg kg^{-1}; $NaHCO_3$)	4	25	2
MI + ARS (0.3 mg kg^{-1}; $NaHCO_3$)	5	25	2
MI + ARS (1 mg kg^{-1}; $NaHCO_3$)	12	25	2
MI + LY (0.3 mg kg^{-1})+ ARS (1 mg kg^{-1}; $NaHCO_3$)	10	25	2
MI + U0126 (0.2 mg kg^{-1}) + ARS (1 mg kg^{-1}; $NaHCO_3$)	11	25	2
MI + IPC	12	25	2
MI + LY (0.3 mg kg^{-1}) + IPC	7	25	2
MI + U0126 (0.2 mg kg^{-1})+ IPC	5	25	2
MI + DHA (mg kg^{-1}; 10 % DMSO)	6	25	2
Sham Vehicle (1 ml kg^{-1})(Western blot samples)	7	N/A	N/A
MI Vehicle (1 ml kg^{-1}) (Western blot samples)	9	25	2
MI + ARS (1 mg kg^{-1}) (Western blot samples)	10	25	2
Sham Vehicle + ARS (1 mg kg^{-1}; $NaHCO_3$) (Samples for pharmacokinetic)	4	N/A	N/A
MI Vehicle + ARS (1 mg kg^{-1}; $NaHCO_3$) (Samples for pharmacokinetic)	4	25	2

ARS = Artesunate; **DHA** = Dihydroartesunate; **I =** Ischaemia; **IPC** = Ischaemic preconditioning; **R** = Reperfusion;
LY = LY294002; **N/A** = Not applicable;

Western Blot Analysis

Western blot analysis was carried out as previously described in chapter 3, with only slight modifications. A total of sixty µg of total protein was loaded. In addition to the already mentioned antibodies in chapter 3, membranes were also incubated with primary rabbit anti-total STAT3, dilution 1:1000, anti-pSTAT3 Tyr^{705}, dilution 1:1000.

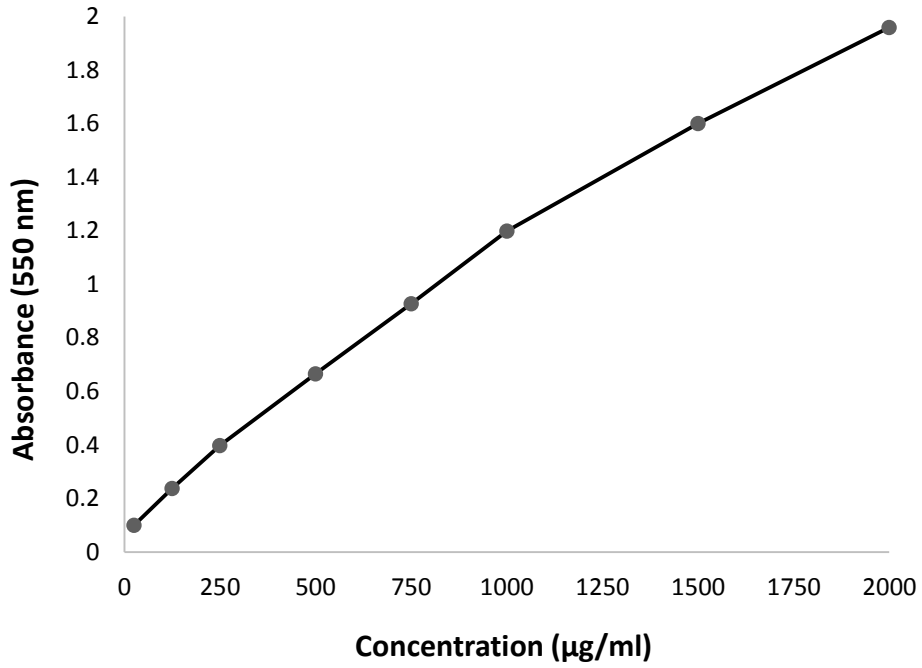


Figure 4.2.2. A representative standard protein curve for western blot analysis

Materials

Unless otherwise stated, all compounds used in this study were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). Thiopentone sodium (Intraval Sodium®) was obtained from Rhône Mérieux Ltd. (Harlow, Essex, UK). Unless otherwise stated, all stock solutions were prepared in non-pyrogenic saline (0.9 % NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK). ARS was obtained from Guilin Pharmaceutical Co. (Guilin, Guangxi, China). LY and U0126 were obtained from Tocris Bioscience (Bristol, UK). Antibodies against pAkt (Ser^{473}) and total Akt were from Cell Signalling Technology (Beverly, MA, USA) and against pSTAT3 (Tyr^{705}) and total STAT3 were purchased from New England Biolabs Ltd (Hitchen, Hertfordshire, UK). Antibodies against pGSK-3β (Ser^{9}), total GSK-3β, eNOS, anti-goat, anti-mouse and anti-rabbit Ig horseradish peroxidase-linked whole antibodies were purchased from Santa Cruz

Biotechnology (Santa Cruz, California, USA). Luminol ECL detection reagents were from Amersham (Buckinghamshire, UK).

Statistical Analysis

All data are presented as mean ± SEM of n observations, where n represents the number of animals or samples studied. Haemodynamic parameters were analysed via a two-way analysis of variance (ANOVA) with repeated measures, followed by a Bonferroni post-test. Data without repeated measurements were analysed by one-way ANOVA, followed by a Bonferroni post hoc test for multiple comparisons. $P < 0.05$ was considered statistically significant.

4.3. Results

Alterations in haemodynamic parameters in animals subjected to LAD occlusion and reperfusion

The mean baseline values of MAP in all groups of animals ranged from 101 ± 7.14 to 130 ± 4.06 mmHg, and were not significantly different between groups ($P > 0.05$, Table 4.3.1). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle in either 10 % DMSO or 30 % Cyclodextrin exhibited a progressive decline in MAP [MI Vehicle (DMSO), 106 ± 4.07 to 90 ± 4.63; MI Vehicle (Cyclodextrin), 119 ± 4.90 to 99 ± 5.26 (P > 0.05, Table 4.3.1). Animals undergoing 2 cycles of IPC before occlusion of the LAD or that were pre-treated with LY or U0126 10 min before initiation of IPC did not exhibit significant changes in MAP (P > 0.05, Table 4.3.1). When compared to MI-animals treated with their respective vehicle, bolus treatment of animals with ARS in either 10 % DMSO (0.1, 3, 1 mg kg^{-1}) or30 % Cyclodextrin (0.3, 1 mg kg^{-1}) at the start of reperfusion did not cause any significant changes in MAP ($P > 0.05$, Table 4.3.1).

The mean baseline values of HR in all groups of animals ranged from 370 ± 10.19 to 443 ± 5.33 bpm, and were not significantly different between groups ($P > 0.05$, Table 4.3.1). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle in either 10 % DMSO or 30 % Cyclodextrin exhibited a progressive decline in MAP [MI Vehicle (DMSO), 413 ± 13.21 to 418 ± 12.76; MI Vehicle (Cyclodextrin), 393 ± 14.10 to 421 ± 6.37 (P > 0.05, Table 4.3.1). Animals undergoing 2 cycles of IPC before occlusion of the LAD or that were pre-treated with LY or UO126 10 min prior to initiation of IPC did not exhibit any significant changes in HR when compared to MI Vehicle (DMSO) treated animals ($P > 0.05$, Table 4.3.1). When compared to MI-animals treated with their respective vehicle, bolus treatment of animals with ARS in either [10 % DMSO (0.1, 3, 1 mg kg^{-1}) or 30 % Cyclodextrin (0.3, 1 mg kg^{-1}) at the start of reperfusion did not cause any significant changes in HR ($P > 0.05$, Table 4.3.1).

PRI, a relative indicator of myocardial oxygen consumption, was derived as a product of MAP and HR. The mean baseline values of PRI in all groups of animals ranged from 38 ± 1.95 to 54 ± 1.80 mmHg bpm x 10^3, and were not significantly different between groups ($P > 0.05$, Table 4.3.1). When compared to Sham Vehicle animals, MI-animals treated with either 10 % DMSO or 30% Cyclodextrin exhibited a decline in PRI

($P > 0.05$, Table 4.3.1). Animals undergoing 2 cycles of IPC before occlusion of the LAD or that were pre-treated with LY or U0126 before initiation of IPC did not exhibit significant changes in PRI ($P > 0.05$, Table 4.3.1). When compared to MI-animals treated with their respective vehicle, bolus treatment of animals with ARS in either 10 % DMSO (0.1, 3, 1 mg kg^{-1}) or 30 % Cyclodextrin (0.3, 1 mg kg^{-1}) at the start of reperfusion did not show any significant changes in PRI ($P > 0.05$, Table 4.3.1).

Table 4.3.1. MAP, HR and PRI in animals subjected to LAD occlusion and reperfusion

	Ischaemia					**Reperfusion**		
Group	**0**	**5**	**15**	**20**	**25**	**55**	**85**	**145**
Sham Vehicle (*n* = 6)								
MAP	120 ± 2.86	119 ± 2.32	115 ± 2.73	115 ± 2.07	111 ± 3.13	106 ± 6.09	105 ± 4.02	102 ± 4.83
HR	443 ± 5.33	439 ± 4.11	439 ± 5.89	439 ± 8.92	437 ± 9.95	418 ± 5.22	418 ± 11.71	419 ± 6.32
PRI	53 ± 1.11	52 ± 0.74	50 ± 0.95	50 ± 1.53	49 ± 2.09	44 ± 3.04	44 ± 2.77	43 ± 2.43
MI Vehicle (DMSO) (*n* = 13)								
MAP	106 ± 4.07	112 ± 3.70	106 ± 2.25	103 ± 2.51	100 ± 3.21	98 ± 5.23	92 ± 5.32	90 ± 4.63
HR	413 ± 13.21	433 ± 12.38	428 ± 12.47	424 ± 12.79	407 ± 19.53	409 ± 10.02	414 ± 13.49	418 ± 12.76
PRI	44 ± 2.47	48 ± 2.22	45 ± 1.64	44 ± 1.61	41 ± 2.58	40 ± 2.28	38 ± 2.98	38 ± 2.21
MI + ARS (0.1; DMSO) (*n* =7)								
MAP	130 ± 4.06	128 ± 6.89	127 ± 5.51	126 ± 4.73	126 ± 4.18	118 ± 0.58	104 ± 1.45	101 ± 5.70
HR	417 ± 4.18	433 ± 6.81	422 ± 9.26	419 ± 9.64	418 ± 9.39	441 ± 11.53	429 ± 7.45	438 ± 11.79
PRI	54 ± 1.80	56 ± 3.71	54 ± 3.36	53 ± 3.14	53 ± 2.63	52 ± 1.46	45 ± 0.50	44 ± 2.04
MI + ARS (0.3; DMSO) (*n*=7)								
MAP	125 ± 5.44	126 ± 5.41	119 ± 4.87	116 ± 5.13	115 ± 6.10	94 ± 10.87	89 ± 7.48	87 ± 4.87
HR	426 ± 8.95	443 ± 5.12	432 ± 5.72	426 ± 6.13	422 ± 6.71	416 ± 13.14	413 ± 10.65	405 ± 3.96
PRI	54 ± 2.99	56 ± 2.72	51 ± 2.12	50 ± 2.29	48 ± 2.79	40 ± 5.24	37 ± 3.67	35 ± 2.19

Table 4.3.1. MAP, HR and PRI in animals subjected to LAD occlusion and reperfusion continued

	Ischaemia					Reperfusion		
Group	**0**	**5**	**15**	**20**	**25**	**55**	**85**	**145**
MI + ARS (1; DMSO) (*n* = 7)								
MAP	119 ± 2.15	117 ± 7.75	116 ± 3.07	112 ± 3.87	109 ± 3.41	112 ± 5.97	95 ± 6.66	86 ± 8.69
HR	407 ± 16.90	432 ± 11.42	418 ± 14.96	409 ± 16.78	407 ± 15.94	416 ± 14.05	410 ± 14.47	430 ± 15.30
PRI	48 ± 2.48	51 ± 4.07	49 ± 2.73	46 ± 3.27	45 ± 2.85	47 ± 3.44	39 ± 3.91	37 ± 4.14
MI + IPC (*n* = 9)								
MAP	101 ± 7.14	118 ± 3.70	112 ± 5.33	111 ± 5.12	119 ± 4.12	102 ± 6.12	98 ± 3.67	94 ± 7.13
HR	406 ± 13.39	419 ± 10.08	405 ± 11.74	402 ± 11.54	405 ± 10.52	414 ± 11.59	407 ± 10.14	428 ± 15.71
PRI	41 ± 2.75	49 ± 2.05	46 ± 2.52	45 ± 2.34	48 ± 2.04	42 ± 2.32	40 ± 1.69	41 ± 3.63
MI + LY294002 + IPC (*n* = 7)								
MAP	102 ± 2.82	107 ± 3.34	105 ± 3.53	103 ± 3.71	103 ± 3.20	90 ± 3.52	89 ± 3.35	86 ± 4.02
HR	370 ± 10.19	377 ± 10.43	370 ± 11.25	366 ± 11.15	362 ± 11.17	366 ± 14.41	385 ± 13.97	383 ± 10.73
PRI	38 ± 1.95	40 ± 2.31	39 ± 2.25	38 ± 2.19	37 ± 2.08	33 ± 2.21	34 ± 1.95	33 ± 2.30
MI + UO126 + IPC (*n* = 2)								
MAP	107	114 ± 4.00	111 ± 4.00	112 ± 2.00	107 ± 4.50	96 ± 8.50	90 ± 9.00	85 ± 2.50
HR	353 ± 50.00	380 ± 22.00	383 ± 7.50	381 ± 8.50	379 ± 6.00	370 ± 14.50	366 ± 17.00	360 ± 30.50
PRI	38 ± 5.35	43 ± 4.03	42 ± 2.36	43 ± 1.71	40 ± 2.34	35 ± 4.53	35 ± 4.82	30 ± 3.48
MI Vehicle (Cyclodextrin) (*n* = 6)								
MAP	119 ± 4.90	121 ± 4.65	115 ± 4.64	112 ± 4.00	111 ± 4.26	103 ± 5.67	98 ± 5.40	99 ± 5.26
HR	393 ± 14.10	426 ± 10.83	403 ± 12.73	396 ± 13.74	393 ± 14.20	382 ± 15.01	396 ± 15.88	421 ± 6.37
PRI	47 ± 3.23	52 ± 2.98	46 ± 3.14	45 ± 3.05	44 ± 3.12	40 ± 3.50	39 ± 3.28	42 ± 3.45

Table 4.3.1. MAP, HR and PRI in animals subjected to LAD occlusion and reperfusion continued

	Ischaemia					Reperfusion		
Group	**0**	**5**	**15**	**20**	**25**	**55**	**85**	**145**
MI + ARS (0.3; Cyclodextrin) (*n* = 7)								
MAP	118 ± 5.02	114 ± 9.36	118 ± 4.54	116 ± 4.12	111 ± 4.87	103 ± 6.77	92 ± 6.46	92 ± 4.72
HR	430 ± 19.41	440 ± 21.06	432 ± 19.11	429 ± 17.10	423 ± 16.58	417 ± 12.53	423 ± 11.47	423 ± 8.66
PRI	51 ± 4.10	50 ± 5.45	51 ± 3.57	50 ± 3.14	47 ± 3.15	43 ± 3.93	39 ± 3.65	39 ± 2.52
MI + ARS (1; Cyclodextrin) (*n* =7)								
MAP	107 ± 5.08	121 ± 3.30	114 ± 4.16	106 ± 6.00	109 ± 4.72	95 ± 3.41	89 ± 2.45	94 ± 4.31
HR	419 ± 16.82	439 ± 10.99	417 ± 13.03	406 ± 15.12	406 ± 13.38	397 ± 14.15	409 ± 10.57	397 ± 17.79
PRI	45 ± 2.31	53 ± 1.94	48 ± 2.74	43 ± 3.52	44 ± 2.61	38 ± 2.74	37 ± 1.24	38 ± 3.16

Alterations in MAP (mmHg), HR (bpm), and PRI (mmHg bpm 10^3) in rats subjected to either i) surgical procedure alone and treated with vehicle (Sham Vehicle), ii) 25 min ischaemia and 120 min reperfusion and treated with vehicle [(MI Vehicle) in either 10 % DMSO, 30 % Cyclodextrin or $NaHCO_3$], iii) 25 min ischaemia and 120 min reperfusion and treated at the start of reperfusion with artesunate (MI + ARS) in either 10 % DMSO (0.1, 0.3, 1 mg kg^{-1} i.v.), 30 % Cyclodextrin (0.3, 1 mg kg^{-1} i.v.) or $NaHCO_3$, (0.1, 0.3, 1 mg kg^{-1} i.v.), iv) treated with either LY294002 (MI + LY 0.3 mg kg^{-1} + ARS) or U0126 (MI + U0126 0.2 mg kg^{-1} + ARS) 10 min before ischaemia, followed by 25 min ischaemia and 120 min reperfusion and treated at the start of reperfusion with ARS, v) treated with either vehicle (MI + IPC), LY294002 (MI + LY + IPC) or U0126 (MI + U0126 + IPC) 10 min before 2 cycles of preconditioning followed by 25 min ischaemia and 120 min reperfusion. Data are expressed as means ± S.E.M. for *n* number of observations (two-way analysis of variance with repeated measures, with Bonferroni post-test).

Effect of regional MI and ARS (in $NaHCO_3$) on haemodynamic parameters

The baseline values of MAP in all groups of animals ranged from 104 ± 5.89 to 120 ± 2.86 mmHg, and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.1A). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [MI Vehicle ($NaHCO_3$)] exhibited a progressive decline in MAP from 107 ± 7.41 to 82 ± 13.72 mmHg at the end of the experiment (Figure 4.3.1.1A). Bolus treatment of animals with ARS at doses 0.1 mg kg^{-1}, 0.3 mg kg^{1}, or 1 mg kg^{-1} at the start of reperfusion did not cause any significant changes in MAP when compared to MI-animals treated with vehicle ($NaHCO_3$) ($P > 0.05$, Figure 4.3.1.1A).

Baseline values of HR in all groups ranged from 418 ± 10.00 to 468 ± 14.50 bpm, and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.1B). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [MI Vehicle ($NaHCO_3$)] caused a progressive decline in HR from 433 ± 16.93 to 415 ± 14.91 bpm at the end of the experiment (Figure 4.3.1.1B). Bolus treatment of animals with ARS at doses 0.1 mg kg^{-1}, 0.3 mg kg^{-1}, or 1 mg kg^{-1} at the start of reperfusion did not have a significant effect on the HR when compared to MI-animals treated with vehicle ($NaHCO_3$) ($P > 0.05$, Figure 4.3.1.1B).

Baseline values of PRI in all groups of animals ranged from 44 ± 3.10 to 53 ± 1.11 mmHg min^{-1} 10^3 and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.1C). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [MI Vehicle ($NaHCO_3$)] exhibited a progressive decline in PRI from 46.46 ± 3.86 to 34 ± 5.57 mmHg min^{-1} 10^3 at the end of the experiment (Figure 4.3.1.1C). Bolus treatment of animals with ARS at doses 0.1 mg kg^{1}, 0.3 mg kg^{-1}, or 1 mg kg^{-1} at the start of reperfusion did not have a significant effect on the PRI when compared to MI-animals treated with vehicle ($NaHCO_3$) ($P > 0.05$, Figure 4.3.1.1C).

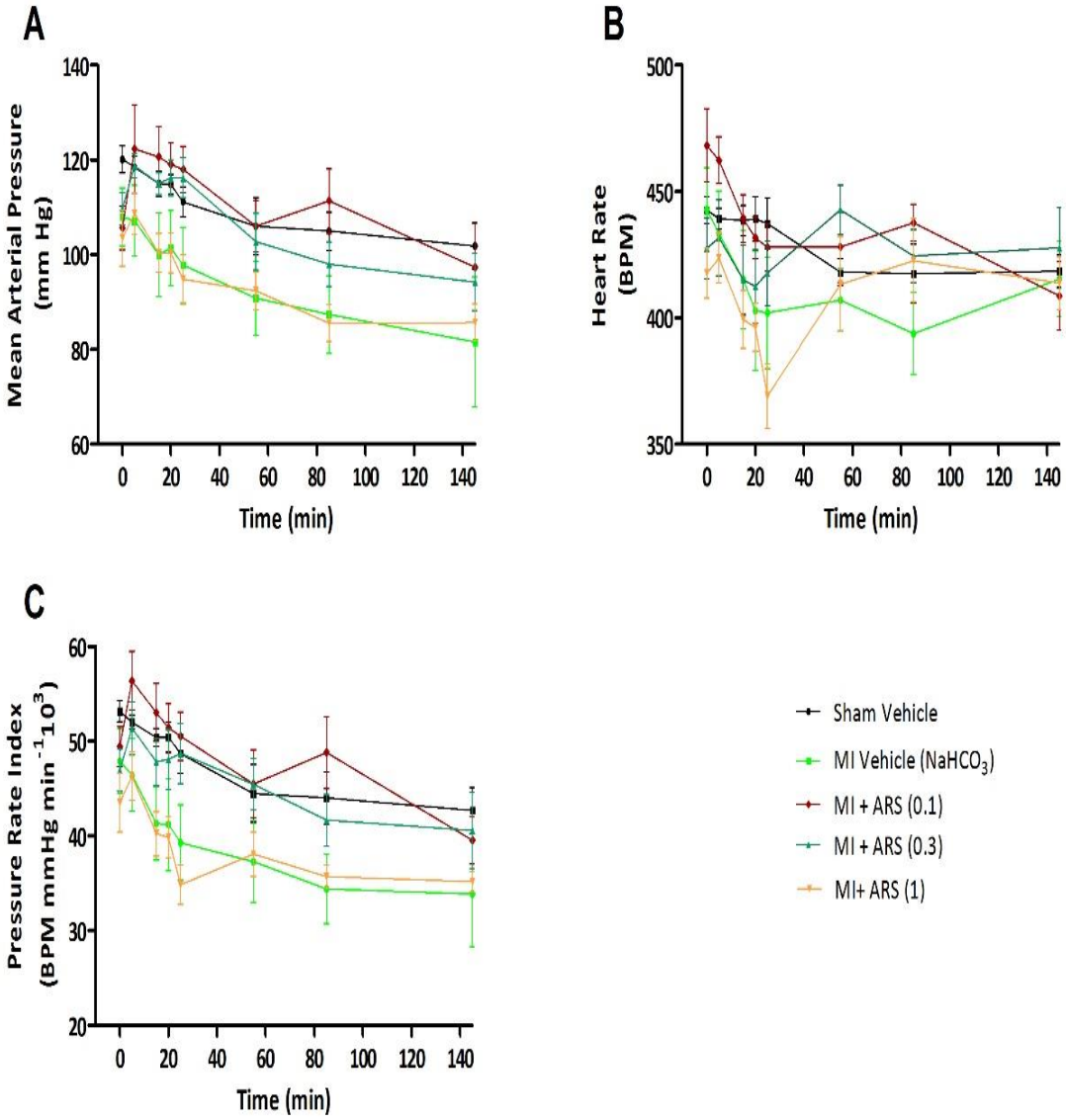


Figure 4.3.1.1. (A) Mean arterial pressure, (B) heart rate, and (C) pressure rate index of rats subjected to surgical procedure alone an treated with vehicle (Sham Vehicle, n = 6), or 25 min ischaemia and 120 min reperfusion and treated with vehicle [MI Vehicle ($NaHCO_3$), n = 5], or 25 min ischaemia and 120 min reperfusion and treated with artesunate in $NaHCO_3$ (ARS 0.1 mg kg^{-1}i.v., n = 3, 0.3 mg kg^{-1}i.v., n = 4, 1 mg kg^{-1}i.v., n = 11), (two-way analysis of variance with repeated measures, with Bonferroni post-test).

Comparison of the effects of using a dose of 1 mg kg^{-1} of ARS in the different vehicle on haemodynamic parameters

The baseline values of mean arterial blood pressure in all groups of animals ranged from 104 ± 5.89 to 120 ± 2.86 mmHg, and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.2A). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle in either 10 % DMSO, $NaHCO_3$ or 30 % Cyclodextrin exhibited a progressive decline in MAP (DMSO, 112 ± 3.70 to 90 ± 4.63; $NaHCO_3$, 107 ± 7.41 to 82 ± 13.72; 30 % Cyclodextrin, 121 ± 4.65 to 99 ± 5.26) mmHg at the end of the experiment (Figure 4.3.1.2A). Bolus treatment of animals with ARS at dose of 1 mg kg^{-1} at the start of reperfusion in all three vehicles did not cause any significant changes in MAP when compared to MI-animals treated with their respective vehicle ($P > 0.05$, Figure 4.3.1.2A).

Baseline values of HR in all groups ranged from 393 ± 14.10 to 443 ± 16.59 bpm, and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.2B). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle in either 10 % DMSO, 30 % Cyclodextrin or $NaHCO_3$ exhibited a progressive decline in HR (DMSO, 433 ± 12.38 to 418 ± 12.76, Cyclodextrin, 426 ± 10.83 to 421 ± 16.37; $NaHCO_3$, 433 ± 16.93 to 415 ± 14.91) bpm at the end of the experiment (Figure 4.3.1.2B). Bolus treatment of animals with ARS at dose of 1 mg kg^{-1} at the start of reperfusion in all three vehicles did not cause any significant changes in HR when compared to MI-animals treated with their respective vehicle ($P > 0.05$, Figure 4.3.1.2B).

Baseline values of PRI in all groups of animals ranged from 44 ± 3.10 to 53 ± 1.11 mmHg min^{-1} 10^3 and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.2C). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle in either 10 % DMSO, 30 % Cyclodextrin or $NaHCO_3$ exhibited a decline in PRI (DMSO, 48 ± 2.22 to 38 ± 2.21; Cyclodextrin, 52 ± 2.98 to 42 ± 3.45; $NaHCO_3$, 46 ± 3.86 to 34 ± 5.57) mmHg min^{-1} 10^3 at the end of the experiment (Figure 1.4C). Bolus treatment of animals with ARS at dose of 1 mg/kg at the start of reperfusion in all three vehicles did not cause any significant changes in PRI when compared to MI-animals treated with their respective vehicle ($P > 0.05$, Figure 4.3.1.2C).

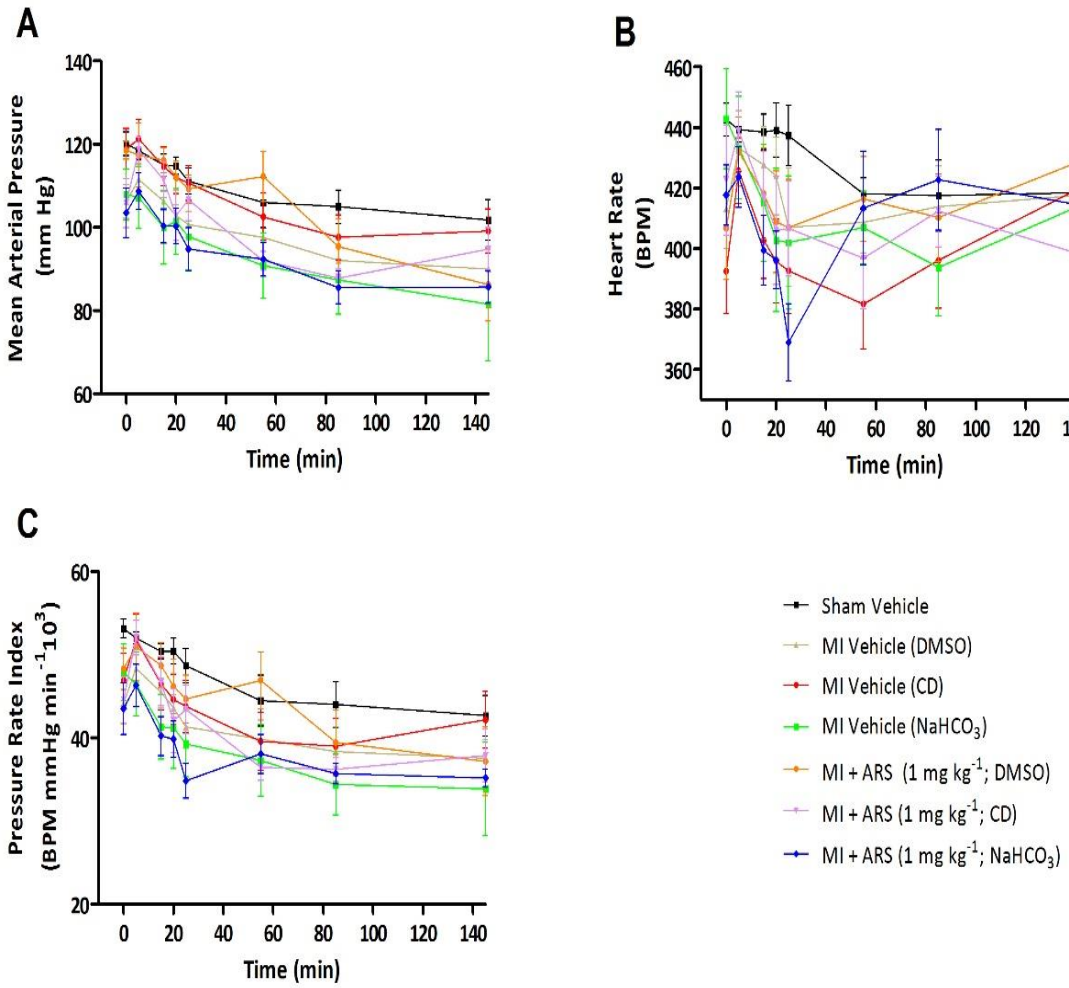


Figure 4.3.1.2. (A) Mean arterial pressure, (B) heart rate, and (C) pressure rate index of rats subjected to surgical procedure alone an treated with vehicle (Sham Vehicle, 10 % DMSO 1 ml/kg i.v., *n* = 6), or 25 min ischaemia and 120 min reperfusion and treated with vehicle (MI Vehicle) in either 10 % DMSO (*n* = 13), 30 % Cyclodextrin (*n* = 7), or $NaHCO_3$ (*n* = 5), or 25 min ischaemia and 120 min reperfusion and treated with artesunate (ARS) in either 10 % DMSO (1 mg kg^{-1}i.v., *n* = 7), 30 % Cyclodextrin (1 mg kg^{-1}i.v., *n* = 6), or $NaHCO_3$ (1 mg kg^{-1}i.v., *n* = 11), (two-way analysis of variance with repeated measures, with Bonferroni post-test).

Effect of inhibition of survival pathways in the ARS-induced effects on haemodynamic parameters

The baseline values of MAP in all groups of animals ranged from 100 ± 8.52 to 120 ± 2.86 mmHg, and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.3A). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [MI Vehicle ($NaHCO_3$)] caused a progressive decline in MAP from 107 ± 7.41 to 82 ± 13.72 mmHg at the end of the experiment (Figure 4.3.1.3A). Bolus treatment of animals with ARS (1 mg kg^{-1}) at the start of reperfusion did not cause any significant changes in MAP when compared to MI-animals treated with vehicle ($NaHCO_3$) ($P > 0.05$, Figure 4.3.1.3A). Furthermore, Bolus administration of animals with LY294002 (0.3 mg kg^{-1}) or UO126 (0.2 mg kg^{-1}) 10 min before occlusion of the LAD did not cause any significant changes in MAP ($P > 0.05$, Figure 4.3.1.3A).

Baseline values of HR in all groups ranged from 418 ± 10.00 to 443 ± 16.59 bpm, and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.3B). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [MI Vehicle ($NaHCO_3$)] caused a progressive decline in HR from 433 ± 16.59 to 415 ± 14.91 mmHg at the end of the experiment (Figure 4.3.1.3B). Bolus treatment of animals with ARS (1 mg kg^{-1}) at the start of reperfusion did not cause any significant changes in HR when compared to MI-animals treated with vehicle ($NaHCO_3$) ($P > 0.05$, Figure 4.3.1.3B). Furthermore, Bolus administration of animals with LY294002 (0.3 mg kg^{-1}) or UO126 (0.2 mg kg^{-1}) 10 min before occlusion of the LAD did not cause any significant changes in HR ($P > 0.05$, Figure 4.3.1.3B).

Baseline values of PRI in all groups of animals ranged from 44 ± 3.10 to 53 ± 1.11 mmHg min^{-1} 10^3 and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.3C). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [MI Vehicle ($NaHCO_3$)] exhibited a progressive decline in PRI (46 ± 3.86 to 34 ± 5.57 mmHg min^{-1} 10^3) at the end of the experiment (Figure 4.3.1.3C). Bolus treatment of animals with ARS (1 mg kg^{-1}) at the start of reperfusion did not cause any significant changes in PRI when compared to MI-animals treated with vehicle ($NaHCO_3$) ($P > 0.05$, Figure 4.3.1.3C). Furthermore, Bolus administration of animals with LY294002 (0.3 mg kg^{-1}) or UO126 (0.2 mg kg^{-1}) 10 min before occlusion of the LAD did not cause any significant changes in PRI ($P > 0.05$, Figure 4.3.1.3C).

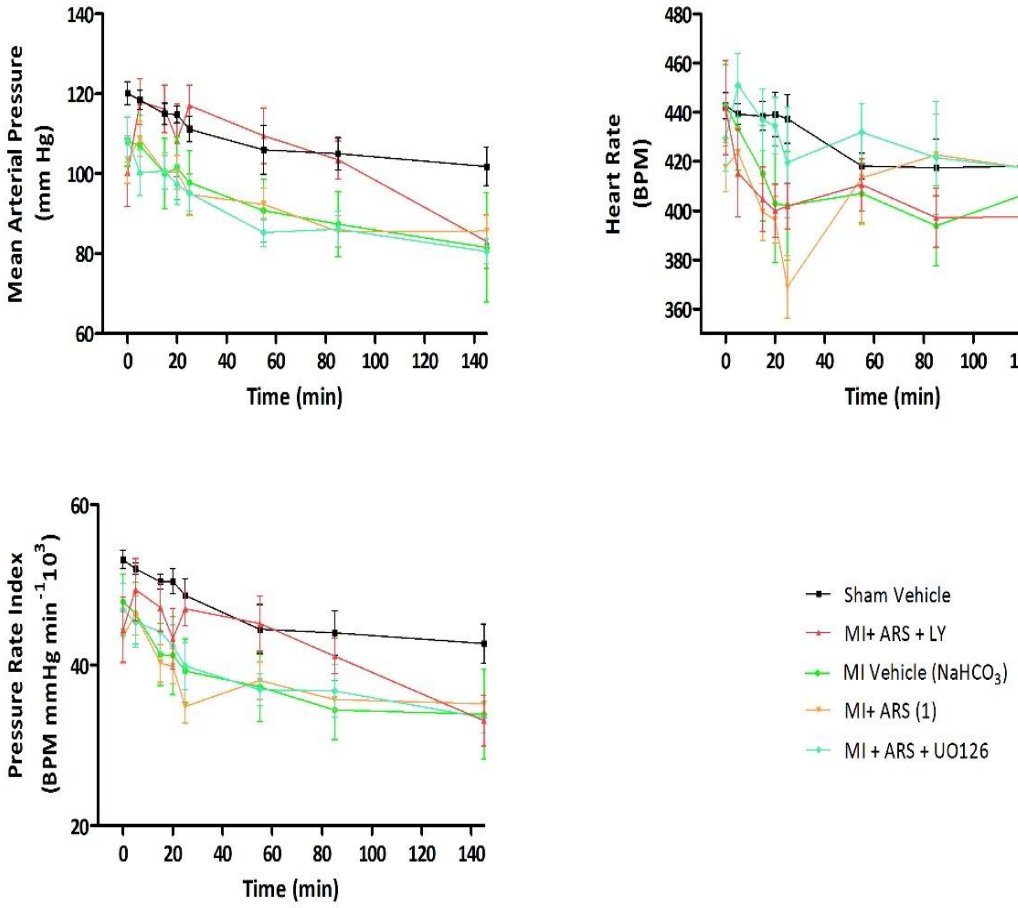


Figure 4.3.1.3. (A) Mean arterial pressure, (B) heart rate, and (C) pressure rate index of rats subjected to surgical procedure alone and treated with vehicle (Sham Vehicle, *n* = 6), or 25 min ischaemia and 120 min reperfusion and treated with vehicle in $NaHCO_3$ [MI Vehicle ($NaHCO_3$) *n* = 5], or 25 min ischaemia and 120 min reperfusion and treated with artesunate in $NaHCO_3$ [MI + ARS (1 mg kg^{-1}i.v.), *n* = 11] at the start of reperfusion, or pre-treated with either LY294002 or UO126 10 min before ischaemia, followed by 25 min of ischaemia and 120 min reperfusion and treated at the start of reperfusion with artesunate in $NaHCO_3$ (MI + ARS + LY 0.3 mg kg^{-1}i.v., *n* = 8; MI + ARS + UO126 0.2 mg kg^{-1}i.v., *n* = 10), (two-way analysis of variance with repeated measures, with Bonferroni post-test).

Effect of DHA on haemodynamic parameters

The baseline values of MAP in all groups of animals ranged from 106 ± 4.07 to 130 ± 4.06 mmHg, and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.4A). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [MI Vehicle (DMSO)] exhibited a progressive decline in MAP (112 ± 3.70 to 90 ± 4.63 mmHg at the end of the experiment (Figure 4.3.1.4A). Bolus treatment of animals with ARS (0.1 mg kg^{-1}) resulted in a significant change in MAP when compared to MI Vehicle (DMSO) only at the 25 min point (126 ± 4.18 vs. 101 ± 3.21). Whereas, bolus treatment DHA (0.1 mg kg^{-1}) upon reperfusion did not cause any significant changes in MAP when compared to MI-animals treated with vehicle (DMSO) ($P > 0.05$, Figure 4.3.1.4A).

Baseline values of HR in all groups ranged from 413 ± 13.21 to 449 ± 10.59 bpm, and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.4B). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [MI Vehicle (DMSO)] exhibited a progressive decline in HR (433 ± 12.38 to 418 ± 12.76) bpm at the end of the experiment (Figure 4.3.1.4B). Bolus treatment of animals with either ARS (0.1 mg kg^{-1}) or DHA (0.1 mg kg^{-1}) upon reperfusion did not cause any significant changes in HR when compared to MI-animals treated with vehicle (DMSO) ($P > 0.05$, Figure 4.3.1.4B).

Baseline values of PRI in all groups of animals ranged from 44 ± 2.47 to 54 ± 1.80 mmHg min^{-1} 10^3 and were not significantly different between groups ($P > 0.05$, Figure 4.3.1.4C). When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [(MI Vehicle (DMSO)] exhibited a progressive decline in PRI (48 ± 2.22 to 38 ± 2.21 mmHg min^{-1} 10^3) at the end of the experiment (Figure 4.3.1.4C). Bolus treatment of animals with either ARS (0.1 mg kg^{-1}) or DHA (0.1 mg kg^{-1}) upon reperfusion did not cause any significant changes in PRI when compared to MI-animals treated with vehicle (DMSO) ($P > 0.05$, Figure 4.3.1.4C).

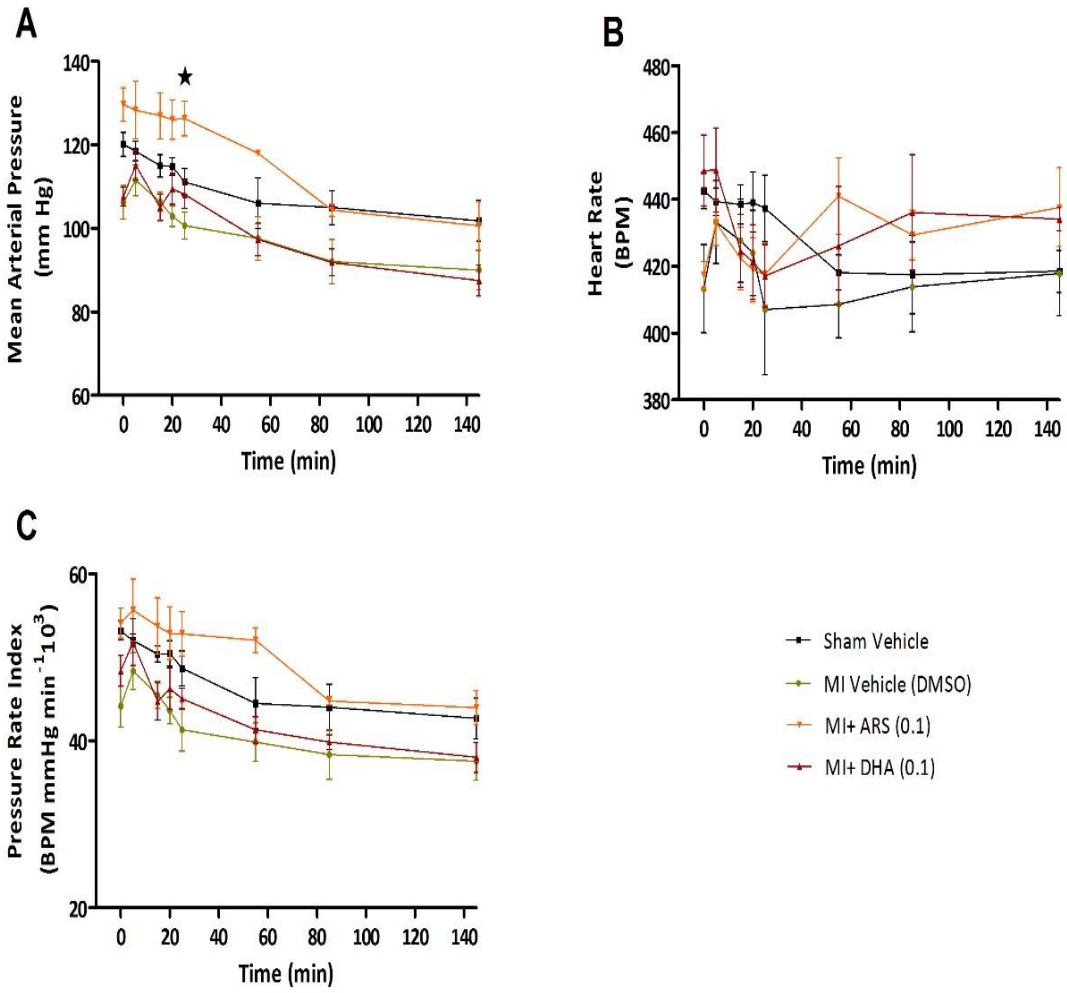


Figure 4.3.1.4 (A) Mean arterial pressure, (B) heart rate, and (C) pressure rate index of rats subjected to either surgical procedure alone and treated with vehicle (Sham, n = 6), or 25 min ischaemia and 120 min reperfusion and treated with either vehicle in 10 % DMSO (MI Vehicle, n = 13), artesunate (MI + ARS, 0.1 mg kg^{-1}, n = 3) or dihydroartemisinin (MI + DHA, 0.1 mg kg^{-1}, n = 6) at the start of reperfusion. ★$P < 0.05$ vs. MI Vehicle (DMSO), (two-way analysis of variance with repeated measures, with Bonferroni post-test).

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Effect on the area at risk

There was no significant difference in the area at risk (AAR) between any of the groups studied (Table 4.3.2.).

Table 4.3.2. List of all the experimental groups with their respective AAR, expressed as a percentage of the left ventricle.

Group	*n*	Area at risk % of left ventricle
Sham vehicle (1 ml kg^{-1}; 10 % DMSO)	6	54.67 ± 1.96
MI Vehicle (1 ml kg^{-1}; 10 % DMSO)	14	51.26 ± 1.59
MI Vehicle (1 ml kg^{-1}; 30 % Cyclodextrin)	7	52.29 ± 1.23
MI Vehicle (1 ml kg^{-1}; $NaHCO_3$)	5	55.20 ± 3.12
MI + ARS (0.1 mg kg^{-1}; 10 % DMSO)	3	49.00 ± 3.79
MI + ARS (0.3 mg kg^{-1}; 10 % DMSO)	7	51.86 ± 2.12
MI + ARS (1 mg kg^{-1}; 10 % DMSO)	7	50.00 ± 2.10
MI + ARS (0.3 mg kg^{-1}; 30 % Cyclodextrin)	7	48.00 ± 4.08
MI + ARS (1 mg kg^{-1}; 30 % Cyclodextrin)	7	49.43 ± 1.23
MI + ARS (0.1 mg kg^{-1}; $NaHCO_3$)	4	47.25 ± 2.72
MI + ARS (0.3 mg kg^{-1}; $NaHCO_3$)	5	49.80 ± 4.77
MI + ARS (1 mg kg^{-1}; $NaHCO_3$)	12	48.17 ± 2.44
MI + LY (0.3 mg kg^{-1})+ ARS (1 mg kg^{-1}; $NaHCO_3$)	10	53.30 ± 1.51
MI + U0126 (0.2 mg kg^{-1}) + ARS (1 mg kg^{-1}; $NaHCO_3$)	11	52.34 ± 2.48
MI + IPC	12	50.42 ± 3.14
MI + LY (0.3 mg kg^{-1}) + IPC	7	50.14 ± 2.09
MI + U0126 (0.2 mg kg^{-1})+ IPC	5	44.20 ± 3.84
MI + DHA (mg kg^{-1}; 10 % DMSO)	6	51.00 ± 2.37

ARS = Artesunate; DHA = Dihydroartemisinin;
IPC = Ischaemic preconditioning; LY = LY294002; **MI** = Myocardial infarction

The area at risk (AAR) in rats subjected to either i) surgical procedure alone and treated with vehicle (Sham Vehicle), ii) 25 min ischaemia and 120 min reperfusion and treated

with vehicle [(MI Vehicle) in either 10 % DMSO, 30 % Cyclodextrin or $NaHCO_3$], iii) 25 min ischaemia and 120 min reperfusion and treated at the start of reperfusion with artesunate [(MI + ARS) in either 10 % DMSO (0.1, 0.3, 1 mg kg^{-1} i.v.), 30 % Cyclodextrin (0.3, 1 mg kg^{-1} i.v.) or $NaHCO_3$ (0.1, 0.3, 1 mg kg^{-1} i.v.), iv) treated with either LY294002 (MI + LY 0.3 mg kg^{-1} + ARS) or U0126 (MI + U0126 0.2 mg kg^{-1} + ARS) 10 min before ischaemia, followed by 25 min ischaemia and 120 min reperfusion and treated at the start of reperfusion with ARS, v) treated with either vehicle (MI + IPC), LY294002 (MI + LY + IPC) or U0126 (MI + U0126 + IPC) 10 min before 2 cycles of preconditioning followed by 25 min ischaemia and 120 min reperfusion, or vi) 25 min ischaemia and 120 min reperfusion and treated at the start of reperfusion with DHA (MI + DHA 0.1 mg kg^{-1}). Data are expressed as means ± S.E.M. for *n* number of observations (one-way analysis of variance with Bonferroni post-test).

Effect of ARS on the infarct size, in animals that underwent transient I/R injury

Animals subjected to surgical procedure alone (Sham Vehicle) exhibited an infarct size of approximately 6 %. When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle [MI Vehicle (DMSO)] demonstrated a significant increase in infarct size ($P < 0.05$, Figure 4.3.2A). When compared with MI animals treated with vehicle, administration of ARS at doses of 0.1 and 0.3 mg kg^{-1}, at the start of reperfusion, had no significant effect on infarct size ($P > 0.05$, Figure 4.3.2A). Whereas, when compared to MI animals treated with vehicle (MI Vehicle), administration of ARS at a higher dose of 1 mg kg^{-1} at the start of reperfusion, caused a significant reduction in infarct size ($P < 0.05$, Figure 4.3.2A).

When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle (MI Vehicle - 30 % CD) demonstrated a significant increase in infarct size ($P < 0.05$, Figure 4.3.2B). When compared with MI animals treated with vehicle (MI Vehicle- 30 % CD), administration of ARS at a dose of 0.3 mg kg^{-1}, at the start of reperfusion, had no significant effect on infarct size ($P > 0.05$, Figure 4.3.2B). When compared to MI animals treated with vehicle (MI Vehicle - 30 % CD), administration of ARS at a dose of 1 mg kg^{-1} at the start of reperfusion, caused a significant reduction in infarct size ($P < 0.05$, Figure 4.3.2B).

When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle (MI Vehicle – $NaHCO_3$) demonstrated a significant increase in infarct size ($P < 0.05$, Figure 4.3.2C). When compared with MI animals treated with vehicle (MI Vehicle – $NaHCO_3$), administration of ARS at a dose of 0.1 mg kg^{-1}, at the start of reperfusion, had no significant effect on the infarct size ($P > 0.05$, Figure 4.3.2C). When compared to MI animals treated with vehicle (MI Vehicle – $NaHCO_3$), administration of ARS at a dose of 0.3 mg kg^{-1} at the start of reperfusion, attenuated the increase in infarct size ($P < 0.05$, Figure 4.3.2C), while a higher dose of 1 mg kg^{-1} of ARS had an even greater effect ($P < 0.05$, Figure 4.3.2C).

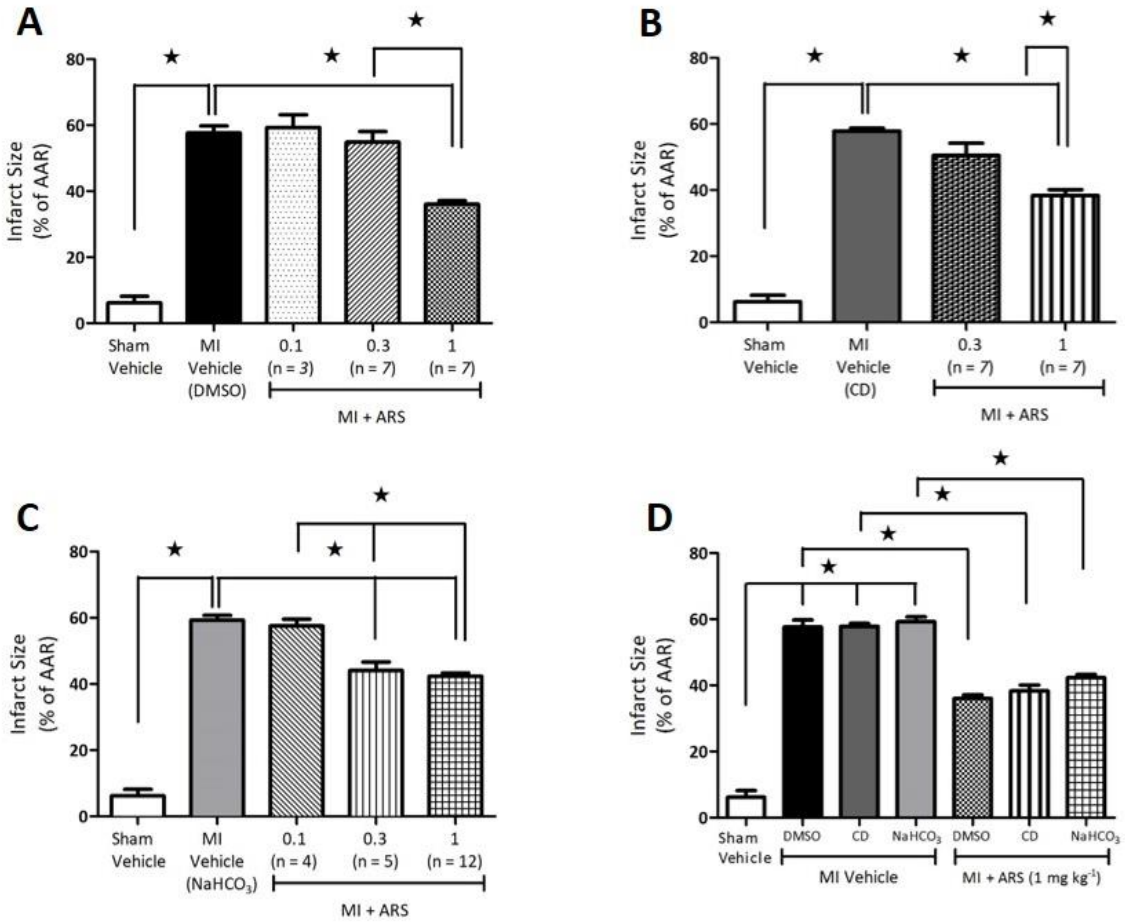

Figure 4.3.2. Infarct size in animals that underwent transient I/R injury. Rats were subjected to surgical procedure alone and treated with vehicle (Sham Vehicle, *n* = 6), or 25 min ischaemia and 120 min reperfusion and treated with vehicle (MI Vehicle) in either 10 % DMSO (panel A, *n* = 14), 30 % Cyclodextrin (CD, panel B, *n* = 7) and $NaHCO_3$ (panel C, *n* = 5), or 25 min ischaemia and 120 min reperfusion and treated at the start of reperfusion, with artesunate (MI + ARS) in either 10 % DMSO (0.1, 0.3, 1 mg kg^{-1} i.v., panel A), 30 % CD (0.3, 1 mg kg^{-1} i.v., panel B) and $NaHCO_3$ (0.1, 0.3, 1 mg kg^{-1} i.v., panel C). Panel D shows a combined figure of MI Vehicle in 10 % DMSO, 30 % CD and $NaHCO_3$ against their respective treatment groups with artesunate (1 mg kg^{-1} i.v). Data are expressed as means ± S.E.M. for *n* number of observations. ★ $P < 0.05$.

Effect of ARS on the infarct size, in animals that underwent transient I/R injury following inhibition of survival pathways

To determine the role of activating components of the RISK survival pathway; PI3K/Akt and ERK 1/2 in the cardioprotective effects of ARS, rats were treated with LY294002 and U0126, a PI3K and ERK 1/2 inhibitor, respectively, 10 min prior to initiation of 25 min ischaemia (followed by 2 h of reperfusion). Animals subjected to surgical procedure alone (Sham Vehicle) exhibited an infarct size of approximately 6 %. When compared to Sham Vehicle animals, animals treated with vehicle (MI Vehicle – $NaHCO_3$), demonstrated a significant increase in infarct size ($P < 0.05$, Figure 4.3.3.1) to ~ 60 %. When compared with MI animals treated with vehicle (MI Vehicle – $NaHCO_3$), administration of artesunate (ARS) at a dose of 1 mg kg^{-1}, at the start of reperfusion, attenuated the increase in infarct size ($P < 0.05$, Figure 4.3.3.1) to ~ 40 %. This effect afforded by ARS was abolished following the administration of LY294002 and U0126, inhibitors of the PI3K/Akt and ERK 1/2 pathway, respectively ($P < 0.05$, Figure 4.3.3.1)

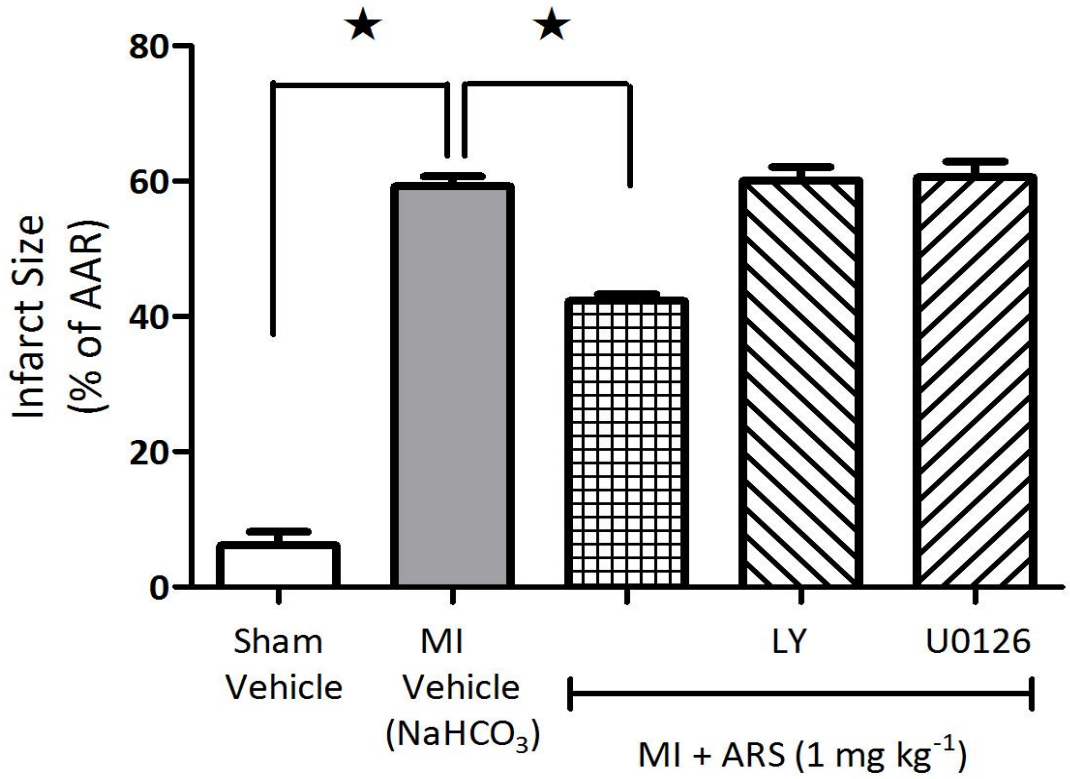

Figure 4.3.3.1 Effect of inhibition of components of the RISK pathway on infarct size in animals that underwent transient I/R injury. Rats were subjected to surgical procedure alone and treated with vehicle (Sham Vehicle, *n* = 6), or 25 min ischaemia and 120 min reperfusion and treated with vehicle [MI Vehicle $(NaHCO)_3$, *n* = 5], or 25 min ischaemia and 120 min reperfusion and treated at the start of reperfusion, with either artesunate (MI + ARS 1 mg kg^{-1} i.v., *n* = 12), or pre-treated with either LY294002 or U0126 10 min before ischaemia, followed by 25 min ischaemia and 120 min reperfusion and treated at the start of reperfusion, with artesunate (MI + ARS + LY 0.3 mg kg^{-1} i.v., *n* = 10; MI + ARS + U0126 0.2 mg kg^{-1} i.v., *n* = 11). Data are expressed as means ± S.E.M. for *n* number of observations. ★ $P < 0.05$ vs. MI Vehicle ($NaHCO_3$).

Effect of inhibition of the RISK pathway on infarct size, in animals that underwent transient I/R injury subsequent to IPC

To confirm that activation of the RISK pathway is pivotal in mediating the IPC-induced cardioprotection, rats were treated with LY and U0126, PI3K/Akt and ERK 1/2 inhibitors respectively, 10 min prior to initiating the IPC stimulus. Animals subjected to surgical procedure alone (Sham Vehicle) exhibited an infarct size of approximately 6 %. When compared to Sham Vehicle animals, those subjected to LAD occlusion and treated with vehicle (MI Vehicle - 10 % DMSO), demonstrated a significant increase in infarct size ($P < 0.05$, Figure 4.3.3.2). This was reduced significantly when animals went through 2 cycles of IPC before occlusion of the LAD ($P < 0.05$, Figure 4.3.3.2). This effect afforded by IPC was abolished following the administration of LY and U0126 which were both given 10 min before the initiation of IPC ($P < 0.05$, Figure 4.3.3.2).

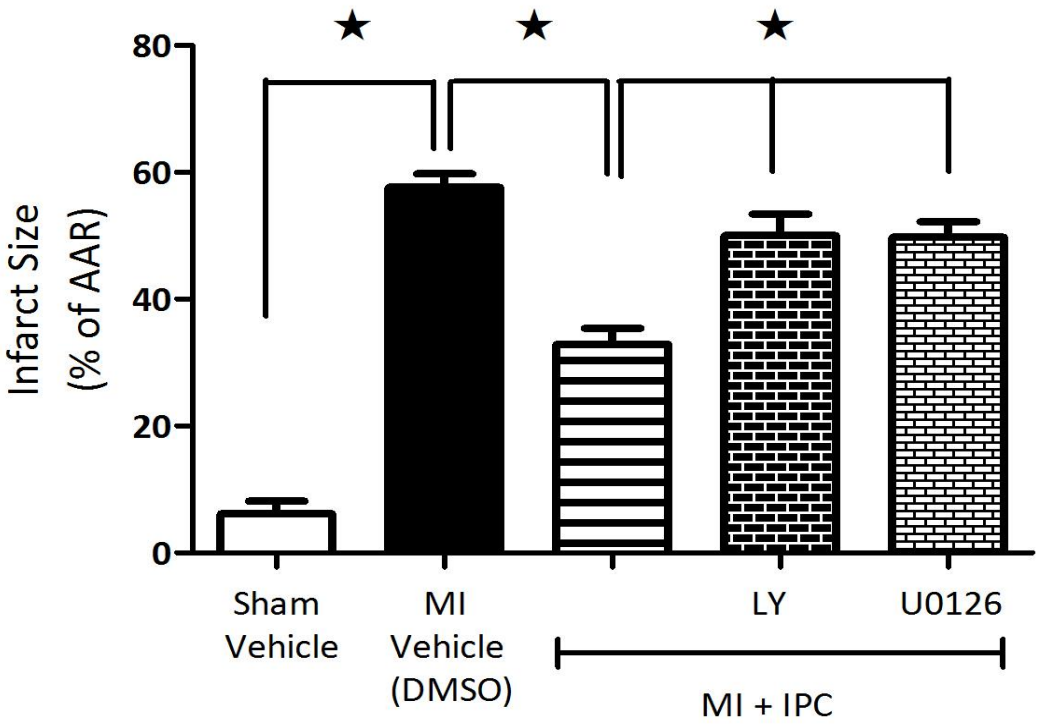

Figure 4.3.3.2 Effect of inhibition of components of the RISK pathway on infarct size in animals that underwent transient I/R injury subsequent to an IPC stimulus. Rats were subjected to surgical procedure alone and treated with vehicle (Sham Vehicle, *n* = 6), or 25 min ischaemia and 120 min reperfusion and treated with vehicle [MI Vehicle (DMSO), *n* = 14], or 2 cycles of preconditioning followed by 25 min ischaemia and 120 min reperfusion and treated with vehicle (MI + IPC *n* = 12), or pre-treated with either LY29400 (MI + IPC + LY 0.3 mg kg^{-1} i.v., *n* = 7) or U0126 (MI + IPC + U0126, 0.2 mg kg^{-1} i.v., *n* = 5) 10 min before the 2 cycles of preconditioning followed by 25 min ischaemia and 120 min reperfusion. Data are expressed as means ± S.E.M. for *n* number of observations. ★ $P < 0.05$ vs. MI Vehicle (DMSO).

Effect of ARS on the phosphorylation of Akt and GSK-3β, in the hearts of animals that underwent transient I/R injury

In order to explore which downstream effectors are involved in mediating the ARS-induced cardioprotection, following activation of the survival pathways mentioned above, western blot analysis was carried out in hearts of animals subjected to LAD occlusion and reperfusion. When compared to hearts from Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle (MI Vehicle) demonstrated no change in the phosphorylation of Akt on Ser^{473} and GSK-3β on Ser^{9} ($P > 0.05$, Figures 4.3.4.1A and B). Administration of ARS at the start of reperfusion to animals subjected to LAD occlusion, resulted in a significant increase in the phosphorylation of Akt on Ser^{473} and GSK-3β on Ser^{9} ($P < 0.05$, Figures 4.3.4.1A and B).

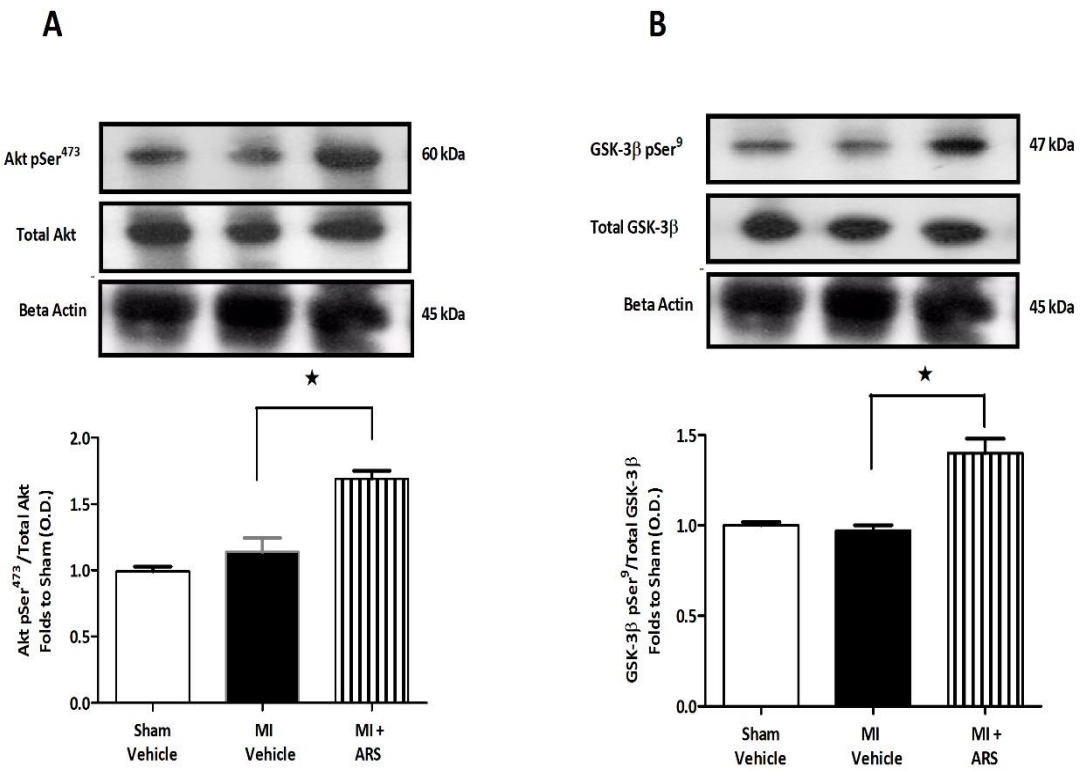

Figure 4.3.4.1. Effect of ARS on the phosphorylation of Akt and GSK-3β on Ser^{473} and Ser^{9} residue, respectively, in the hearts of animals that underwent transient I/R injury. Each immunoblot is from a single experiment and is representative of four separate experiments in which rats were subjected to surgical procedure alone and treated with vehicle (Sham Vehicle, n = 4), or 25 min ischemia and 120 min reperfusion and treated with either vehicle (MI Vehicle, n = 4), or ARS (MI + ARS, n = 4) at the start of reperfusion. Densitometric analysis of the bands is expressed as relative optical density of Akt and GSK-3β phosphorylation at Ser^{473} and Ser^{9}, corrected for the corresponding Akt and GSK-3β content and normalised using the related sham-operated band. Data are expressed as means ± S.E.M. for n number of observations. ★P < 0.05 vs. MI Vehicle. (Western blot generated by M. Collino, University of Turin)

Effect of ARS on the nuclear translocation of the p65 NF-κB subunit in the hearts of animals that underwent transient I/R injury

When compared to hearts of Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle (MI Vehicle) exhibited significant increases in the nuclear translocation of the p65 subunit ($P < 0.05$, Figure 4.3.4.2) indicating activation of NF-κB. Administration of ARS at the start of reperfusion to animals subjected to LAD occlusion significantly attenuated the increase in nuclear translocation of p65 and, hence, inhibition of activation of NF-κB in the heart ($P < 0.05$, Figure 4.3.4.2).

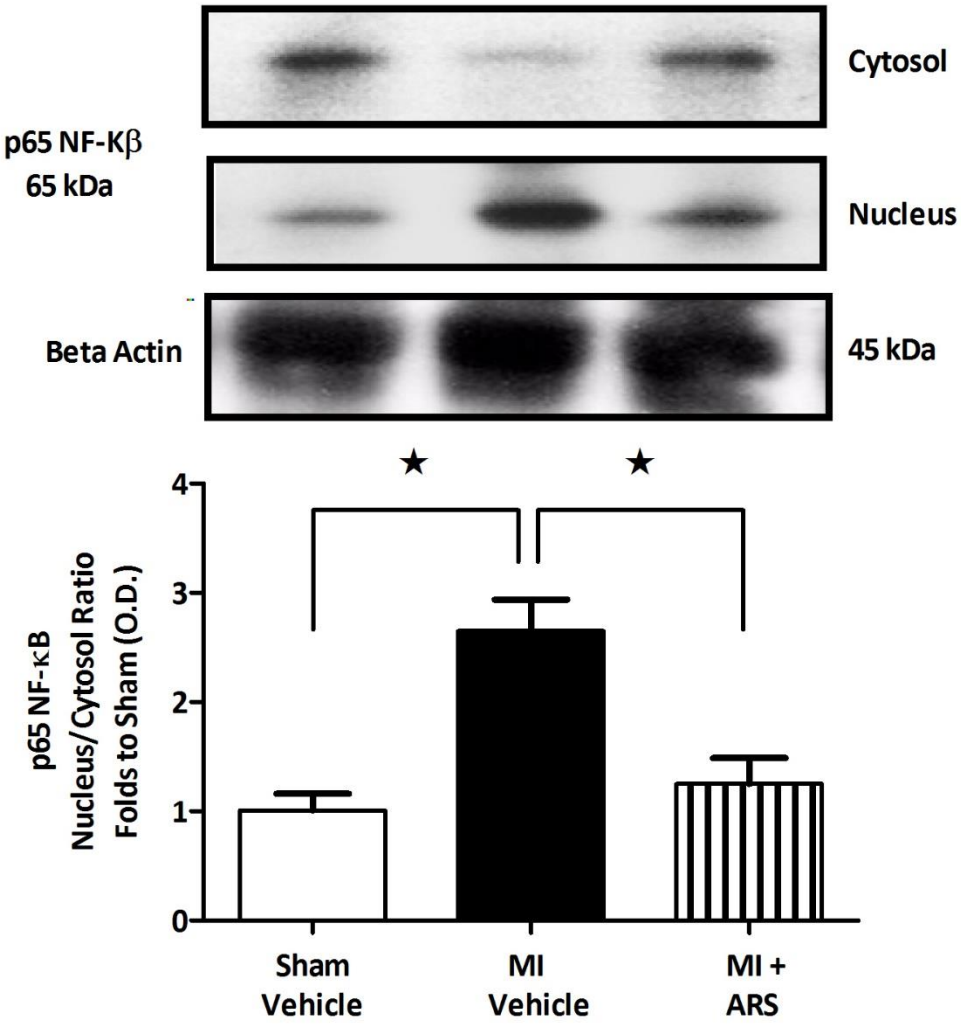

Figure 4.3.4.2. Effect of ARS on NF-κB in the hearts of animals that underwent transient I/R injury. Each immunoblot is from a single experiment and is representative of four separate experiments in which rats were subjected to surgical procedure alone and treated with vehicle (Sham Vehicle, n = 4), or 25 min ischemia and 120 min reperfusion and treated with either vehicle (MI Vehicle, n = 4), or ARS (MI + ARS, n = 4) at the start of reperfusion. NF-κB translocation from the cytosol to the nucleus was evaluated measuring NF-κB p65 subunit levels in both cytosol and nuclear fractions and expressing the results as nucleus/cytosol ratio**.** Data are expressed as means ± S.E.M. for n number of observations. ★ $P < 0.05$ vs. MI Vehicle. (Western blot generated by M. Collino, University of Turin)

Effect of ARS on the expression of eNOS in the hearts of animals that underwent tr ient I/R injury

When compared to hearts from Sham Vehicle animals, animals subjected to LAD oc sion and treated with vehicle (MI Vehicle) demonstrated no change in the expression of NOS on Ser^{1177} ($P > 0.05$, Figure 4.3.4.3). Administration of ARS at the start of re fusion to animals that underwent LAD occlusion however, resulted in a significant in ase in the expression of eNOS on Ser^{1177} ($P < 0.05$, Figure 4.3.4.3).

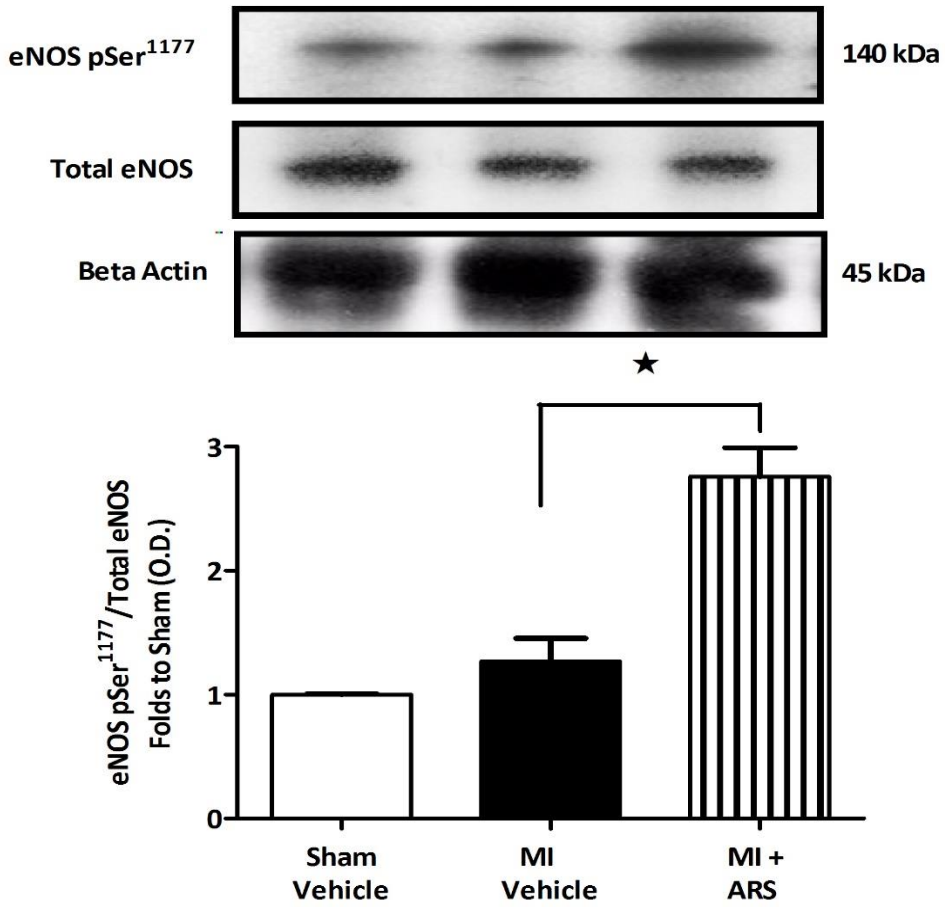

Figure 4.3.4.3. Effect of ARS on the expression of eNOS in the hearts of animals that underwent transient I/R injury. Each immunoblot is from a single experiment and is representative of four separate experiments in which rats were subjected to surgical procedure alone and treated with vehicle (Sham Vehicle, $n = 4$), or 25 min ischemia and 120 min reperfusion and treated with either vehicle (MI Vehicle, $n = 4$), or ARS (MI + ARS, $n = 4$) at the start of reperfusion. Densitometric analysis of the bands is expressed as relative optical density of eNOS and corrected for the corresponding total eNOS content and normalized using the related sham band. Data are expressed as means ± S.E.M. for n number of observations. ★ $P < 0.05$ vs. MI Vehicle. (Western blot generated by M. Collino, University of Turin)

Effect of ARS on the phosphorylation of STAT3, in the hearts of animals that underwent transient I/R injury

STAT3 is a vital mediator in the SAFE pathway and is known to mediate cardioprotection when activated by mechanical procedures (e.g. pre-and postconditioning) as well as various pharmacological interventions. To elucidate whether STAT3 is also involved in the ARS-induced cardioprotection, western blot analysis was carried out in hearts of animals subjected to LAD occlusion and reperfusion. When compared to hearts from Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle (MI Vehicle) demonstrated no change in the phosphorylation of STAT3 ($P > 0.05$, Figures 4.3.4.4). Administration of ARS at the start of reperfusion to animals subjected to LAD occlusion, resulted in a significant increase in the phosphorylation of STAT3 ($P < 0.05$, Figures 4.3.4.4).

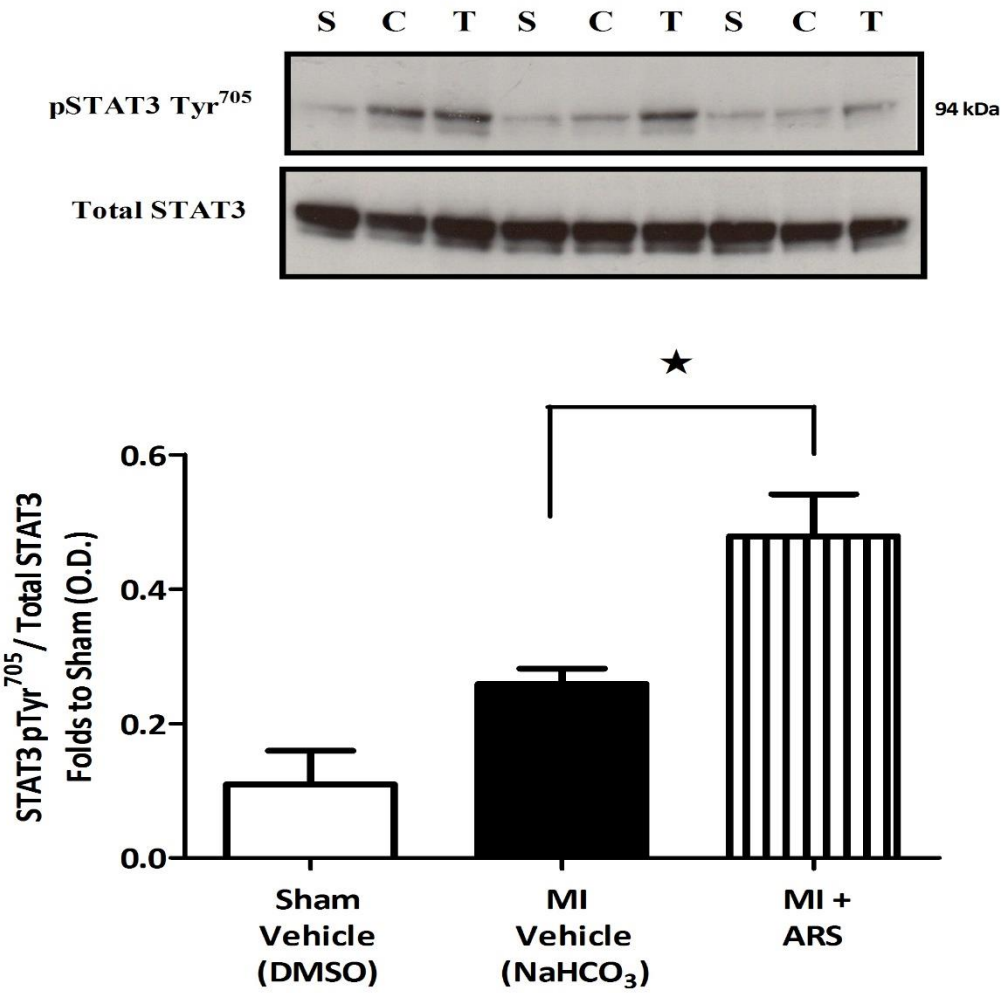

Figure 4.3.4.4. Effect of ARS on the expression of STAT3 in the hearts of animals that underwent transient I/R injury. Each immunoblot is from a single experiment and is representative of six separate experiments in which rats were subjected to surgical procedure alone and treated with vehicle [Sham Vehicle (S), n = 3], or 25 min ischemia and 120 min reperfusion and treated with either vehicle [MI Vehicle (C), n = 5], or ARS [MI + ARS (T), 1 mg kg^{-1} i.v., n = 6] at the start of reperfusion. Representative blot showing densitometric analysis of the bands, expressed as relative optical density of STAT3 and corrected for the corresponding total STAT3 content and normalized using the related sham band. Data are expressed as means ± S.E.M. for n number of observations. ★ $P < 0.05$ vs. MI Vehicle.

Plasma concentration-time profiles of ARS and its metabolite DHA

Maximal plasma concentrations (C_{max}) of ARS and DHA, 39.7 and 223 ng ml^{-1} respectively, were detected at a T_{max} of 10 min (or less) in sham vehicle rats (Figure 4.3.4.5A). Plasma concentrations of ARS and DHA began to fall by 20 and 125 min, respectively.

Rats that underwent MI demonstrated a C_{max} of 67.9 and 187.3 ng ml^{-1} for ARS and DHA respectively, at a T_{max} of 10 min (Figure 4.3.4.5B). Plasma concentrations of ARS and DHA began to fall by 35 and 65 min, respectively.

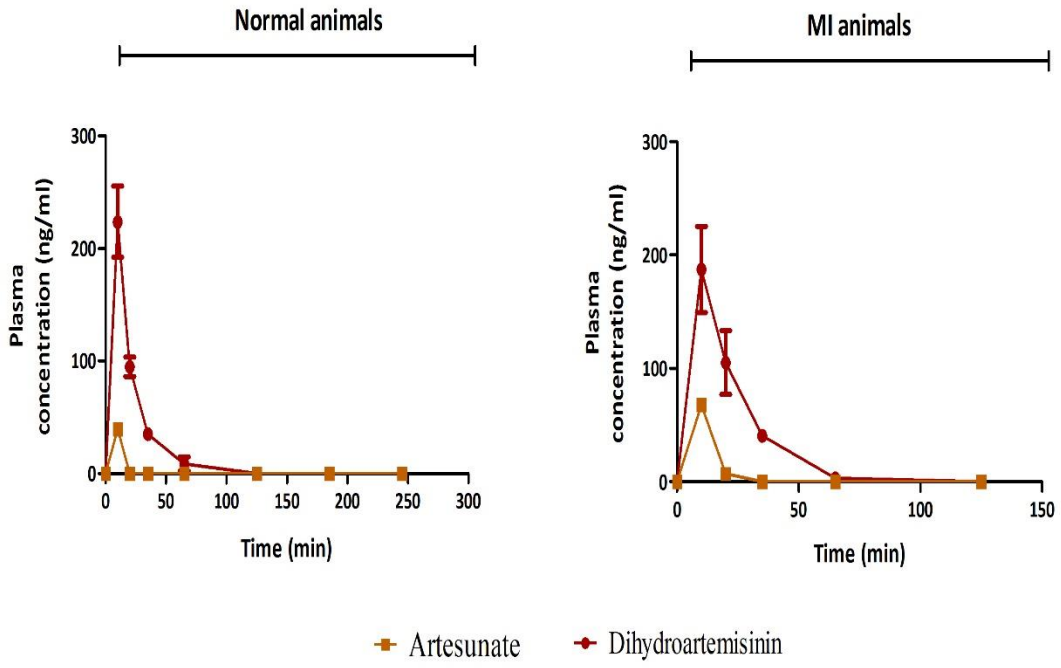

Figure 4.3.4.5. Plasma concentration-time profiles of ARS and DHA following a single intravenous (i.v.) dose of ARS. Rats were either subjected to surgical procedure alone (A) and treated with artesunate (1 mg kg^{-1} i.v., *n* = 4), or 25 min ischaemia and 120 min reperfusion (B) and treated with artesunate (1 mg kg^{-1} i.v., *n* = 4). Data are expressed as means ± S.E.M. for *n* number of observations. (Analysis performed by Prof. Laurent Decosterd's lab, Centre Hospitalier Universitaire Vaudois)

Effect of DHA on the infarct size in animals that underwent transient I/R injury

To determine whether DHA, the active metabolite of many artemisinin derivatives, exerts cardioprotective effects, rats were treated with DHA at the start of reperfusion. Due to the poor solubility of DHA in water and oils (Li *et al*., 2009) a dose of 0.1 mg kg^{-1} was used since it was not possible to fully dissolve the drug down to any other concentration, using various vehicles suitable for i.v. administration. Therefore here I compare it to a similar dose of ARS (in DMSO) which was shown previously not to be effective in reducing the infarct size (Figure 4.3.2 A). Animals subjected to surgical procedure alone (Sham Vehicle) exhibited an infarct size of approximately 6 %. When compared to Sham Vehicle animals, animals subjected to LAD occlusion and treated with vehicle (MI Vehicle - 10 % DMSO), demonstrated a significant increase in infarct size ($P < 0.05$, Figure 4.3.5A). Administration of ARS to rats that underwent MI at a dose of 0.1 mg kg^{-1} had no significant effect on infarct size ($P > 0.05$, Figure 4.3.5). Whereas, the administration of DHA at dose of 0.1 mg kg^{-1} caused a significant reduction in infarct size ($P < 0.05$, Figure 4.3.5).

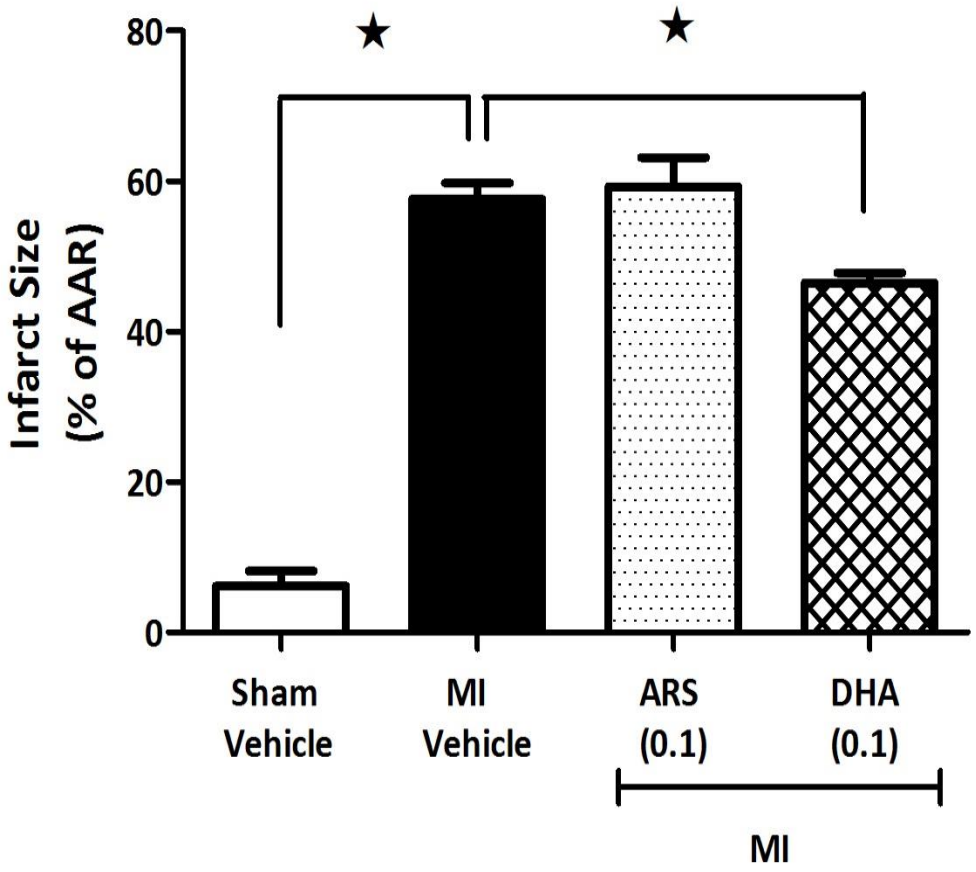

Figure 4.3.5. Infarct size in animals that underwent transient I/R injury. Rats were subjected to either surgical procedure alone and treated with vehicle (Sham, n = 6), or 25 min ischaemia and 120 min reperfusion and treated with either vehicle in 10 % DMSO (MI Vehicle, n = 14), ARS (MI + ARS, 0.1 mg kg^{-1}, n = 3) or DHA (MI + DHA, 0.1 mg kg^{-1}, n = 6) at the start of reperfusion. ★P < 0.05 vs. MI Vehicle.

4.4. Discussion

Ischaemic heart disease remains the leading cause of morbidity and mortality in the Western world. Although several interventions aimed at reducing the I/R injury have shown efficacy in experimental studies, they continue to fail to translate in a clinical setting, a reason believed to be due to the inaccurate timing of the intervention. Thus, there continues to be a growing need to uncover novel translatable interventions that could lessen the global impact of this disease on society.

I report here for the first time that rats treated with ARS at a dose of 1 mg kg^{-1} at the start of reperfusion results in a significant reduction in infarct size, resulting from myocardial I/R injury.

I demonstrate that an intravenous injection of ARS (1 mg kg^{-1}) administered in various types of vehicles (10 % DMSO, 30 % CD, and $NaHCO_3$) exhibited protection, i.e. reduction in infarct size in a rat model of transient I/R. It is clear, however, that the solubility in 10 % DMSO confers the biggest reduction in infarct size compared with the other two vehicles. DMSO, an amphipathic molecule with a high polar domain and two apolar methyl groups, rendering it soluble in both aqueous and organic media, is a widely used solvent for hydrophobic drugs (Guimarães *et al*., 2010). Studies have shown it to exhibit various protective roles, including antioxidant (Guimarães *et al*., 2010) to anti-inflammatory (DeForge *et al*., 1992; Kelly *et al.,* 1994; Chang *et al*., 2001). However, DMSO is associated with several systemic side effects such as diastolic and systolic hypertension (Hameroff *et al*., 1981), bradycardia (Santos *et al*., 2003), heart block (Shlafer *et al.,* 1976; Rapoport *et al.,* 1991) and rarely cardiac arrest (Baum *et al*., 1992). Although formulation of ARS in DMSO yielded the most effective reduction in infarct size, it must be taken into account the translatable ability of drug administration in the clinical setting. As an injectable formulation of ARS in $NaHCO_3$ was commercially available, this formulation was used for a large number of the studies in this chapter.

PI3Ks, and their downstream target serine/threonine kinase Akt, are a conserved family of signal transduction enzymes, involved in the regulation of inflammatory responses, cellular activation and apoptosis (Cantley, 2002). This pathway is thought to serve as an endogenous negative feedback regulator and/or compensatory mechanism limiting pro-inflammatory and apoptotic events in response to I/R injury (William *et al*.,

2006; Ha *et al*., 2008). Activation of the PI3K/Akt survival pathway has been implicated in preventing cardiac myocyte apoptosis (Matsui *et al*., 1999; Aikawa *et al*., 2000; Negoro *et al.*, 2001) and in protecting the myocardium against I/R injury (Fujio *et al*., 2000). I demonstrate here that PI3K inhibition by LY, a specific PI3K inhibitor, abolished the ARS-induced cardioprotection. In addition, western blotting studies probing for phosphorylated Akt protein, show Akt the downstream kinase of PI3K becomes phosphorylated at Ser^{473}, following treatment with ARS. This data confirms that the ARS-induced cardioprotection is mediated through a PI3K/Akt-dependent mechanism. Activation of this pathway is also the key driver of the cardioprotective effects afforded by ischaemic preconditioning.

Akt phosphorylates eNOS (Dimmeler *et al*., 1999; Fulton *et al*., 1999; Michell *et al*., 1999) and the resulting eNOS activation has anti-apoptotic (Kim *et al*., 1997), anti-inflammatory (Lamas *et al*., 1998) and antioxidant effects (Motterlini *et al*., 1996). I demonstrate that the cardioprotection afforded by ARS is partly due to an increase in phosphorylation of eNOS at Ser^{1177}, secondary to activation of the PI3K/Akt pathway. Although this effect on eNOS would need to be confirmed by using a pharmacological inhibitor such as N(5)-(1-Iminoethyl)-L-ornithine HCL to see if there is a reversal of effect of ARS, it's in line with various studies showing that activation of the PI3K-Akt-eNOS pathway plays a role in cardioprotection; i) ischaemic post-conditioning in a remodelled rat myocardium was shown to be via increased phosphorylation of Akt-downstream targets such as eNOS, secondary to activation of the PI3K/Akt pathway (Zhu *et al*., 2006), and ii) using a model of splanchnic artery occlusion, protection conferred by Akt was through phosphorylation of eNOS (Roviezzo *et al*., 2007).

The serine/threonine kinase GSK-3 plays a vital role in glycogen metabolism, via the phosphorylation and inactivation of glycogen synthase (Embi *et al*., 1980). The two known isoforms, GSK-3α and GSK-3β, also regulate various cellular processes such as survival and apoptosis (Frame & Cohen, 2001; Song *et al.*, 2009). Phosphorylation of GSK-3β at Ser^{9} by Akt results in its inactivation (Cross *et al*., 1995). Inactivation of GSK-3β plays a key role in the cardioprotection afforded by various pharmacological agents in the setting of I/R injury. Yin and colleagues demonstrated that adrenomedullin protects against apoptosis-induced by I/R injury through activation of an Akt-GSK-3-caspase signalling pathway (Yin *et al*., 2004). The cardioprotection afforded by bradykinin is

associated with modulation of mPTP opening secondary to inhibition of GSK-3β, via activation of the PI3K/Akt pathway (Park *et al*., 2006). Intermedin-mediated cardioprotection in I/R injury is also associated with inhibition of the mitochondrial-mediated myocardial apoptosis, secondary to activation of the Akt/ GSK-3β signalling pathway (Song *et al.,* 2009). I clearly show in this chapter that ARS activates the PI3K/Akt pathway resulting in the phosphorylation of GSK-3β at Ser^{9}.

NF-κB is a downstream target of GSK-3β, as this kinase regulates the activation of NF-κB at the level of the transcriptional complex (Hoeflich *et al.,* 2000; Steinbrecher *et al.,* 2005; Dugo *et al.,* 2006). Under physiological conditions, NF-κB is found complexed with a family of inhibitory proteins in the cytoplasm known as I-κB (Baueuerle & Baltimore, 1988). Activation and, thus, liberation of NF-κB is a consequent step following the phosphorylation of IκB, which triggers its polyubiquitination and subsequent proteosomal degradation. Once liberated, NF-κB rapidly translocates to the nucleus, where it regulates inflammatory gene expression (Li & Stark, 2002). Inhibition of NF-κB by GSK-3β is a potential mechanism of cardioprotection (Gao *et al*., 2008). GSK-3β inhibition by 4-benzyl-2-methyl-1, 2, 4-thiadiazolidine-3, 5-dione (TDZD-8) conferred cardioprotection in rats undergoing 30 min ischaemia followed by 6 h reperfusion, an effect associated with inhibition of NF-κB. Recently, Collino and colleagues demonstrated GSK-3β inhibition with either insulin or TDZD-8 was associated with a significant reduction of the nuclear NF-κB activity, in diabetic rats subjected to I/R injury (Collino *et al*., 2009). Blockade of NF-κB activation by NF-κB decoys (Morishita *et al*., 1997; Sawa *et al*., 1997), non-anticoagulant heparin (Thourani *et al*., 2000), a vitamin E-like antioxidant (Altavilla *et al*., 2000), and pyrrolidine dithiocarbamate (Muller *et al*., 2000) have been reported to attenuate myocardial I/R injury and to improve cardiac functional recovery (Sawa *et al*., 1997). I demonstrate in this chapter that treatment with ARS prevents the translocation of the p65 subunit of NF-κB from the cytosol to the nucleus.

MAPK's play a pivotal role in a diverse repertoire of biological process such as metabolism, survival and apoptosis (Rose *et al*., 2010). Members of the MAPK cascade have been implicated in regulating myocyte survival after I/R injury and oxidative stress (Lips *et al*., 2004). Indeed, ERK 1/2 signalling has been identified as one of the major components of the RISK pathway. As a result, many investigators have shown that activation of the ERK pathway by various stimuli leads to cardioprotection during

reperfusion. Inhibition of ERK signalling was demonstrated to increase daunomycin-induced apoptosis in cultured neonatal rat cardiomyocytes, whereas p38 MAPK is involved in the induction of cardiomyocyte apoptosis (Zhu *et al*., 1999). Treatment of isolated hearts with the MAPK/ERK kinase inhibitor PD98059 prior to global ischaemia/reoxygenation attenuated functional recovery and, hence, worsened I/R injury caused (Yue *et al*., 2000). Lips and colleagues demonstrated that although ERK 1 nullizygous gene-targeted mice were resistant to I/R injury, ERK $2^{+/-}$ gene-targeted mice exhibited enhanced infarction areas, suggesting ERK 2 signalling is a vital as a mediator of cardioprotection (Lips *et al*., 2004). Furthermore, more recently, a study by Lee and colleagues demonstrated ARS to attenuate the LPS-induced inflammatory response in microglial BV2 cells via activation of ERK 1/2 (Lee *et al*., 2012). Using a specific ERK 1/2 inhibitor, U0126, I show that activation of this pathway is vital in the cardioprotection conferred by ARS. Activation of this pathway is also shared with the IPC-induced cardioprotection.

The activation of the SAFE pathway which, comprise of TNF-α and STAT3 as the key players is now a well-recognised independent 'RISK-free' pathway conferring protection in ischaemic pre- and postconditioning (Lecour *et al*., 2005; Suleman *et al*., 2008; Lacerda *et al*., 2009). STAT3 is a latent transcription factor, originally identified as a mediator of cytokine signalling (Kishimto *et al*., 1994), is now known to play a role in various cell functions from proliferation (Shirogane *et al*., 1999), differentiation (Minami *et al*., 1996; Nakajima *et al*., 1996) to survival (Catlett-Falcone *et al*., 1999; Bromberg *et al.,* 1999). Oshima and colleagues demonstrated using cardiac-specific transgenic mice expressing constitutively active STAT3 and exposed to I/R injury to exhibit a dramatic reduction in infarct size when compared with non-transgenic littermates (Oshima *et al.,* 2005). Recently, it was shown that sphingosine-1 phosphate-induced reduction in myocardial infarct size was dependent on STAT3 and Akt activation (Somers *et al*., 2012). I demonstrate that there is a significant up-regulation in STAT3 activation in hearts that were subjected to transient I/R and treated with ARS when compared to control animals.

Is ARS itself the active component? or does it function as an i.v. injectable pro-drug, which is converted to an active metabolite? Studies have shown that ARS becomes rapidly hydrolysed *in vivo* to DHA, which is a common active metabolite (in malaria) shared by the artemisinin derivatives (White, 1994). Intravenous ARS has a half-life of

less than 15 min in man, as it is metabolised through esterase-catalysed hydrolysis to DHA (Gautam *et al*., 2009). This conversion occurs rapidly following i.v. administration, indicated by the swift decline in ARS concentrations in the early post-dose period (Morris *et al*., 2011). DHA metabolism occurs through conjugation by the UDP-glucuronosyltransferase system, with UGT1A9 and UGT2B7 being the main responsible isoforms (Llett *et al*., 2002). The metabolism of DHA occurs more slowly with an average half-life estimates for DHA following i.v. ARS administration of 1 – 2 h (Morris *et al*., 2011). I showed that peak plasma concentrations of both ARS (39.7 ng ml^{-1}) and DHA (223 ng ml^{-1}) were achieved in the rat within 5 min of the injection of ARS. This demonstrates a very rapid conversion of ARS to DHA in the rat, and this metabolism was unaltered in rats subjected to myocardial I/R. The peak plasma concentrations seen here in the sham treated rats are much lower than those exhibited in healthy human volunteers, whereby, a similar dose of ARS (1 mg kg^{-1}) resulted in a peak plasma concentration of ARS and DHA of around 6128 ng ml^{-1} and 800 ng ml^{-1} , respectively (Li *et al*., 2009). The efficacy of ARS in malaria has been attributed to i) attaining a high rapid initial C_{max}, and ii) its swift hydrolysis to DHA (Batty *et al*., 1998; Navaratnam *et al*., 2000; Li *et al*., 2009). This could mean that DHA is more potent than ARS in malaria and potentially other conditions. I show here that a dose of 0.1 mg kg^{-1} or DHA caused a significant reduction in infarct size, while the same dose of ARS was not effective. This indicates that DHA is the effective mediator of the cardioprotective effects of ARS (although I cannot exclude that ARS has at least some cardioprotective effects in its own right).

Limitations of the study

A major limitation of my study was the method of quantifying the infarct size. In this study the tetrazolium method (NBT stain) was used to determine the infarct size of various animal groups. This method although is widely considered a low cost, easily performed, reliable and reproducible method (Csonka *et al*., 2010), I found was not only very time consuming but due to the fact that at time visual interpretation of heart pieces may be subjective in cases of poor viable/non-viable contrast, it made the process quite laborious. Due to the fact that measuring the infarct size for each animal consumed over an hour, I was limited to performing myocardial infarction surgery on 4 a time. This, in addition to the time limitation I had I not only couldn't increase the *n* number for certain study groups but also couldn't perform extra experiments for certain study groups such

as determining the effect of ARS alone in sham treated animals. Although I don't expect there to be any adverse effect of ARS in sham treated animals, since various studies done on healthy human volunteers using the same dose I have here, have demonstrated that ARS was well tolerated, exhibiting no adverse effects including no deleterious haemodynamic or ECG effects (Li *et al*., 2009; Miller *et al*., 2012). Nevertheless, as part of good experimental practice, it is always vital to include sham groups treated with drug just to confirm that the drug doesn't have adverse effects besides those effects that you are measuring in your study.

Conclusion

I report here for the first time that the administration of ARS at the onset of reperfusion attenuates the myocardial injury associated with I/R. The observed beneficial effects of ARS are associated with i) activation of the PI3K/Akt/ERK 1/2 (RISK) pathway, ii) activation of eNOS, iii) inhibition of GSK-3β, iv) inhibition of NF-κB, and v) activation of the STAT3 (SAFE) pathway. Thus, ARS may represent a novel highly translatable approach for the therapy of acute MI in man.

Chapter 5

General Discussion

5.1 Overview

Protein kinases have long been integrated in various signal transduction pathways to help regulate cardiac function in both health and disease. Not only are they involved in maintaining cardiac function, there is strong evidence that the PI3K/Akt pathway mediates cardioprotection. Indeed, decreased myocardial Akt activation is characteristic of sepsis, and prevention of Akt activation ameliorates cardiovascular dysfunction (Ha *et al.,* 2008). In addition, activation of PI3K/Akt by ischaemic preconditioning (Li *et al*., 2011) or with the use of pharmacological agents such as insulin (Baines *et al*., 1999) or erythropoietin (EPO) (Kim *et al*., 2008) is crucial for salvaging the ischaemic/reperfused myocardium.

The research presented here has explored this common cardioprotective pathway, namely the PI3K/Akt pathway, in animal models of sepsis-induced cardiac dysfunction and acute myocardial I/R injury, respectively, utilising two clinically established pharmacological agents, namely EPO and artesunate (ARS). The main findings of this thesis are:

i. A 24 h model of CLP was set up in aged (8 months old) C57BL/6 mice, employing fluid resuscitation and antibiotic therapy at 6 h and 12 h post-surgery. In contrast to the young mice (2 months old), the aged mice exhibited a reproducible degree of renal dysfunction, liver injury and most notably cardiac dysfunction. Thus, it was evident that susceptibility to develop cardiac dysfunction increased with age (chapter 2).

ii. Subsequently, the study to investigate the effect of EPO on cardiac dysfunction was carried out in a model of endotoxaemia in young mice and polymicrobial sepsis in aged mice. I demonstrated for the first time that administration of EPO (1000 IU kg^{-1}) 1 h post endotoxaemia or CLP improved cardiac contractility, measured by improvements in echocardiographic parameters (EF, FS and FAC), an effect that was seen in both models, to a similar extent (chapter 3).

   This cardioprotective effect was associated with phosphorylation and, thus, activation of Akt, which led to; i) inhibition of the activation of GSK-3β and NF-κB, ii) reduced expression of NF-κB driven gene transcription of pro-inflammatory mediators such as IL-1β; and iii) phosphorylation and, subsequent activation of eNOS. All of these effects were secondary to activation of the EpoR-

βcR heterocomplex, as these effects of EPO were lost in βcR KO mice. Furthermore, the observed increases in EF afforded by EPO were not independent of changes in end-diastolic diameter and volume, hence, diastolic relaxation was not affected by EPO (chapter 4).

iii. Moreover, ARS an established antimalarial drug was used to investigate its effects in an acute model of myocardial I/R in the rat. I demonstrate for the first time that a single bolus of low dose ARS (1 mg kg^{-1}) given at the time of reperfusion resulted in a significant and substantial reduction in myocardial infarct size. This cardioprotective effect of ARS was associated with the activation of the PI3K/Akt and ERK 1/2 (RISK pathway) and STAT-3 (SAFE pathway) (chapter 4). The involvement of the PI3K/Akt pathway in this effect was confirmed by using a pharmacological inhibitor against PI3K with western blot analysis detecting phosphorylated Akt at Ser^{473}. This correlates with various studies showing that pharmacological agents administered at the time of reperfusion as well as an ischaemic preconditioning stimulus confers cardioprotection by activation of the RISK pathway (Baines *et al*., 1999; Hausenloy *et al*., 2005; Kim *et al.,* 2008). Specifically, I have demonstrated that the reduction in infarct size observed following treatment with ARS was associated with activation of i) Akt which led to inhibition of the activation of GSK-3β and NF-κB and activation of eNOS and ii) activation of ERK 1/2. Additionally, western blot analysis probing for the phosphorylation and thus activation of STAT-3 at Tyr^{705} confirmed that activation of this pathway is necessary for the cardioprotective effects of ARS in this model (chapter 5).

Furthermore, the studyshows that ARS is rapidly metabolised in the rat to DHA, and that this metabolism is not affected by myocardial I/R. This supports the notion that DHA is the active metabolite of many artemisinin derivatives. Indeed, injection of DHA (0.1 mg kg^{-1}) at the start of reperfusion does reduce infarct size while a similar dose of ARS did not.

5.2 A need for a new model?

Most existing animal models provide at best a crude approximation of human sepsis as most fail to take into account that the outcome in the clinical setting is generally

influenced by various factors such as genetic background, therapeutic interventions and therapeutic care, age and pre-existing conditions. Indeed, it is evident that many therapeutic interventions after being tested in *in vitro* and *in vivo* studies in both healthy animals and animal models of disease have failed to translate to human subjects, highlighting the urgent need for an improved, more representative sepsis model, which can help expedite this process. The involvement of various responses ranging from inflammatory to coagulation in the sepsis-induced response underlay the rationale for the mediator-modulating approach which, dominated the development of new therapies for sepsis (Cohen, 2002; Marshall, 2003). Examples include targeting ad blocking the early pro-inflammatory cytokine TNF-α and the modulation of NO production. Prophylactic administration of anti-TNF-α monoclonal antibody protected Gram-negative bacteraemic baboons against hypotension, organ failure and death (Tracey *et al.*, 1987) and endotoxaemic mice against the lethal effects of endotoxaemia (Beutler *et al*., 1985), respectively. Likewise, administration of monoclonal anti-TNF-α in various studies conferred protection when given as a treatment post bacterial insult (Hinshaw *et al.*, 1990; Hinshaw *et al*., 1992; Hinshaw *et al*., 1994). Subsequently, clinical trials followed which proved to either be without success (Buras *et al*., 2005) or were halted early due to harm (Fisher *et al*., 1996). With the association of excess NO production as a vital player in the pathogenesis of vascular hyporeactivity and septic shock came along efforts which not only showed a raise in blood pressure (Kilbourn *et al*., 1990; Nava *et al*., 1991), but also an increase in mortality (Cobb *et al*., 1992, Fukatsu *et al*., 1996, Strand *et al*., 1997) in various animal models. Nevertheless, a multi-center double-blind study of a nonspecific NO synthase inhibitor, N(G)-mono-methyl-l-arginine (546C88) to septic shock patients, led to termination of a phase III study due to increased mortality (Lopez *et al*, 2004). These are just two of many examples, which were tried and tested in experimental models before being implemented in clinical trials unsuccessfully. So why the disparity? Possible explanations include timing and dosage of intervention and diseases severity (Buras *et al*., 2005). Although the time of onset in patients is often unknown with patients presenting in hospital with a clinically manifest sepsis state after days have elapsed, the majority of animal models receive therapeutic intervention either before or within a few hours of the microbial or toxin insult (Dyson & Singer, 2009). Sepsis is characterised by a pro-inflammatory phase followed by a compensatory anti-inflammatory response and immunosuppression, with the latter increasing the susceptibility of critically ill patients

to nosocomial infection (Hotchkiss & Karl, 2003). Thus, not only does targeting early pro-inflammatory mediators such as TNF-α provide only a small window of opportunity for therapeutic intervention, but also controlling the extent of inhibition is vital as many of these mediators play central roles in host defence (Dyson & Singer, 2009). Furthermore, the role that dosing plays in the lack of translation from bench to bedside is seen in the NO synthase inhibitor in patients (Lopez *et al.*, 2004). Although N (G)-mono-methyl-l-arginine was titrated against blood pressure, the values attained were considerably higher than that achieved with catecholamines in the placebo group. Additionally, the increased survival demonstrated by the quartile of patients that received a low dose this NO synthase inhibitor compared to control patients was overshadowed by a resulting 'opposite effect in survival (e.g. an increase in mortality)' in patient group receiving moderate-to-high doses (Lopez *et al*., 2004). Moreover, the animal models of sepsis themselves are known to range in terms of complexity and degree of translation to the clinical setting. This can be attributable to various factors which, for simplicity could be divided into 'variation between human and experimental animal populations' i.e. choice of species and gender and 'physiologic and pharmacologic support in animal models' i.e. fluid resuscitation, antibiotics and analgaesia/anaesthesia (Dyson & Singer, 2009) as well as gender and co-morbidities.

I set about trying to improve and increase the clinical relevance of the CLP model to provide a better platform to test the drug of interest, EPO, in the treatment of sepsis-induced cardiac dysfunction. It was evident that there was an increased susceptibility of C57BL/6 mice to develop a reproducible degree of renal dysfunction, liver injury and most notably cardiac dysfunction, with age. This is in line with the clinical situation, as both incidence and severity of sepsis and septic shock increases in the older population (Girard *et al.,* 2005).

However, although I believe that having such a model, which employs a clinically feasible fluid resuscitation and antibiotics therapy strategy, is a step forward in cementing the translatable ability of using EPO for the treatment of cardiac dysfunction, it does not come without its limitation. Firstly, I only used male mice in this 24 h model, which is not akin to the clinical setting, where 40 % of septic patients are female (Wichmann *et al*., 2000). In addition, it is estimated that more than 50 % of septic patients present with at least one co-morbid condition (Moss, 2005), influencing outcome among both acutely ill patients (Knaus *et al*., 1991) and patients with sepsis (Pittet *et al*., 1993). Indeed, recent

investigators have reported that untreated type 1 and 2 diabetes exacerbates the sepsis-induced inflammation and subsequent mortality in CLP (Osuchowski *et al*., 2010; Jacob *et al*., 2008). In addition, Doi and colleagues showed that pre-existing renal disease worsens outcome following CLP (Doi *et al.*, 2008).

Therefore, incorporating co-morbidity conditions into my current model is one approach by which mimicking clinical sepsis could be improved. Moreover, the mice utilised in this aged CLP model are inbred, a common model system used for various studies to help exclude the inter-subject variability, thus making results more reproducible. However, I do not believe that using only inbred mice is an accurate model for human conditions, whereby the population exhibits heterogeneity in terms of their septic response. Thus, for future experiments, it would be interesting to investigate the response of outbred mice undergoing the same approach I took in the CLP model described in this thesis.

5.3 Cardioprotection by EPO

Over the years, the use of EPO has gained momentum for its use as a cardioprotective agent, specifically in reducing injury of the heart following I/R (Calvillo *et al.*, 2003; Cai *et al*., 2003; Sato *et al*., 2010). In addition, although EPO has been demonstrated to exhibit tissue protective effects in sepsis studies ranging from attenuation of acute kidney dysfunction (Coldewey *et al*., 2013) to improvement in skeletal muscle microcirculation (Kao *et al.*, 2011) and survival (Aoshiba *et al*., 2009), none have focused on exploiting the existing cardioprotective effects of EPO in a model of sepsis-induced cardiac dysfunction. I show for the first time that EPO's cardioprotective effects do extend to protection against the cardiac dysfunction accompanying sepsis. It is imperative that this cardioprotective effect is dependent on activation of the proposed 'tissue protective' receptor as opposed to the haematopoietic receptor. Thus, targeting the EpoR-βcR heterocomplex could be a potential therapeutic strategy to treat the cardiac dysfunction associated with sepsis.

As with all studies, there are certain limitations, which need to be addressed and improved on. Firstly, the timing of drug administration currently stands as 1 h post endotoxaemia or CLP. Although treatment is given post insult, it can still be considered

as an early intervention, which can fail to translate effectively in the clinical setting whereby patients more often present with sepsis once the disease has progressed past the early stages. Thus, it would be natural to try and see if EPO still exhibits these cardioprotective effects when given at a much later time point following the induction of endotoxaemia or polymicrobial sepsis. A second limitation may be the potentially high dose of EPO used in this study, which, although is considered to be less than that used in many other clinical studies (Corwin *et al*., 2007; Napolitano *et al.*, 2008). could lead to activation of the haematopoietic receptor and thereby, increase the risk of hypertension and/or thrombovascular events (Brines & Cerami, 2008). In its haematopoietic role, EPO circulates at concentrations in the low picomolar range (1–7 pmol L^{-1}; reviewed in Jelkmann , 2007) and the affinity of the homodimeric receptor for EPO is 100–200 pmol L^{-1} (Brines & Cerami, 2008). In contrast to its hormonal role, the local concentrations of EPO in its tissue-protective roles are higher and the affinity of the receptor lower (1-20 nmol L^{-1}) (Brines & Cerami, 2008), requiring a brief exposure to EPO to elicit a continual response. Thus, in order to achieve tissue-protective dose, higher doses of EPO are needed, as demonstrated by various studies (Abdelrahman *et al*., 2004; Kao *et al*., 2007; Mitra *et al.*, 2007). Although this exceeds a minimum level required for tissue protection, the continued maintenance of serum levels above the haematopoietic range, administration of EPO clinically is typically associated with excessive production of erythrocytes (Brines & Cerami, 2008). Therefore, it is still strongly advisable to try and have an alternative way of separating EPO tissue protective effects from its haematopoietic effects.

Taking the last point into account, it is clear that the translatable ability of EPO into clinical practice is hindered by the expected caution regarding the classic properties of EPO to stimulate red blood cell production and, thus, increase the risk of thrombosis. For instance, in a multi-center clinical trial, Corwin and colleagues demonstrated that critically ill trauma patients treated with EPO exhibited significant survival benefit at 29 and 140 days and this effect was associated with an increase in clinically relevant thrombovascular events (Corwin *et al*., 2007). The desire to eliminate the 'cross-talk' between both the haematopoietic and tissue-protective systems when utilising exogenous EPO, prompted a search to develop molecules that would retain the tissue-protective properties of EPO, without stimulating erythropoiesis. Chemical modifications of EPO (e.g. carbamylated EPO) or the generation of peptides that mimic the structure of EPO have resulted in the generation of proteins/peptides that are tissue-protective, without

causing erythropoiesis. Pyroglutamate surface B helix peptide (pHBSP) is an 11-amino acid peptide, the structure of which, is modelled on the three-dimensional structure of helix B of EPO, has been shown to confer protection in models of haemorrhagic shock (Patel *et al*., 2011), renal I/R (Patel *et al*., 2012) and myocardial I/R (Ahmet *et al*., 2011) which, is an effect believed to be mediated via activation of the EpoR-βcR heterocomplex. Therefore, the next step would entail determining whether pHBSP, can confer cardioprotection in my model of CLP-induced cardiac dysfunction.

5.4 Not just an antimalarial

Similar to EPO, ARS has over the years gone from being used just as an anti-malarial agent to one that might be used under conditions where inflammation is required to be blocked (Xu *et al*., 2007; Lee *et al*., 2012). I demonstrate for the first time that ARS exhibits cardioprotective effects in an *in vivo* model of rat I/R. I believe that this is an exciting area to develop further, with the potential of ARS to be translated into the clinical setting, as the doses used here to reduce myocardial I/R are lower than the doses used safely in patients with severe malaria (2.4 mg kg^{-1}) (Dondorp *et al*., 2005; Dondorp *et al*., 2010). I also show that either ARS or DHA can be administered at the time of reperfusion, which could indicate that ARS may be given as an adjunctive therapy to current reperfusion strategies such as thrombolysis and primary coronary angioplasty to provide further cardioprotection in the setting of an acute myocardial infarction. Moreover, with the strong evidence that pharmacological activation of pro-survival (e.g. PI3K/Akt) pathways at the time of reperfusion induces cardioprotection through the phosphorylation of various downstream effectors which, converge on the mitochondrial mPTP, the next step would be to investigate whether the ARS-induced activation of the RISK and SAFE pathway converges on the mPTP (Davidson *et al*., 2006; reviewed in Miura & Tanno, 2012). Subsequently, although I have shown that ARS exhibits cardioprotective potential in an acute (2 h) model of myocardial I/R, I would also like to extend these observations to a recovery (chronic) model of regional myocardial I/R in order to elucidate whether the observed cardioprotective effect of ARS are sustained after longer reperfusion periods. This would also enable me to investigate the effect of ARS in infarct size (measured by histology) and on the functional impairment (evaluation by echocardiography) associated with myocardial infarction.

Conclusion

This thesis set out to investigate the potential of EPO and ARS that have previously demonstrated efficacy in protecting different animal models of disease, in their role in activating a well-known survival pathway shown to confer cardioprotection in various models of cardiac injury/dysfunction. I believe that the work reported in this thesis i) advances our knowledge of the tissue protective effects of EPO to sepsis-induced cardiac dysfunction and ii) brings to light the novel use of an antimalarial agent in the protection against myocardial I/R injury. However, as mentioned above, the models used in these studies are not without imperfections and prior to commencing clinical trials, further experiments in large animal models of disease (sepsis and MI) ideally with co-morbidities and clinically relevant pharmacological interventions (e.g. resuscitation and antibiotics in sepsis) are warranted. It should be noted that the MHRA has approved the data contained in this thesis together with the relevant background literature as 'complete preclinical package' for future phase II clinical trials aimed at evaluating the efficacy of ARS in patients with acute MI and other disorders (letter from MHRA to Prof C Thiemermann, October 2013).

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Erythropoietin attenuates acute kidney dysfunction in murine experimental sepsis by activation of the β-common receptor

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The β-common receptor (βcR) plays a pivotal role in the nonhematopoietic tissue-protective effects of erythropoietin (EPO). Here we determined whether EPO reduces the acute kidney injury (AKI) caused by sepsis and whether this effect is mediated by the βcR. In young (2 months old) C57BL/6 wild-type and βcR knockout mice, lipopolysaccharide caused a significant increase in serum urea and creatinine, hence AKI. This AKI was not associated with any overt morphological alterations in the kidney and was attenuated by EPO given 1 h after lipopolysaccharide in wild-type but not in βcR knockout mice. In the kidneys of endotoxemic wild-type mice, EPO enhanced the phosphorylation of Akt, glycogen synthase kinase-3β, and endothelial nitric oxide synthase, and inhibited the activation of nuclear factor-κB. All these effects of EPO were lost in βcR knockout mice. Since sepsis is more severe in older animals or patients, we tested whether EPO was renoprotective in 8-month-old wild-type and βcR knockout mice that underwent cecal ligation and puncture. These older mice developed AKI at 24 h, which was attenuated by EPO treatment 1 h post cecal ligation and puncture in wild-type mice but not in βcR knockout mice. Thus, activation of the βcR by EPO is essential for the observed reduction in AKI in either endotoxemic young mice or older mice with polymicrobial sepsis, and for the activation of well-known signaling pathways by EPO.

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Despite a substantially improved knowledge of the pathophysiology of sepsis, the treatment of this condition is still a clinical challenge. To date, therapies have been mostly supportive and all specific therapeutic approaches, except early administration of antibiotics and 'early goal-directed therapy,'[1,2] have failed to be translated successfully into the clinical setting. Hence, new pharmacological strategies are urgently needed to improve this condition. 'In sepsis, some organs can escape relatively unscathed, whereas others become affected both early and severely.'[3] The kidney is one such organ that can be affected early with the development of acute kidney injury (AKI). This dysfunction is one of the most frequent and serious complications of sepsis and septic shock.[4] According to the Acute Kidney Injury Network consensus conference in 2007, AKI is diagnosed 'as an abrupt (within 48 hours) reduction in kidney function defined as an absolute increase in serum creatinine ($\geqslant$26.4 μmol/l), a percentage increase in serum creatinine ($\geqslant$1.5-fold from baseline), or a reduction in urine output ($\leqslant$0.5 ml/kg per h for more than 6 h).'[5] It is interesting to note that this definition does not take into consideration histological evidence of tissue injury and is solely based on rise in creatinine or fall in urinary output, even though the word 'injury' may imply the existence of morphological evidence of injury.[6]

Interestingly, the incidence of AKI increases with the severity of sepsis, occurring in 19% of patients with sepsis, 23% patients with severe sepsis, and 51% patients with septic shock.[7] Sepsis occurs more frequently and severely in older patients[8] and there is some evidence that the elderly septic patient is more susceptible to the development of AKI.[9] Similarly, in animal models of experimental sepsis, systemic inflammation and the release of proinflammatory cytokines are greater in older animals,[10–12] and the severity of septic

AKI was recently reported to be age dependent in female mice.[13] It has been proposed that the increase in the severity of sepsis in aging animals is secondary to changes in gut flora.[14] Emerging evidence suggests that the pathogenesis of septic renal dysfunction involves distinct mechanisms when compared with a nonseptic etiology.[15] There is evidence in humans that AKI caused by sepsis is not associated (or at least solely explained) with significant morphological changes such as extensive tubular necrosis.[6,16] The proposed pathophysiological mechanisms of sepsis-associated AKI include systemic vasodilatation, intrarenal vasoconstriction, inflammation, and bioenergetics failure.[6,7,15,17,18]

The pleiotropic hormone erythropoietin (EPO) is known to possess organ-protective properties that are independent from its well-established hematopoietic effects.[19] Endogenous EPO is primarily produced by renal cortical fibroblasts, and the fact that erythropoietin receptors (EPO-Rs) are expressed in glomerular, endothelial, and tubular epithelial cells,[20,21] suggests that endogenous EPO may function in a paracrine manner to limit the extent of injury after a noxious stimulus.[20] Furthermore, studies have proposed that the tissue-protective effects of EPO are mediated through a 'tissue-protective' receptor that is pharmacologically distinct from the classical hematopoietic EPO-R.[22] This tissue-protective receptor is a heterocomplex composed of EPO-R and the β-common receptor (βcR) that exhibits a lower affinity for EPO.[23] Over the years, EPO has been shown to be protective in various animal models of injury including cerebral ischemia,[24,25] myocardial ischemia,[26–28] and renal injury.[29–31] Indeed, in a model of bilateral renal artery occlusion in the rat, EPO inhibits apoptotic cell death, promotes renal functional recovery, and enhances tubular epithelial regeneration.[20]

It should be noted that the effects of EPO in sepsis-induced AKI are controversial.[32–37] Some studies report no beneficial effect of EPO in models of endotoxemia in the rat or pig.[32,33] In contrast, other studies have shown that EPO exerts beneficial effects in experimental sepsis. In 2007, Mitra *et al.*[35] reported for the first time that pretreatment with EPO reduces renal dysfunction in the mouse. Moreover, administration of EPO given 1 h after endotoxin or cecal ligation and puncture (CLP) reduces renal dysfunction and mortality in mice.[36] de Souza *et al.*[37] recently reported their extensive investigation that pre- or post-treatment with high-dose EPO (4000 IU/kg) exhibited anti-inflammatory effects and improved survival as well as renal function in a rat model of sepsis. However, none of the above (positive) studies investigated the role of βcR in either the observed effects or the signaling events initiated by EPO. This study investigates the effects of EPO and, specifically, the role of βcR in renal dysfunction caused by endotoxemia (lipopolysaccharide (LPS)) or polymicrobial sepsis (CLP).

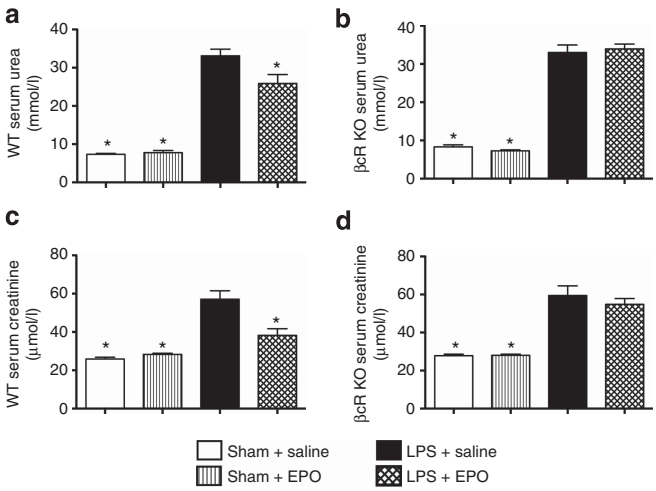

Figure 1 | Effect of erythropoietin (EPO) on renal dysfunction in endotoxemic wild-type (WT) and β-common receptor (βcR) knockout (KO) mice. (**a**, **b**) Serum urea and (**c**, **d**) serum creatinine were measured 18 h subsequent to sham operation or lipopolysaccharide (LPS) administration. Mice received either LPS (9 mg/kg intraperitoneally (i.p.)) or vehicle (5 ml/kg 0.9% saline i.p.). At 1 h after induction of endotoxemia, mice were treated with either EPO (1000 IU/kg EPO subcutaneously (s.c.)) or vehicle (10 ml/kg 0.9% saline s.c.). (**a**, **c**; WT mice): sham + saline ($n = 3$), sham + EPO ($n = 3$), LPS + saline ($n = 9$), and LPS + EPO ($n = 10$). (**b**, **d**; βcR KO mice): sham + saline ($n = 3$), sham + EPO ($n = 3$), LPS + saline ($n = 9$), and LPS + EPO ($n = 20$). Data are expressed as means ± s.e.m. for n number of observations. *$P < 0.05$ vs. LPS + saline of WT or KO animals, respectively.

RESULTS

Effect of EPO on renal dysfunction in endotoxemic wild-type and βcR knockout mice

EPO administration had no effect on serum urea and creatinine levels of sham wild-type (WT) and βcR knockout (KO) mice (Figure 1). When compared with sham mice treated with saline, WT mice subjected to 18 h of endotoxemia demonstrated a significant increase in both serum urea (7.3 ± 0.27 vs. 33.1 ± 1.75 mmol/l, $P < 0.05$; Figure 1a) and serum creatinine (25.9 ± 0.92 vs. 57.1 ± 4.72 μmol/l, $P < 0.05$; Figure 1c), indicating the development of AKI. Administration of EPO significantly attenuated the rise in serum urea (33.1 ± 1.75 vs. 25.9 ± 3.2 mmol/l, $P < 0.05$; Figure 1a) and creatinine (57.1 ± 4.72 vs. 38.2 ± 2.47 μmol/l, $P < 0.05$; Figure 1c). To investigate the role of βcR in LPS-induced AKI for the beneficial properties of EPO reported above, we have evaluated the effect of EPO on serum urea and creatinine in βcR KO mice. When compared with sham βcR KO mice treated with saline, βcR KO mice subjected to 18 h of endotoxemia demonstrated a significant increase in serum urea (8.3 ± 0.55 vs. 33.0 ± 1.94 mmol/l, $P < 0.05$; Figure 1b) and creatinine (27.8 ± 0.81 vs. 59.4 ± 5.06 μmol/l, $P < 0.05$; Figure 1d). Most notably, the renal dysfunction caused by endotoxemia in βcR KO mice was not attenuated by EPO (urea 33.0 ± 1.94 vs. 34 ± 1.27 mmol/l, $P > 0.05$; Figure 1b; creatinine 59.4 ± 5.06 vs. 54.9 ± 3.06 μmol/l, $P > 0.05$; Figure 1d).

Effects of endotoxemia and/or EPO on renal morphology and immunohistochemical staining for cleaved caspase-3 (CC-3) and kidney injury molecule-1 (Kim-1)

To gain a better understanding of the mechanisms underlying the observed renal dysfunction in endotoxemia, we carried

out an extensive histological analysis in all experimental groups. Evaluation by light microscopy (hematoxylin and eosin staining) revealed that there was no overt morphological evidence of proximal tubular epithelial cell injury, interstitial edema, interstitial inflammation, vasculopathy, or glomerular abnormality in any of the study groups (Figure 2a–f). Therefore, the results of the hematoxylin and eosin pathology evaluation produced no significant treatment-related changes. To further investigate the potential role of apoptosis in the observed renal dysfunction, we analyzed all sections for the active fragment of caspase-3, which plays a key role in apoptosis. Immunohistochemical analysis for CC-3 revealed that CC-3 staining was characterized by generally minimal or very minimal staining of individual or small clumps of cells predominantly within the medulla, but scattered positively stained cells were also visible throughout the kidney within glomeruli/tubulointerstitial spaces and blood vessels. Overall, we found no staining in sham-operated animals and a small increase in staining in all animals subjected to endotoxemia with no significant treatment-related changes ($P > 0.05$, Figure 2g). To further investigate the potential role of proximal tubular injury in the observed renal dysfunction, we analyzed all sections for Kim-1, an early biomarker of proximal tubular injury. Kim-1 staining was characterized by generally diffuse staining of the whole kidney with positive staining presenting as areas of multifocal to diffuse increased staining intensity within the pars recta region of the kidney. Compared with sham WT animals, evaluation of Kim-1 immunohistochemistry showed slightly increased staining in the endotoxemic WT mice, which appeared reduced after treatment with EPO. However, the magnitude of changes was small. This evaluation was, therefore, repeated by blind re-reading of groups by the original study pathologist and by a second pathologist (data

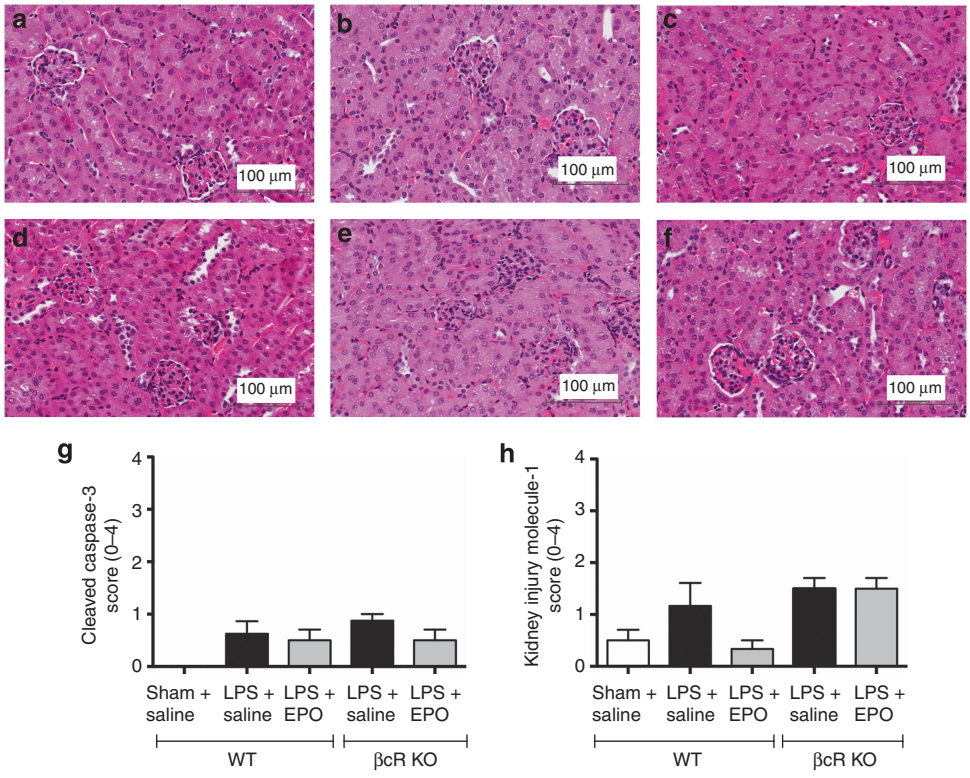

Figure 2 | Effects of endotoxemia and/or erythropoietin (EPO) on renal morphology (hematoxylin and eosin (HE) staining) and immunohistochemical staining for cleaved caspase-3 (CC-3) and kidney injury molecule-1 (Kim-1). (**a–f**) Morphological evaluation (HE staining) and immunohistochemistry (IHC)-based scoring of (**g**) CC-3 and (**h**) Kim-1 were performed in kidneys obtained from mice 18 h after sham operation or lipopolysaccharide (LPS) administration. Mice received either LPS (9 mg/kg intraperitoneally (i.p.)) or vehicle (5 ml/kg 0.9% saline i.p.). At 1 h after induction of endotoxemia, mice were treated with either EPO (1000 IU/kg EPO subcutaneously (s.c.)) or vehicle (10 ml/kg 0.9% saline s.c.). (**a–f**) Evaluation of HE-stained specimens revealed that there was no overt morphological evidence of proximal tubular epithelial cell injury, interstitial edema, interstitial inflammation, vasculopathy, or glomerular abnormality in any of the study groups and no treatment-related changes could be observed. Wild-type (WT) mice: (**a**) sham + saline ($n = 4$), (**b**) LPS + saline ($n = 3$), and (**c**) LPS + EPO ($n = 3$). β-Common receptor (βcR) knockout (KO) mice: (**d**) sham + saline ($n = 4$), (**e**) LPS + saline ($n = 4$), and (**f**) LPS + EPO ($n = 4$). (**g**) Analysis of CC-3 sections revealed no staining in sham-operated animals and a small increase in staining in all animals subjected to endotoxemia with no significant treatment-related changes in the CC-3 score ($P > 0.05$). (**h**) The results of the Kim-1 IHC pathology evaluation showed slightly increased staining in the endotoxemic WT mice, which appeared reduced after treatment with EPO. There was no treatment-related change in the βcR KO mice. Overall, none of the observed changes in Kim-1 IHC scores were statistically significant ($P > 0.05$).

not shown) to confirm that there was indeed a small difference. There was no treatment-related change in the βcR KO mice. Overall, none of the observed changes in Kim-1 immunohistochemistry were statistically significant ($P > 0.05$; Figure 2h).

Effect of EPO on the phosphorylation of Akt, glycogen synthase kinase-3β (GSK-3β), and endothelial nitric oxide synthase (eNOS), and on the nuclear translocation of the p65 nuclear factor (NF)-κB subunit in the kidneys of endotoxemic WT and βcR KO mice

To gain a better insight into the potential mechanism(s) underlying the observed beneficial effects of EPO, we investigated the effects of this hormone on cell signaling pathways in the kidneys of WT and βcR KO mice, known to confer tissue protection or to inhibit inflammation. When compared with kidneys of sham mice treated with saline, kidneys of WT and βcR KO mice subjected to LPS demonstrated no change in the total Akt content and the phosphorylation of Akt on Ser^{473} (Figure 3a), total GSK-3β content and the phosphorylation of GSK-3β on Ser^{9} (Figure 3b), or total eNOS content and phosphorylation of eNOS on Ser^{1177} (Figure 3c). Treatment of endotoxemic WT mice with EPO, however, resulted in a substantial increase in the phosphorylation of serine residues on Akt (Figure 3a), GSK-3β (Figure 3b), and eNOS (Figure 3c). In contrast, administration of EPO to endotoxemic βcR KO mice caused no change in the

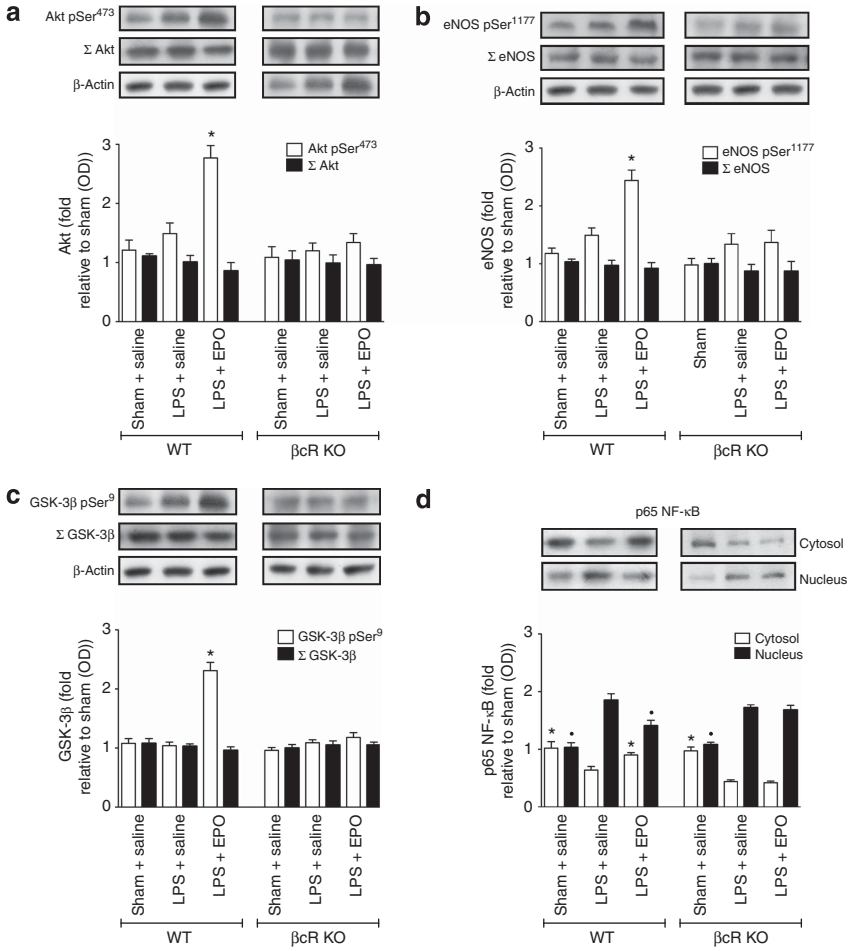

Figure 3 | Effect of erythropoietin (EPO) on the phosphorylation of Akt, glycogen synthase kinase-3β (GSK-3β), and endothelial nitric oxide synthase (eNOS), and on the nuclear translocation of the p65 nuclear factor (NF-κB) subunit in the kidneys of wild-type (WT) and β-common receptor (βcR) knockout (KO) mice with endotoxemia. Mice received either lipopolysaccharide (LPS; 9 mg/kg intraperitoneally (i.p.)) or vehicle (5 ml/kg 0.9% saline i.p.). At 1 h after induction of endotoxemia, mice were treated with either EPO (1000 IU/kg EPO subcutaneously (s.c.)) or vehicle (10 ml/kg 0.9% saline s.c.). Each band is from a single western blot experiment that is representative of three separate experiments. Data are expressed as means ± s.e.m. for *n* number of observations. $*P < 0.05$ versus LPS + saline of WT or KO animals, respectively (white bars). $\bullet P < 0.05$ vs. LPS + saline of WT or KO animals respectively (black bars). (**a**–**c**) Densitometric analysis of the bands (semiquantitative western blot analysis) is expressed for all groups studied as relative optical density (OD) of (**a**) phosphorylated Akt ($pSer^{473}$) and the corresponding total Akt content (Σ Akt); (**b**) phosphorylated GSK-3β ($pSer^{9}$) and the corresponding total GSK-3β content (Σ GSK-3β); and (**c**) phosphorylated eNOS ($pSer^{1177}$) and the corresponding total eNOS content (Σ eNOS). All values were corrected for the corresponding β-actin and normalized using the related sham-operated band. (**d**) Densitometric analysis of the bands is expressed for all groups studied as relative OD for NF-κB p65 subunit levels in both cytosolic and nuclear fractions normalized using the related sham-operated band.

phosphorylation of Akt (Figure 3a), GSK-3β (Figure 3b), or eNOS (Figure 3c).

When compared with kidneys from sham mice treated with saline, the kidneys of endotoxemic WT and βcR KO mice exhibited significant increases in the nuclear translocation of the p65 NF-κB subunit (Figure 3d), indicating the activation of NF-κB. Treatment of endotoxemic WT mice with EPO resulted in a significant reduction in nuclear translocation of p65 and, hence, inhibition of NF-κB activation in the kidney (Figure 3d). In contrast, administration of EPO to endotoxemic βcR KO mice did not attenuate the nuclear translocation of p65 caused by LPS (Figure 3d).

Development of renal dysfunction in aging mice following CLP

The development of renal dysfunction in our short and severe model of polymicrobial sepsis via CLP is strongly age dependent (Figure 4). Compared with sham-operated animals (26.3 ± 0.72 μmol/l), we could not determine a significant renal dysfunction (determined by changes in serum creatinine) 24 h after CLP in 2-month-old (25.7 ± 1.13 μmol/l) and 5-month-old (38.1 ± 6.2 μmol/l) WT mice that underwent CLP surgery. However, when compared with sham-operated mice, 8-month-old WT mice that underwent CLP surgery developed a significant and reliable increase in serum creatinine (26.3 ± 0.72 vs. 84.6 ± 7.34 μmol/l, $P<0.05$).

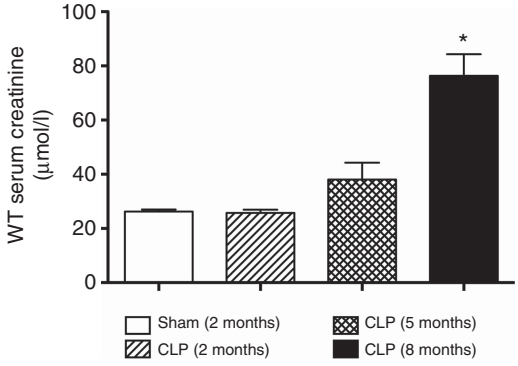

Figure 4 | The development of renal dysfunction in aging mice following cecal ligation and puncture (CLP). Serum creatinine levels were measured 24 h subsequent to sham operation or CLP surgery in wild-type (WT) mice at different ages (2, 5, and 8 months). Data are expressed as means ± s.e.m. for *n* number of observations. *$P<0.05$ versus sham. Sham (2 months, $n=3$); CLP (2 months, $n=3$); CLP (5 months, $n=5$); and CLP (8 months, $n=10$).

Effect of EPO on renal dysfunction in WT and βcR KO mice that underwent CLP

When compared with sham-operated WT mice, 8-month-old WT and βcR KO mice that underwent CLP surgery demonstrated a significant increase in serum creatinine (WT mice 26.3 ± 0.72 vs. 76.3 ± 8.01 μmol/l, $P<0.05$, Figure 5a; KO mice 23.2 ± 6.44 vs. 82.1 ± 11.77 μmol/l, $P<0.05$, Figure 5b). Administration of EPO to septic WT mice significantly attenuated the rise in serum creatinine (76.3 ± 8.01 vs. 49.9 ± 6.54 μmol/l, $P<0.05$; Figure 5a). Most notably, the creatinine increase caused by CLP in βcR KO mice was not attenuated by EPO (creatinine 82.1 ± 11.77 vs. 71.4 ± 10.81 μmol/l, $P>0.05$; Figure 5b).

Detection of *Csf2rb* mRNA expression in medulla and cortex of the kidneys from WT sham mice

To gain a better insight into where the βcR was expressed in the mouse kidneys, we determined the *Csf2rb* (encodes the murine βcR) gene expression in cortex and medulla. We could clearly detect the presence of βcR mRNA in both cortex and medulla (Figure 6). Comparison of the consensus sequence of our PCR product with the published *Csf2rb* gene (accession no. NM_007780.4) revealed 100% sequence identity.

DISCUSSION

Here we demonstrate that administration of 1000 IU/kg EPO subcutaneously 1 h after LPS administration or CLP attenuates AKI. Most notably, we describe for the first time that the observed beneficial effects of EPO in WT mice

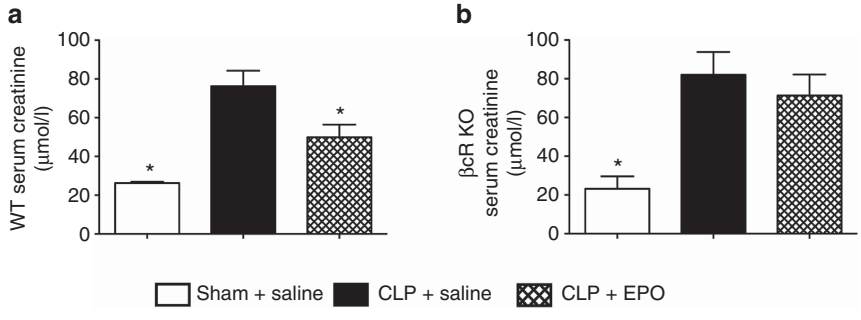

Figure 5 | Effect of erythropoietin (EPO) on renal dysfunction in septic wild-type (WT) and β-common receptor (βcR) knockout (KO) mice. Serum creatinine levels were measured 24 h subsequent to sham operation (no cecal ligation and puncture (CLP)) or CLP surgery in (**a**) WT and (**b**) βcR KO mice. At 1 h after CLP surgery, mice were treated subcutaneously (s.c.) with either EPO (1000 IU/kg EPO s.c.) or vehicle (10 ml/kg 0.9% saline s.c.). (**a**; WT mice): sham + saline ($n=3$), sham + EPO ($n=3$), CLP + saline ($n=10$), and CLP + EPO ($n=12$). (**b**; βcR KO mice): sham + saline ($n=3$), CLP + saline ($n=6$), and CLP + EPO ($n=20$). Data are expressed as means ± s.e.m. for *n* number of observations. *$P<0.05$ versus CLP + saline of WT or βcR KO animals, respectively.

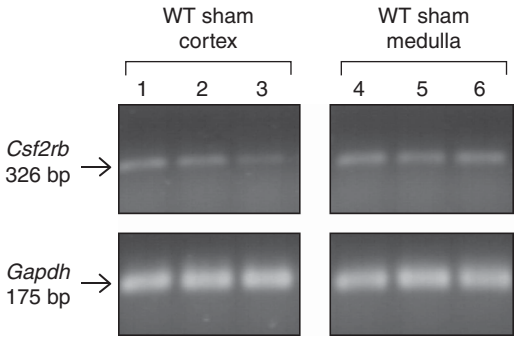

Figure 6 | Detection of *Csf2rb* gene expression in kidney medulla and cortex. The *Csf2rb* gene encodes the mouse colony stimulating factor 2 receptor β (βcR). Messenger RNA was extracted from medulla or cortex of wild-type (WT) sham mice ($n = 3$) and subjected to reverse transcription. PCR amplification of a *Csf2rb* gene fragment and a fragment of the murine *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) gene (mRNA extraction and gel loading control) was performed using the cDNA from medulla or cortex of the kidneys as template. When separated in a 1% agarose gel, amplicons with a calculated molecular size of 326 bp for the *Csf2rb* fragment could be detected for both kidney cortex (lanes 1–3) and medulla (lanes 3–6). Intensity of the 175 bp *Gapdh* band in lanes 1 to 6 confirmed comparative loading of the gel.

subjected to either endotoxemia or sepsis were not observed in mice that lacked βcR. In 2004, Brines *et al.*[23] proposed that the tissue-protective effects of EPO and its derivatives might be mediated (at least in part) by the βcR functionally associated with EPO-R generating an EPO-R/βcR complex.[38,39] The βcR is a signal transduction subunit that is shared by the α-chain subunits of the interleukin-5, interleukin-3, and granulocyte macrophage colony-stimulating factor receptors.[40] The α-chains are able to bind their ligand with low affinity, but the heteroreceptors are not able to signal in the absence of the βcR. On the other hand, the βcR does not measurably bind a ligand by itself, but it amplifies a signal once a ligand has bound.[41,42] The βcR is not required for erythropoiesis, as βcR KO mice have normal erythrocyte maturation.[23] In mice, βcR mRNA was expressed in both the cortex and medulla of the kidney. Our finding that the renoprotective effects of EPO in endotoxemia/sepsis are lost in βcR KO mice supports our hypothesis that βcR is essential for the beneficial effects of EPO in sepsis.

There is now increasing evidence that the renal dysfunction associated with sepsis in patients is secondary to functional rather than structural changes.[6,18] Although some morphological abnormalities have been reported in kidneys from patients who died from sepsis, these changes were relatively small and are unlikely to account for the profound renal dysfunction observed in these patients.[18] For instance, despite a high prevalence of AKI (65%) in 20 patients who died from sepsis, only one patient had evidence of kidney necrosis, as reported by Hotchkiss *et al.*[16] Furthermore, no renal tubular or glomerular cell apoptosis was seen in any of these patients. Thus, renal histology did not reflect the severity of renal injury indicated by the observed decrease in kidney function.[16] We show here that mice subjected to endotoxemia for 18 h have a profound renal dysfunction in the absence of overt morphological alterations or even increases in biomarkers of apoptosis (CC-3) or proximal tubular injury (Kim-1) in the kidney. As EPO did not affect any of the above (minimal) signs of injury, it is unlikely that the reduction in renal dysfunction afforded by EPO in sepsis is secondary to prevention of renal injury.

What then are the signaling events that are activated by EPO via the βcR? There is evidence that the beneficial effects of EPO are secondary to the activation of the survival kinase Akt.[43–46] Akt is a member of the phosphoinositide 3-kinase signal transduction enzyme family that regulates cellular activation, inflammatory responses, chemotaxis, and apoptosis.[47] When phosphorylated by its upstream regulator, phosphoinositide-dependent kinase, Akt modulates cell survival and growth.[47] We document here that EPO causes the activation of Akt in WT mice subjected to endotoxemia, whereas the activation of this pathway by EPO was lost in βcR KO mice. The above findings support the view that both βcR and the activation of Akt are essential for the reported beneficial effects of EPO.

What, then are the downstream targets of Akt? There is good evidence that activation of Akt results in Ser^9 phosphorylation of GSK-3β.[48–50] 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 and is regulated by multiple signaling pathways including the Akt pathway that inhibits this kinase by causing Ser^9 phosphorylation.[48,49] Specific inhibitors of GSK-3β activation attenuate the AKI caused by co-administration of LPS and peptidoglycan[51] and improve survival in endotoxemia.[52] Agents that activate the PI3K/Akt pathway[48] also inhibit GSK-3β. We have previously reported that insulin activates Akt, inhibits GSK-3β, and reduces AKI caused by LPS and peptidoglycan.[53] We report here that EPO enhances the phosphorylation of the Ser^9 residue of GSK-3β, resulting in the inhibition of this kinase. This effect of EPO was lost in βcR KO mice.

Downstream of GSK-3β, several studies have now reported an association between GSK-3β and NF-κB activity *in vitro*[54,55] and *in vivo*.[51,53] Treatment of tumor necrosis factor-α-stimulated hepatocytes with a specific GSK-3β inhibitor resulted in a decrease in the NF-κB-dependent gene transcription.[56] NF-κB is a transcription factor that plays an important role in regulating expression of genes encoding mediators of local and systemic inflammation, such as cytokines, chemokines, cell adhesion molecules, apoptotic factors, and others.[57] The protective effects of inhibitors of GSK-3β in endotoxemia/sepsis have been attributed to inhibition of NF-κB secondary to either phosphorylation of Ser^{536} on the p65 subunit of NF-κB[51] or to prevention of the association of cAMP response element-binding (CREB) with p65.[52] In addition, GSK-3β may also inhibit the activation of

NF-κB by phosphorylating and degrading IκBα, which is required to prevent NF-κB translocation.[55] All of these effects may contribute to the reported beneficial effects of inhibitors of GSK-3β in sepsis. We report here that EPO prevents the nuclear translocation of p65 and, hence, the activation of NF-κB. This effect of EPO was lost in βcR KO mice. All of the above findings support the view that EPO enhances the activation of Akt, resulting in inhibition of GSK-3β and inhibition of the activation of NF-κB in a βcR-dependent manner.

In addition to inhibiting the activation of GSK-3β, activation of Akt results in the phosphorylation of eNOS on Ser^{1177}, which in turn causes activation of eNOS, resulting in an enhanced formation of NO in the microcirculation. There is evidence that EPO enhances the phosphorylation of serine residues on eNOS, resulting in its activation.[37,58,59] In endothelial cells, EPO caused eNOS activation, whereas inhibition of EPO-R or βcR by neutralizing antibodies or small interfering RNA abolished the EPO-induced NO formation.[59] Most notably, inhibition of the βcR abolished the EPO-induced increase and the phosphorylation of eNOS, Akt, Src, or Janus kinase 2.[59] These findings support the view that βcR plays a key role in the activation of eNOS by EPO in endothelial cells.[59] We report here that the increase in Ser^{1177} phosphorylation of eNOS by EPO in the kidney is lost in βcR KO mice. While our study was underway, de Souza *et al.*[37] also showed that the attenuation of sepsis-induced AKI by EPO in the rat is associated with an increased expression of eNOS and inhibition of the activation of NF-κB, which the authors attributed to an anti-inflammatory and endothelial protective effect. In line with this study, Rodrigues *et al.*[17] reported recently in the same animal model that a 24-h pretreatment with continuous erythropoietin receptor activator protected against sepsis-induced AKI at 24 h, which was in part attributable to a suppression of the inflammatory response. Interestingly, their results revealed that renal EPO-R expression, which was downregulated in their model of sepsis, was preserved by pretreatment with continuous erythropoietin receptor activator.

In conditions associated with sepsis, activation of eNOS is beneficial as the enhanced formation of NO causes local vasodilation, inhibits adhesion of platelets and neutrophils, and regulates angiogenesis.[60,61] Agents that release NO or enhance the formation of endogenous NO may attenuate excessive intrarenal vasoconstriction and reduce renal dysfunction.[62,63] Thus, activation of eNOS (possibly secondary to activation of Akt) may contribute to the beneficial effects of EPO reported here.

All of the above findings support the view that βcR is essential for the following effects of EPO in mice with endotoxemia: (1) reduction of AKI, (2) activation of Akt, (3) activation of eNOS, (4) inhibition of GSK-3β, and (5) inhibition of the activation of NF-κB.

In order to investigate whether the βcR is also essential for the effects of EPO in polymicrobial sepsis, we developed a model of CLP-induced AKI. We found that the degree of renal dysfunction increased with the age of the animals. We were unable to document a significant degree of AKI at 24 h after CLP in young (2 and 5 months old) mice, whereas 8-month-old mice exhibited a very large increase in creatinine within 24 h after CLP. Most notably, administration of EPO (1 h after CLP) attenuated the AKI caused by polymicrobial sepsis in aging mice. In order to investigate the role of the βcR in this effect of EPO, we bred aged-matched βcR KO mice. Using these animals, we demonstrate that the beneficial effects of EPO were lost in 8-month-old βcR KO mice.

In conclusion, our findings show convincingly for the first time that βcR is essential for EPO-mediated attenuation of AKI in experimental sepsis as well as for all of the signaling events traditionally associated with the well-documented beneficial effects of EPO.

MATERIALS AND METHODS

Additional details on the methods are provided in the Supplementary Material online.

Animals

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 Stationary Office and the Guide for the Care and Use of Laboratory Animals of the National Research Council. This study was carried out on 73 male WT C57BL/6 mice (Harlan Laboratories, Wyton, UK) and 73 male βcR KO mice (B6.129S1-*Csf2rb*^{tm1Cgb}/J) on a C57/BL-6J genetic background (bred and maintained at Queen Mary University of London Biological Services Unit) receiving a standard diet and water *ad libitum*. We employed young (2 months old) mice for the model of endotoxemia and old (8 months old) mice for the model of polymicrobial sepsis.

Endotoxemia

In the model of endotoxemia-induced AKI, 2-month-old mice received either LPS (9 mg/kg in 5 ml/kg 0.9% saline intraperitoneally) or vehicle (5 ml/kg 0.9% saline intraperitoneally). At 1 h after induction of endotoxemia, mice were treated either with EPO (1000 IU/kg recombinant human EPO in 10 ml/kg 0.9% saline) or vehicle (10 ml/kg 0.9% saline subcutaneously). Sham mice were not subjected to LPS injection, but were otherwise treated in the same way. At 18 h, the experiment was terminated and kidney and blood samples were collected for quantification of organ injury. WT mice and βcR KO mice were randomly allocated into eight different groups.

Polymicrobial sepsis

The detailed CLP procedure is described in the Supplementary Material online. We evaluated the susceptibility of aging mice (2, 5, and 8 months old) to develop AKI 24 h after CLP. Finally, we established a model of severe polymicrobial sepsis in 8-month-old male WT mice that developed reliable renal dysfunction 24 h after CLP. This model was used to verify the effects of EPO on renal function obtained in the model of endotoxemia following the same treatment protocol.

Quantification of organ injury

Blood and kidney samples were harvested at the end of the experiment and processed as described in the Supplementary Material online for evaluation of serum creatinine/urea, western blot analysis, and histological evaluation (Supplementary Figure S1 online).

Western blot analysis

We assessed the degree of phosphorylation of Akt on Ser^{473}, GSK-3β on Ser^{9}, eNOS on Ser^{1177}, and the nuclear translocation of the p65 subunit of NF-κB. Semiquantitative western blot analysis was carried out as described previously[64] and outlined in detail in the Supplementary Material online.

Csf2rb mRNA expression in the medulla and cortex of kidneys from WT sham mice

Details are available in the Supplementary Material online.

Statistical analyses

All values described in the text and figures are presented as 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 one-way analysis of variance followed by Dunnett's *post hoc* test. A P-value of <0.05 was considered to be statistically significant.

DISCLOSURE

MB and AC are officers of Araim Pharmaceuticals and currently hold stocks/shares in the company. All the other authors declared no competing interests.

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An abstract entitled 'Erythropoietin reduces the acute kidney injury in experimental sepsis via activation of the β-common receptor' was recently presented at the 35th Annual Conference on Shock in Miami.

SUPPLEMENTARY MATERIAL

Figure S1. Immunohistochemical (IHC) scores of cleaved caspase-3 (CC-3) and kidney injury molecule-1 (Kim-1) staining in mouse kidneys.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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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 heteroceptors, 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

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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 Langerdorff-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. pyroglutamate surface B helix peptide (pHBSP)] show tissue-protective effects, but do not mediate erythropoiesis, suggesting that these agonists are specific for the tissue-protective receptor. These agonists potentially do not give rise to the complications associated with EPO treatment, such as thrombosis or hypertension. In light of the data presented here, targeting the 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, 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

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

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

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).

mice, endotoxemia in βcR KO mice resulted in a significant reduction in percentage EF, FS and FAC (P<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 (P>0.05) (Fig. 2B-D).

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

Effect of EPO on the phosphorylation of Akt, GSK-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) or eNOS on Ser1177 (P>0.05) (Fig. 3C). Administration of EPO to endotoxemic WT mice, however, resulted in a significant increase in the phosphorylation of Akt on Ser473 (P<0.05) (Fig. 3A), GSK-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).

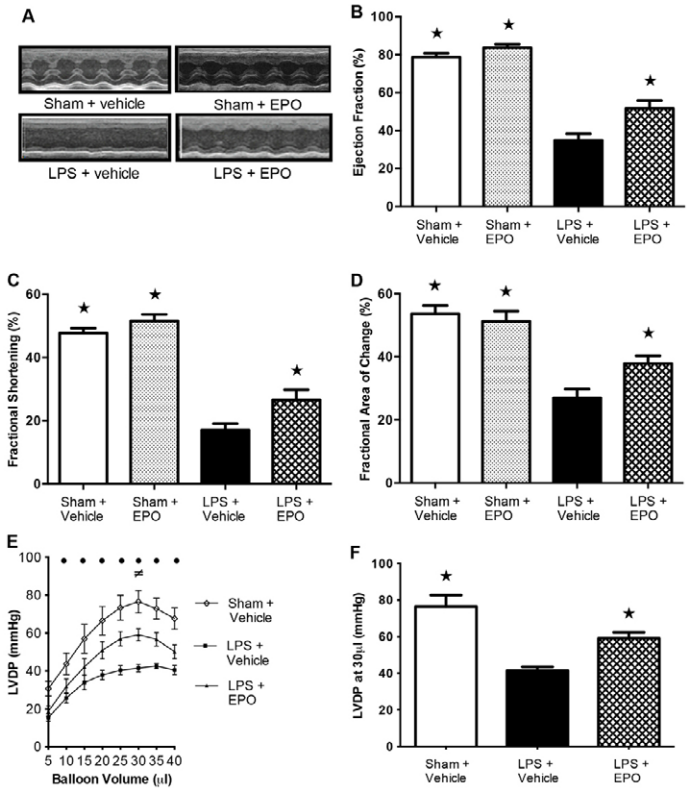

Fig. 1. Effect of erythropoietin (EPO) on the cardiac dysfunction in endotoxemic wild-type mice. (A-D) Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) 18 hours subsequent to sham-operation or lipopolysaccharide (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.

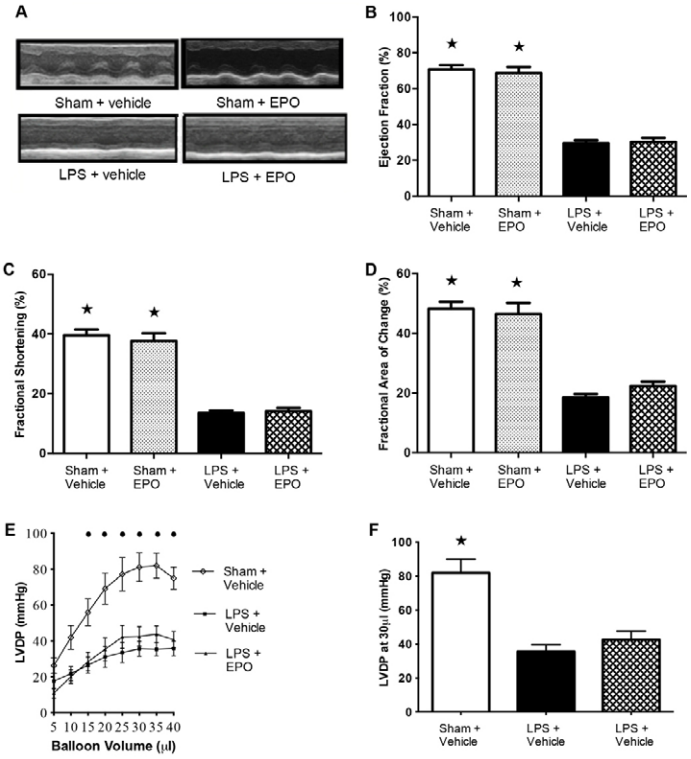

Fig. 2. Effect of erythropoietin (EPO) on the cardiac dysfunction in endotoxemic β-common receptor knockout (βcR KO) mice. (A-D) Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D) 18 hours subsequent to sham-operation 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.

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

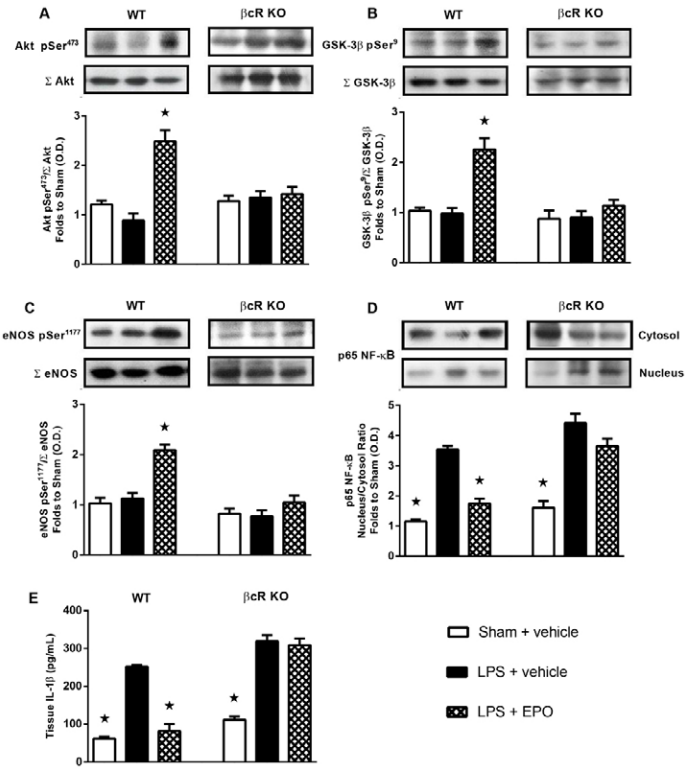

Fig. 3. Effect of erythropoietin (EPO) on signalling pathways and IL-1β expression in the hearts of wild-type and β-common receptor knockout (βcR KO) mice with endotoxemia. Mice received either lipopolysaccharide (LPS; 9 mg/kg i.p.) or vehicle (5 ml/kg 0.9% saline i.p.). At 1 hour after induction of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

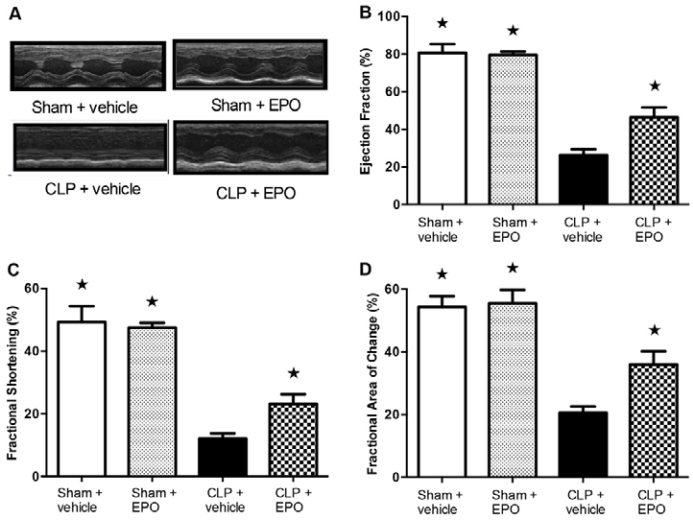

Fig. 4. Effect of erythropoietin (EPO) on the cardiac dysfunction in wild-type mice that underwent cecal ligation and puncture (CLP). Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change(D) in wild-type mice 24 hours subsequent to CLP surgery. At 1 hour after induction of CLP, mice were treated either with EPO (1000 IU/kg s.c.) or vehicle (10 ml/kg 0.9% saline s.c.). (B-D) Sham + vehicle (*n*=3); sham + EPO (*n*=3); CLP + vehicle (*n*=9); CLP + EPO (*n*=10). Data are expressed as means ± s.e.m. for *n* number of observations. ★*P*<0.05 versus CLP + 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.

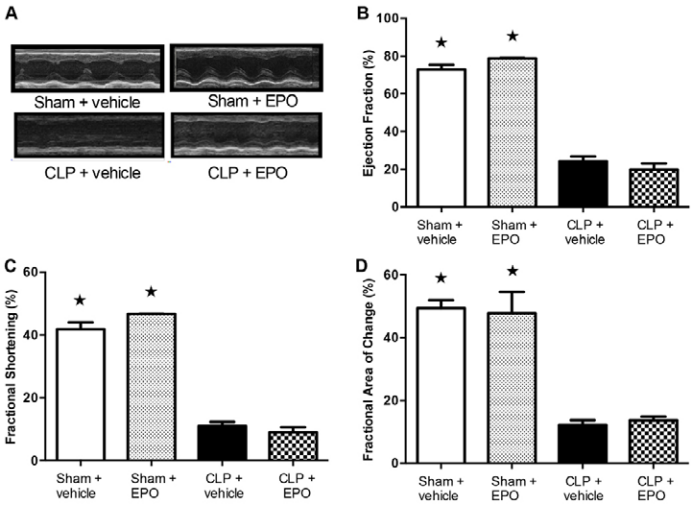

Fig. 5. Effect of erythropoietin (EPO) on the cardiac dysfunction in β-common receptor knockout (βcR KO) mice that underwent cecal ligation and puncture (CLP). Representative M-mode echocardiograms (A), and percentage ejection fraction (B), fractional shortening (C) and fractional area of change (D)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

Akt is a member of the phosphoinositide 3-kinase (PI3K) signal transduction enzyme family and regulates cellular activation, inflammatory responses, chemotaxis and apoptosis (Cantley, 2002). When phosphorylated by its upstream regulator, phosphoinositide-dependent kinase, Akt modulates cell survival and growth (Cantley, 2002). In our study, EPO caused a significant increase in phosphorylation of Akt on Ser473 (resulting in activation of this kinase), an effect that was lost in 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 (Dimmeler et al., 1999; Fulton et al., 1999) and cardiomyocytes (Burger et al., 2006). There is evidence that EPO induces nitric oxide (NO) production and eNOS phosphorylation (Su et al., 2011; Souza et al., 2012; Kao et al., 2011), and that its cardioprotective effects are, in part, mediated by the upregulation of eNOS, demonstrated *in vitro* (Burger et al., 2006; Rui et al., 2005) and *in vivo* (Rui et al., 2005). Inhibition of the 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

(Senftleben and Karin, 2002). Treatment of TNFα-stimulated hepatocytes with a specific GSK-3β inhibitor results in decreased NFκB-dependent gene transcription (Schwabe and Brenner, 2002). This study also indicated four potential phosphorylation sites for GSK-3β on the NFκB 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 NFκB-dependent proinflammatory genes, along with a reduced phosphorylation of Ser536 on the NFκB p65 subunit. In addition, GSK-3β might also inhibit the activation of NFκB by phosphorylating and degrading IκBα, which is required to prevent NFκB translocation (Takada et al., 2004). In our study, EPO attenuated the activation of NFκB caused by endotoxemia in the heart, an effect that was lost in βcR KO mice. NFκB 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 TNFα, 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 endotoxemic 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.'

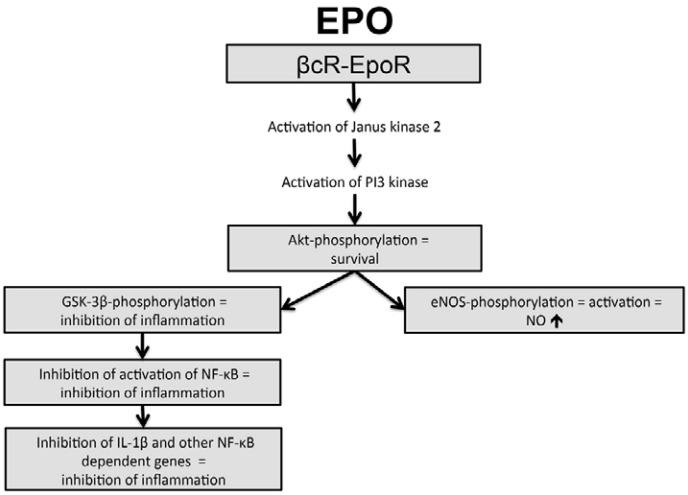

Fig. 6. Schematic overview of the signalling events modulated by erythropoietin (EPO) in the hearts of endotoxemic wild-type mice. Activation of βcR-EpoR by EPO is associated with (a) activation of the phosphoinositide 3-kinase (PI3K)-Akt signalling pathway, which results in the inhibition of the activation of glycogen synthase kinase-3β (GSK-3β), which is known to suppress the activation of nuclear factor-κB (NFκB), resulting in a blunted expression of the NFκB-driven gene transcription of pro-inflammatory mediators such as interleukin-1β (IL-1β), and (b) activation of the PI3K-Akt signalling pathway, which results in the increased activation of endothelial nitric oxide synthase (eNOS) and thus the enhanced formation of nitric oxide (NO). The signalling steps that were analyzed in our study are highlighted in light grey.

Conclusions

Our results show for the first time that the administration of EPO reduces the impaired systolic contractility associated with sepsis. In endotoxemia the observed beneficial effects of EPO are associated with: (a) activation of Akt, (b) inhibition of GSK-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 (Imipenem/Cilastin; 20 mg/kg). Based on previous evidence and preliminary data, an 18-G needle was used with the double puncture technique in order to generate cardiac dysfunction during the early phase of sepsis (24 hours). Briefly, mice were anesthetized i.p. with 1.5 ml/kg of a ketamine (100 mg/ml)/xylazine (20 mg/ml) solution in a 2:1 ratio. Buprenorphine was injected additionally to provide adequate analgesia. The rectal temperature of the animals was maintained at 37°C with a homeothermic blanket. The abdomen was opened via a 1.5 cm midline incision, and the cecum exposed. The cecum was ligated just below the ileocecal valve and punctured at both opposite ends. After a small amount of fecal matter was extruded from both ends, the cecum was placed back in its anatomical position. The abdomen was sutured; 1 ml of Ringer's solution was given for resuscitation s.c. and the mice were placed back in their cages. Antibiotic therapy and analgesia was administered 6 hours after surgery and every 12 hours after that. We evaluated the susceptibility of mice of increasing ages (2, 5 and 8 months old) to develop cardiac dysfunction 24 hours after CLP. Finally, we established a model of severe polymicrobial sepsis in 8-month-old male WT mice, which developed reliable cardiac dysfunction 24 hours after CLP.

Assessment of cardiac function *in vivo* (echocardiography)

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

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

Cardiac function was assessed in mice by the isolated Langendorff-perfused heart *ex vivo* as reported previously (Kapoor et al., 2010). At 16-18 hours after the administration of LPS, mice were anesthetized and heparinized (heparin sodium, 1000 IU/100 g, i.p.). Following thoracotomy, the heart was excised and rapidly transferred to ice-cold Krebs-Henseleit buffer (KHB), containing (in mmol/l) NaCl 118, KCl 3.8, $MgSO_4$ 1.19, $NaHCO_3$ 25, $CaCl_2$ 1.25, KH_2PO_4 1.18, sodium pyruvate 5, and glucose 10; equilibrated with 95% O_2/5% CO_2 (pH 7.4). Aortic cannulation was performed and hearts were perfused immediately with filtered KHB, gassed continuously with 95% O_2/5% CO_2 and maintained at 37°C. Hearts were retrogradely perfused in a non-recirculating Langendorff mode. The flow was measured and could be adjusted using a flow meter to achieve a coronary perfusion pressure of 75±5 mmHg. A small water-filled polyethylene balloon was carefully inserted into the LV via a small incision in the left atrium made near the opening for the pulmonary vein. The hearts were electrically paced at ~590 beats per minute via a silver electrode attached to the wall of the right atrium and then allowed to stabilise for at least 10 minute before any experimental protocols were carried out. Alterations in isovolumic LVDP in response to 5 µl incremental intraventricular loading of the balloon up to 40 µl were assessed. Pressure volume curves were generated to assess LVDP.

Western blot analysis

Briefly, mouse heart samples were homogenized in 10% homogenization buffer and centrifuged at 1500 ***g*** for 5 minutes at 4°C. Supernatants were removed and centrifuged at 18,600 ***g*** at 4°C for 40 minutes to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer and centrifuged at 18,600 ***g*** for 20 minutes at 4°C. The resulting supernatants containing nuclear proteins were carefully removed, and protein content was determined on both nuclear and cytosolic extracts using a bicinchoninic acid (BCA) protein assay following the manufacturer's directions (Therma Fisher Scientific, Rockford, IL). Proteins were separated by 8% sodium dodecyl sulphate-PAGE (SDS-PAGE) and transferred to a polyvinyldenedifluoride (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-peNOS 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.

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

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SUPPLEMENTARY MATERIAL

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Figure S1

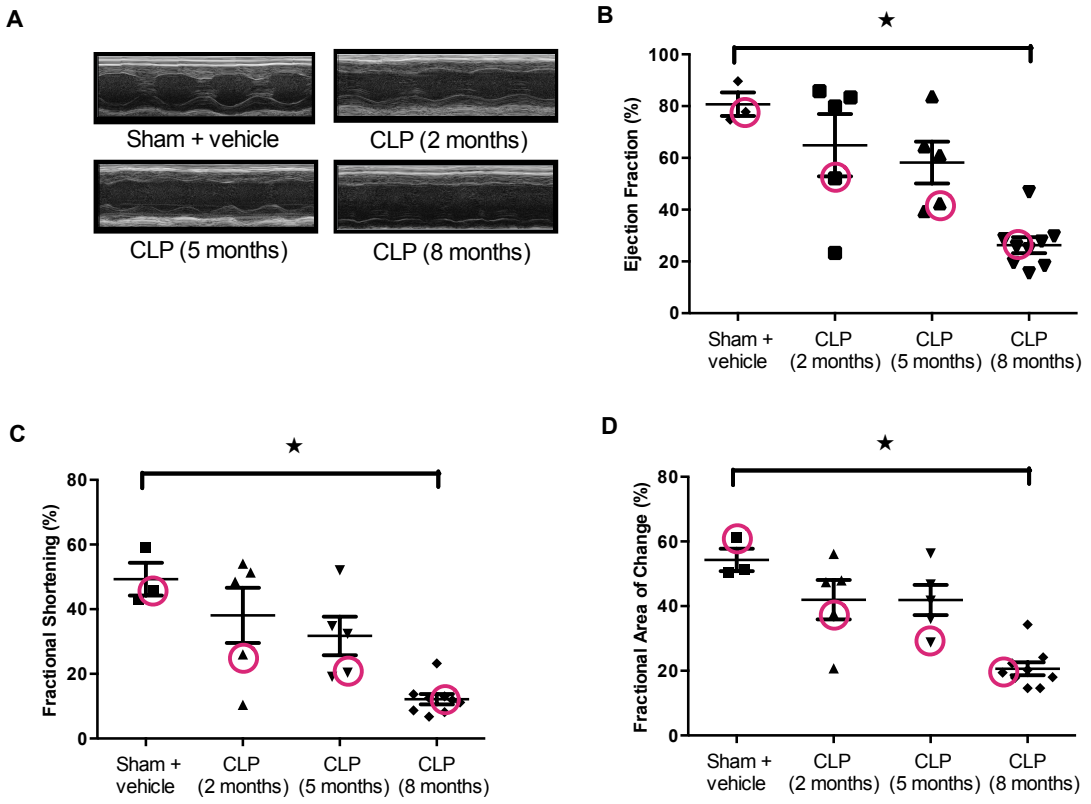

Figure S1: The development of cardiac dysfunction in aging wild-type mice following cecal ligation and puncture (CLP). (A) Representative M-mode echocardiograms, percentage (B) ejection fraction, (C) fractional shortening and (D) fractional area of change 24 h subsequent to CLP surgery. Sham + vehicle, (n = 3), CLP 2 months (n = 5); CLP 5 months (n = 5); CLP 8 months (n = 9). Red circles indicate animals used for representative M-mode images seen in (A). Data are expressed as means ± SEM. for n number of observations. ★ $P < 0.05$.