Cardioprotection afforded by targeting guanylyl cyclase during early reperfusion

A thesis submitted to Cardiff University in accordance with the requirements for the degree of PHILOSOPHIÆ DOCTOR

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Declaration

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Summary

Guanylyl cyclase - cyclic guanosine monophosphate (cGMP) signalling has been demonstrated to play an important role in the endogenous cardioprotective signalling of the myocardium during early reperfusion. It is proposed that infarct limitation is afforded by elevating cGMP and activating protein kinase G and its distal targets.

It was hypothesised that increasing the activity of soluble guanylyl cyclase (sGC) would limit myocardial ischaemia-reperfusion injury. Primarily using the rat isolated perfused heart method, the experiments reported in this thesis investigate the role of exogenous targeting of sGC during early reperfusion, specifically exploring targeting different redox states of the enzyme and their effects on myocardial infarct size. The novel sGC stimulator BAY 41-2272 and activator BAY 60-2770 were selected to investigate this hypothesis.

Both administration of BAY 41-2272 and BAY 60-2770 during early reperfusion significantly limited infarct size compared to controls. This was associated with elevated total tissue cGMP levels. Inhibition of nitric oxide could not completely abrogate this protection, but exogenous perfusion of nitric oxide along with BAY 41-2272 showed synergistic action. Oxidation of the prosthetic haem group by ODQ abrogated the protection afforded by BAY 41-2272 but potentiated the protection afforded by BAY 60-2770. Targeting both the reduced and oxidised forms of sGC together did not afford additive protection, in fact it reduced the protection afforded compared to the individual treatments. Preliminary data also suggest that targeting the particulate form of guanylyl cyclase increases activity of Akt signalling during early reperfusion suggesting common signalling between soluble and particulate guanylyl cyclase.

These data suggest that targeting sGC during early reperfusion can afford cardioprotection by limiting infarct size. The relationship between cGMP elevation and infarct size needs to be investigated further. Nevertheless, these studies suggest that sGC may be a tractable target for the therapeutic management of acute myocardial infarction.

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List of publications

Manuscripts

Bice JS, Keim Y, Stasch J-P, Baxter GF. NO-independent production of cGMP limits infarct size in a rat model of ischaemia-reperfusion injury (In preparation).

Abstracts

Bice JS, Baxter GF (2009). NO-independent activation of sGC by BAY 41-2272 at reperfusion limits infarct size. *pA2 Online* *(http://www.pa2online.org/abstract/abstract.jsp?abid=29170&period=41)*

Bice JS, Baxter GF (2010). NO-independent stimulation of sGC limits infarct size when administered at reperfusion. *Exp Clin Cardiol* **15** (3): P42

Bice JS, Baxter GF (2011). Targeting of soluble guanylyl cyclase limits infarct size in a model of acute myocardial infarction. *BMC Pharmacology* **11**(Suppl 1)**:** P8.

Abbreviations

5-HD	5-hydroxy decanoate
ACh	acetylcholine
AMI	acute myocardial infarction
ANF	atrial natriuretic factor
ANP	atrial natriuretic peptide
AR	area at risk
ATP	adenosine triphosphate
AUC	area under the curve
BAY 41-2272	3-(4-amino-5-cyclopropylpyrimidine-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo(3,4-b)pyridine
BAY 58-2667	4-(4-carboxybutyl)(2-((4-phenethylbenzol) oxy)phenethyl)amino)methyl)benzoic acid
BAY 60-2770	4-((4-carboxybutyl) [2- (5-fluoro-2-([4'-(trifluoromethyl) biphenyl-4-yl]methoxy)phenyl)ethyl] amino}methyl)benzoic acid
BCA	bicinchoninic acid
BNP	brain natriuretic peptide
BPM	beats per minute
cAMP	adenosine-3', 5'-cyclic monophosphate
CAO	coronary artery occlusion
CFR	coronary flow rate
cGMP	guanosine-3', 5'-cyclic monophosphate
CHD	coronary heart disease
CHIP	carboxyl-terminus of HSP70 interacting protein
CHO	Chinese hamster ovarian
CNBD	cyclic nucleotide binding domain
CNP	c-type natriuretic peptide
C-PTIO	2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, monopotassium salt
CSA	cyclosporin-A
CTEPH	chronic thromboembolic pulmonary hypertension
CVD	cardiovascular disease
CYPD	cyclophilin D
DETA-NO	diethylenetriamine NONOate
DMSO	dimethyl sulphoxide
ECG	electrocardiogram
EDRF	endothelium derived relaxing factor
eNOS	endothelial nitric oxide synthase
EPR	electro paramagnetic resonance
FRET	fluorescence resonance energy transfer
GC	guanylyl cyclase
GSK3β	glycogen synthase kinase 3 beta
GSNO	S-Nitrosoglutathione
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
HR	heart rate
HRP	horseradish peroxidase

HSP	heat shock protein
HUVEC	human umbilical vein endothelial cell
I	infarct
IBMX	3,7-Dihydro-1-methyl-3-(2-methylpropyl)1H-purine-2,6-dione
iNOS	inducible nitric oxide synthase
IPC	ischaemic preconditioning
JAK	Janus kinase
K_{ATP}	adenosine triphosphate-sensitive potassium channel
KO	knockout
LDCA	left descending coronary artery
L-NAME	Nω-Nitro-L-arginine methyl ester
LPS	lipopolysaccharide
LV	left ventricle
LVDP	left ventricular developed pressure
LVEDP	left ventricular end diastolic pressure
MAPK	mitogen activated protein kinase
mPTP	mitochondrial permeability transition pore
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOA	nitric oxide analyser
NOC-9	6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine
NOS	nitric oxide synthase
NP	natriuretic peptide
NPR	natriuretic peptide receptor
NSB	non specific binding
OBC	ozone based chemiluminescence
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PAH	pulmonary arterial hypertension
PDE	phosphodiesterase
pGC	particulate guanylyl cyclase
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PKG	protein kinase G
PLB	phospholamban
PPCI	primary percutaneous coronary intervention
PVDF	polyvinylidene fluoride
RIA	radioimmunosorbent assay
RISK	reperfusion injury salvage kinase
ROS	reactive oxygen species
RPP	rate pressure product
RV	right ventricle
SAFE	survivor activating factor enhancement
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	sarcoplasmic endoplasmic reticulum calcium ATPase
sGC	soluble guanylyl cyclase
SNAP	*S*-Nitroso-*N*-acetylpenicillamine
SOD	superoxide dismutase

SR	sarcoplasmic reticulum
STAT	signal transduction activator of transcription
STEMI	ST elevation myocardial infarction
TCA	trichloroacetic acid
VF	ventricular fibrillation
VPB	ventricular premature beat
YC-1	3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole

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Chapter 1
General Introduction

1 Introduction

1.1 Heart disease

Cardiovascular disease (CVD) causes more deaths worldwide than any other disease. The World Health Organisation (WHO) reports that there were over 17 million deaths in 2008 attributable to CVD (Mendis S, 2011). Nearly 8 million of these deaths were as a direct result of coronary heart disease (CHD), disease that results in insufficient blood supply to the myocardium.

CHD deaths are most often caused by sudden rupture of an atherosclerotic plaque. In the United Kingdom, mortality due to CHD was over 88,000 in 2008, with most deaths attributed to the 124,000 heart attacks that patients suffer annually in the UK (Mendis S, 2011). Although mortality rate of CHD is decreasing in the UK as a result of improved treatments, diagnosis and primary prevention programmes, the global picture is different. It is predicted that global mortality rate will rise beyond 2015 (Deaton *et al.*, 2011).

It is almost one hundred years since James Herrick delivered his seminal paper on coronary thrombosis. Herrick's paper, presented at the American Association of Physicians on May 14th 1912 was poorly received and attracted little discussion. Herrick had linked clinical observations to post-mortem findings suggesting that coronary thrombosis was not a "tocsin of doom", that is, that it was not always fatal; he argued that it should be possible, and indeed desirable to make the diagnosis during life (Herrick, 1912). Herrick's employment of his theories in his clinical work gradually persuaded others to diagnose thrombotic obstruction of vessels clinically and this was pivotal in laying the foundations of future CHD research (Ross, 1983).

1.2 Coronary artery thrombosis

Coronary thrombosis of a major blood vessel supplying the myocardium leads to myocardial ischaemia, which is described as the sudden and sustained lack of blood flow and associated hypoxia to part of the heart, resulting in permanent damage to the heart tissue. This is clinically diagnosed as acute myocardial infarction (AMI) (Baker *et al.*, 2011; Buja & Weerasinghe, 2008; Fuster *et al.*, 1988). WHO guidelines for clinical diagnosis of AMI suggest that three criteria are satisfied. These are: clinical history of ischaemic like pain of duration 20 minutes, changes in repeated electrocardiograms (ECG), and rise and/or fall of cardiac biomarkers such as troponin and creatine kinases (Lippincott *et al.*, 1979). As a result of the increasing sensitivity of biomarker assays, WHO guidelines were revised in 2000 to give more weighting towards increases in these "soluble markers" (Antman *et al.*, 2000).

The progressive pathology that ultimately results in the rupture of an atherosclerotic plaque, coronary thrombosis and AMI can occur over many decades (Davies & Thomas, 1985). Atherosclerotic lesions form when leukocytes adhere to the endothelial monolayer of an artery, followed by maturation of monocytes into macrophages and their uptake of lipid resulting in the production of foam cells. Smooth muscle cells migrate towards the intima adjacent to the endothelial monolayer, followed by collagen, elastin and proteoglycans. A lipid or necrotic core forms in the centre of the lesion resulting from dead or dying smooth muscle cells, macrophages and an accumulation of cholesterol. A fissure in the fibrous cap of the plaque allows blood coagulation components to interact with tissue factors in the lesion, triggering platelet aggregation, thrombus formation and

occlusion of the vessel lumen. If the occluded vessel is a major artery supplying the myocardium then this can lead to the rapid development of an ischaemic risk zone or zone of jeopardised tissue. The propensity to arrhythmias and decreased left ventricular contractility, which can be fatal, is directly related to the size of the risk zone. Out of approximately 350 patients treated daily in the UK for AMI, over 100 of these patients will die within the first few hours of presentation (Kushner *et al.*, 2009). Those patients who survive initial therapy (discussed later in this chapter) will be left with a degree of irreversible damage to the portion of the myocardium subjected to ischaemia (termed the area at risk (AR), or risk zone) (Shaw & Kirshenbaum, 2008). The portion of the area at risk that has undergone irreversible cell damage is called the infarct.

1.3 Specific cellular processes during myocardial ischaemia

In the ischaemic risk zone, deprived of oxygenated blood flow, hypoxia swiftly leads to cessation of mitochondrial oxidative phosphorylation and ultimately the loss of the major source of adenosine trisphosphate (ATP). Concomitant with the ceasing of oxidative reactions, cytosolic glycogen becomes the major substrate for anaerobic glycolysis, which becomes accelerated (Reimer & Ideker, 1987). This compensatory mechanism is a vain attempt of the myocardium to survive. Unfortunately anaerobic glycolysis can only produce up to 7 % of ATP required for the normal functioning myocardium (Wollenberger & Krause, 1968). Although contractile function of the heart, the major energy demanding process of the myocardium, is suppressed rapidly, ATP demand quickly outstrips production and stores are depleted by half within the first 10 minutes of a severe ischaemic episode (Jennings *et al.*, 1978). Accumulation of H^+ and lactate, as well as

mitochondrial fatty acid metabolism, results in the production and release of reactive oxygen species (ROS) and reactive nitrogen species, both of which contribute to impaired contraction with persistent electrical activity and culminating in ventricular arrhythmias (Buja & Vela, 2008; Thandroyen *et al.*, 1992).

In the first minutes of ischaemic insult, there is a net loss of cytosolic K^+ by efflux, not altering the Na^+/K^+ adenosine triphosphatase (ATPase) initially. This is followed by an increase in free Mg^{2+} and a decrease in total Mg. Eventually the ATP demand to maintain electrochemical gradients cannot be met and results in the inhibition of the Na^+/K^+-ATPase. As a result, there is net Na^+ influx and further K^+ efflux as well as cell swelling due to increased uptake of water and Cl^-. The rise in intracellular Na^+ activates the Na^+/Ca^{2+} exchanger, which addresses the ion imbalance by extruding three Na^+ in exchange for one Ca^{2+}. Changes in the sarcolemma and sarcoplasmic reticulum (SR) come about as result of an increase in cytosolic free $[Ca^{2+}]$, also activating proteases causing alterations in contractile proteins. There is a decrease in Ca^{2+} sensitivity due to phosphate and hydrogen ions, which leads to sustained impairment of contractility despite elevated cytosolic $[Ca^{2+}]$ (Allen & Kurihara, 1982). Ca^{2+} overload leads to cell damage by activating membrane phospholipases, depressing mitochondrial respiration and increasing mitochondrial permeability (see Figure 1.1). During prolonged ischaemic episodes, contracture occurs. The interaction between myosin heads and actin is maintained because of the lack of ATP production (Stapleton & Allshire, 1998) (see 1.31 for more detail).

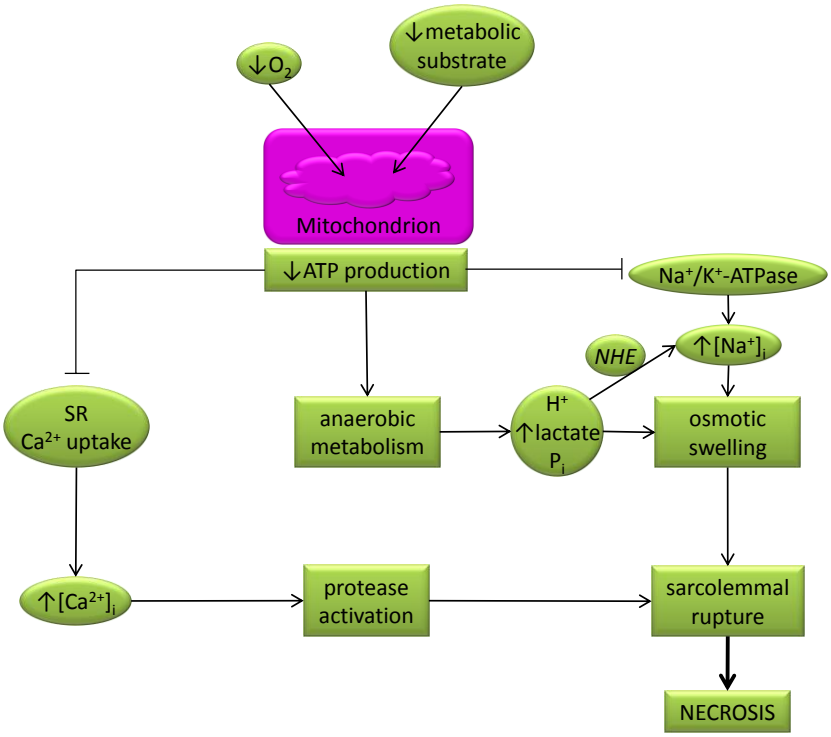

Figure 1.1 *Schematic representation of ionic changes during ischaemia. Reduced molecular oxygen and metabolic substrates results in a reduction in ATP production. SR Ca^{2+} uptake is impaired leading to $[Ca^{2+}]_i$ accumulation. Anaerobic metabolism is associated with intracellular accumulation of inorganic phosphate, lactate, and H^+. Activation of the Na^+/H^+ exchanger (NHE) results in accumulation of intracellular Na^+. Increasing intracellular concentrations of solutes results in osmotic swelling causing sarcolemmal rupture, further exacerbated by the activation of Ca^{2+} dependent proteases and phospholipases. Sustained stimuli leads to oncosis and tissue necrosis (adapted from Ferdinandy et al. (2007)).*

1.4 Cell death during myocardial ischaemic injury

The types of cell death that occur during ischaemia and reperfusion have long been debated. Early literature reported that much of the cellular death that occurs was by oncosis leading to tissue necrosis (Buja & Willerson, 1981; Jennings *et al.*, 1960; Reimer & Ideker, 1987). However, this view has been challenged in more recent studies that have provided evidence that this may not be the case (Bialik *et al.*, 1997; Cheng *et al.*, 1996; Itoh *et al.*, 1995; Ohno *et al.*, 1998). It is now generally accepted that there are three types of cell death that occur during myocardial ischaemia and reperfusion. Apoptosis,

oncosis and autophagy are the three terms used to describe the demise of cells. Necrosis is seen as the final manifestation, at the tissue level, of both oncotic and apoptotic cell death. (Buja & Weerasinghe, 2008; Majno & Joris, 1995).

Oncosis is characterised by its development through exogenous stimuli, as a consequence of energy depletion and/or membrane damage and it accounts for most necrotic damage. Environmental insults that initiate oncotic pathways include hypoxia, inflammatory processes and ischaemia (as described above). In contrast to apoptosis and autophagy, oncosis is described as accidental and passive cell death (Buja & Vela, 2008). Cellular homeostasis is lost due to progressive membrane damage caused by products of activated leukocytes, the complement attack complex (C5b-9) and osmotic fluctuations caused in part by fluctuations in Ca^{2+}. Cell swelling occurs due to uncontrolled influx of water as well as Na^{+} and Ca^{2+}. Ultimately, cell swelling leads to membrane blebbing and cell rupture (Majno & Joris, 1995). Leakage of intracellular components upon membrane rupture in cells undergoing oncosis results in exudative inflammation.

Apoptosis has been described as "programmed cell death", it requires energy and results in cell and nuclear shrinkage and fragmentation without exudative inflammation (Kerr *et al.*, 1972). There are two molecular pathways that lead to apoptotic death, both of which occur in cardiac myocytes. The external pathway utilises cell surface receptors and the intrinsic pathway involves the calcium-dependent organelles, mitochondria and SR. Both pathways involve the activation of caspases (cytosolic aspartate residue-specific cysteine proteases) such as caspase-8, but differ in that the extrinsic pathway is activated by binding of death receptors e.g., FasL, and the intrinsic pathway involves the formation of the

mitochondrial permeability transition pore (mPTP) and cytochrome *c (*Kumar *et al., 2007).* The most recent studies, although in agreement that apoptotic cell death occurs during coronary occlusion, do not agree concerning the extent or time period in which apoptosis occurs. Kajstura *et al.* (1996) reported that apoptotic cell death was the major form of cell death during up to 6 hours coronary occlusion in the rat. Conversely, Fliss *et al.* (1996) and Gottlieb *et al.* (1994) reported that apoptosis occurred only during reperfusion. Scarabelli *et al.* (1999) employed multiple staining techniques to report a time course of apoptosis in specific cell types during ischaemia/reperfusion. They reported that endothelial cells are the predominant cell type affected by apoptosis, and that the cells undergo apoptosis before cardiomyocytes. The percentage of cells undergoing apoptosis, contributing towards ischaemic injury, has been reported between 5 and 33 % (Garg *et al.*, 2003; Takashi & Ashraf, 2000) whilst Bialik *et al.* (1997) report a range between 3 and 12 %. Reported data for apoptotic contribution to ischaemic cell death has, in the main, been confirmed by terminal uridine deoxynucleotidyl transferase staining. It is important to mention that there are numerous reports that suggest that current sensitivity and specificity of this method in determining apoptotic cell death is questionable, which may in part explain the inconsistency in reported results (Bialik *et al.*, 1997; Buja & Vela, 2008; Fliss & Gattinger, 1996).

Autophagy or macro autophagy is a physiological process that results in cells digesting internal organelles or dysfunctional cytoplasmic components via the lysosomal degradative pathway. This process occurs both under physiological and pathophysiological conditions, including myocardial ischaemia (Kanamori *et al.*; Yan *et al.*, 2005). During prolonged

ischaemia autophagy is substantially increased and is said to be pro survival as degraded membrane lipids within autophagosomes are recruited to maintain needed levels of ATP production and protein synthesis (Levine & Klionsky, 2004). Hickson-Bick *et al.* (2008), reported that in neonatal cardiomyocytes, autophagy can protect cells against programmed cell death. They showed that apoptotic markers were increased when lipopolysaccharide (LPS) treated cardiomyocytes were treated with inhibitors of autophagy. This work supports data from an acute hypoxia-reoxygenation model of cardiomyocytes that suggests that autophagy protects against ischaemia-reperfusion injury by selective sequestration of damaged mitochondria, resulting in the limitation of pro-apoptotic factors (Hamacher-Brady *et al.*, 2006).

1.5 Progression of infarct

During ischaemia, myocytes can be classified into three categories: cells that are viable and so far unaffected by ischaemic insult; cells that have been irreversibly damaged and undergone cell death; and those that have been reversibly damaged, which may recover function with timely reperfusion (Reimer *et al.*, 1993). The speed with which myocytes progress through these states, and the ultimate size of the infarct depends on a number of factors. Collateral blood flow and the severity of index ischaemia as well as duration of ischaemia will affect this end point (Reimer *et al.*, 1977). Many non-human primates and pigs have little or no preformed collateral anastomoses between coronary arterial regions, and so occlusion of a coronary artery will result in total or near total ischaemia of the affected region (Fujiwara *et al.*, 1982; Lavallee & Vatner, 1984). This is also the case for most human patients who have very few physiologically occurring anastomoses. The

exception to this would be if physical stimuli such as brief ischaemic episodes (angina pectoris) have occurred historically, resulting in collateral development (Fulton, 1963). In contrast, canine and feline models have been shown to have considerable collateral anastomoses, meaning that occlusion of one coronary artery rarely leads to total ischaemia due to perfusion of the ischaemic risk zone by the collateral circulation (Jennings *et al.*, 1978). Similarly, the guinea pig heart is well documented as having an extensive collateral coronary circulation (Maxwell *et al.*, 1987), so much so that Winkler *et al.* (1984), were unable to detect infarct in guinea pig hearts following 6 hours of coronary artery ligation.

Seminal work published in 1977 by Keith Reimer and colleagues described the first reliable infarct model, reporting the importance of controlling the above mentioned parameters (Reimer *et al.*, 1977). They were the first group to characterise robustly the progression of infarct in a canine model of ischaemia-reperfusion, known as the "wave front phenomenon" (see Figure 1.2). Cell death occurs in a wave like fashion from the subendocardial myocardium progressing toward the subepicardial myocardium over time, with many cells in the subepicardial myocardium surviving up to six hours after coronary artery occlusion. Following 24 hours of coronary artery occlusion, they showed that infarct had become almost transmural. Any tissue that was still viable at this point was associated with adjacent blood vessels perfusing the anterior myocardium. They concluded that the wave front progression of the infarct was a consequence of the collateral blood flow, which is greatest at the epicardial myocardium (Reimer *et al.*, 1977; Reimer *et al.*, 1993).

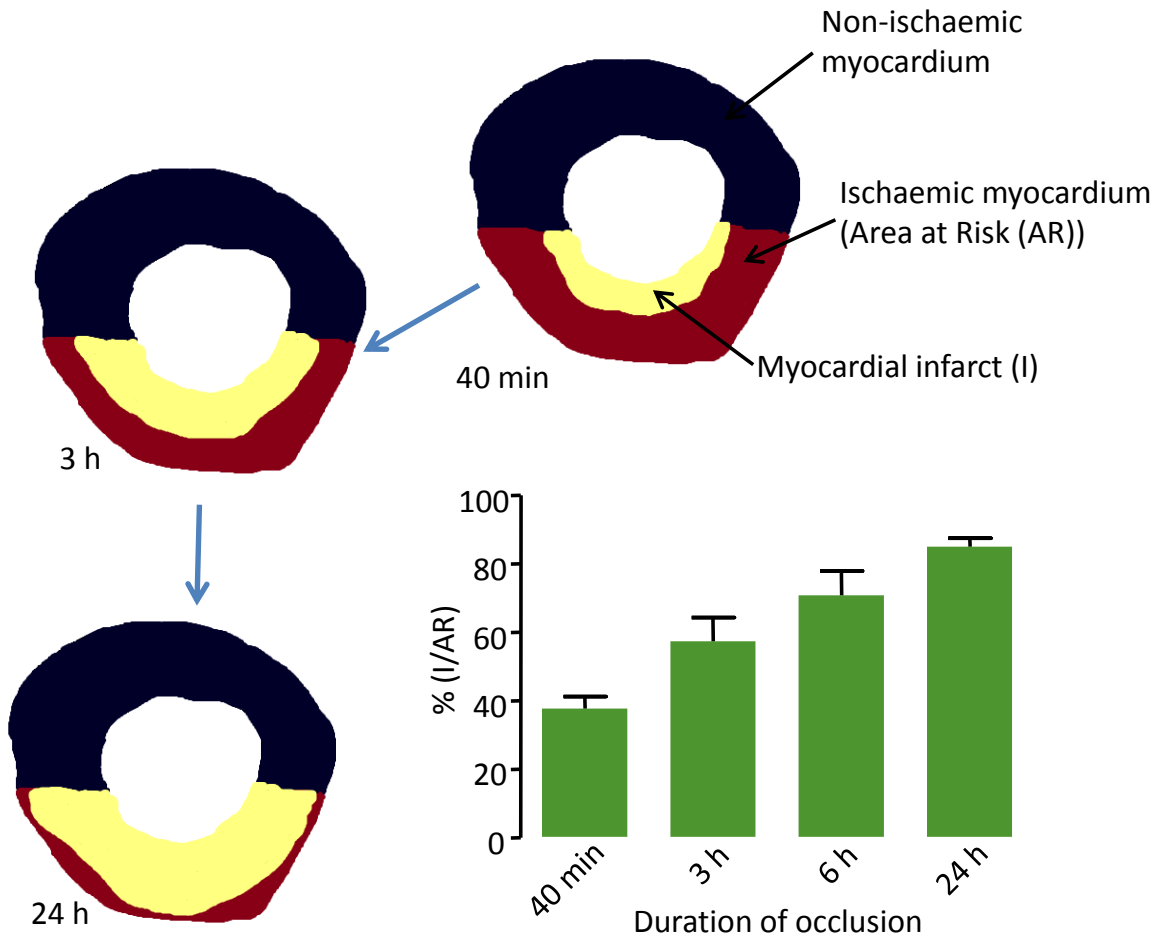

Figure 1.2 *Schematic of the wavefront phenomenon of myocardial necrosis proposed by Reimer and Jennings, (1977). Infarct develops in a wave like fashion from the subendocardial myocardium to the subepicardial myocardium with time. Histogram adapted from Reimer et al. (1977), showing increasing infarct (I) size as a percentage of the area at risk (AR) over time.*

1.6 The collateral circulation

As mentioned above the extent of coronary collateral circulation varies depending on animal model and even between human patients (Kloner *et al.*, 1976; Leshnower *et al.*, 2007; Maxwell *et al.*, 1987; Reimer & Jennings, 1979). The conclusions drawn by the seminal work of Reimer *et al. (1977)* were based partly on the presence of collateral anastomoses. Leshnower *et al.* (2007) have recently investigated the progression of infarct upon coronary artery occlusion in a model that is more akin to species that have limited collateral circulation, or human patients whose collateral circulation is poorly developed. They chose an ovine model because of its consistent paucity of preformed collaterals. In contrast to the work of Reimer and Jennings 30 years earlier, Leshnower and colleagues reported a more evenly distributed infarct throughout the ventricular wall, following 45 minutes coronary artery occlusion. When occlusion time was increased to 1 hour, distribution of the infarct was altered again, the majority of infarct now focused in the mid myocardium, with the least damage in the endocardial myocardium. Although these more recent data highlight differences between the progression of infarction in well-collateralised hearts and those with limited collateral circulation, there is certainly agreement in the relationship between size of infarct and duration of index ischaemia. Interestingly, the most recent studies investigating this phenomenon continue to adopt the view of Reimer and colleagues that the endocardial myocardium is most susceptible to ischaemia and ultimately infarct.

1.7 Reperfusion of the myocardium

The seminal work of Reimer and Jennings (1977), introduced the idea that reperfusion could reduce the total amount of infarction. As mentioned above, increasing the duration of index ischaemia, and hence delaying reperfusion results in larger infarct size. Clinically, treatment for patients presenting with myocardial infarction has changed dramatically over the course of several decades. Historically management of symptoms and rest was the mainstay of treatment, whereas today controlled reperfusion is the preferred choice (Van de Werf *et al.*, 2003).

Although coronary artery bypass and thrombolytic therapy are still used, the treatment of choice for patients presenting with ST-segment elevated acute myocardial infarction (STEMI) is primary percutaneous coronary intervention (PPCI). European guidelines suggest that PPCI by an experienced team of cardiologists is preferential over earlier thrombolytic therapies given by ambulance crews (paramedics) if it can be achieved within 1-2 hours (Van de Werf *et al.*, 2003). Although PPCI requires patients to be transported to specialist facilities, large cross pooled studies have shown that if this is achieved within 2 hours, it is more effective than thrombolysis (Boersma & Group, 2006). Both PPCI and thrombolytic therapies carry risk, the major factor being bleeding (Armstrong *et al.*, 2003). Re-occlusion of the artery can also occur following PPCI, but the risk is much reduced (5 %) in patients fitted with physical stents (Kastrati *et al.*, 2007). The combination of anti-platelet (thrombolytic), and anti-coagulant drugs given to patients treated by both thrombolysis and PPCI puts them at risk of haemorrhagic stroke and major non cerebral bleeding (Armstrong *et al.*, 2003).

1.8 Reperfusion injury

A consequence of the above-described therapies for the treatment of patients presenting with AMI, is that the physical act of restoring blood flow to a previously occluded vessel can cause irreversible damage to the myocardium (Downey & Cohen, 2006; Hearse & Bolli, 1992; Piper *et al.*, 1998). Lethal reperfusion injury is described as irreversible injury leading to cell death that results during reperfusion itself, caused as a direct result of the act of reperfusing the myocardium, beyond damage caused by the preceding ischaemic episode.

In the first few minutes of reperfusion, further metabolic and biochemical insult occurs. The oxygen paradox reported by Hearse *et al.* (1978) describes the requirement for restoration of oxygen to the ischaemic area to allow aerobic respiration to restart. Paradoxically, the sudden return of oxygen allows the re-energisation of the mitochondria, which, in turn, generates detrimental ROS in the first few minutes of reperfusion. In re-addressing the ion homeostasis during early reperfusion, the Na^+/Ca^{2+} exchanger is activated in reverse mode, resulting in an overload of intracellular $[Ca^{2+}]$ uptake and subsequent release by the SR. Intracellular pH is brought back to physiological levels rapidly with wash out of lactic acid and the activation of the Na^+/H^+ exchanger and the Na^+/HCO_3^- symporter. Elevated intracellular $[Ca^{2+}]$ cause several detrimental actions, which result in cell death. Through protease activation, sarcolemmal rupture occurs and through uptake of Ca^{2+} into the mitochondria, mPTP is formed (Halestrap *et al.*, 2004). Release of inflammatory mediators results in recruitment of neutrophils, which cause microvascular plugging, further generation of ROS and release of deleterious

enzymes. Cellular swelling and microvascular plugging and associated interstitial oedema contribute to the no-reflow phenomenon even with an open coronary artery (Schwartz & Kloner, 2011).

Reperfusion injury can manifest in several ways; it can be described as either reversible or irreversible (lethal) reperfusion injury. Vascular injury that results as a consequence of inflammation and endothelial dysfunction around the site of reperfusion are examples of lethal injury. Myocardial stunning, the prolonged contractile dysfunction that occurs after a short period of ischaemia, is reversible over time; however it generally manifests for much longer than the ischaemic episode itself, often days (Hearse & Bolli, 1992). Reperfusion-induced arrhythmias can contribute to either reversible or irreversible reperfusion injury, depending on their severity, displaying as ventricular premature beats (VPB) or in the most severe cases ventricular fibrillation (VF).

The most widely held concept of lethal reperfusion-induced injury is the killing of cells that, prior to reperfusion, were still viable (Basso & Thiene, 2006; Burke & Virmani, 2007). It is generally accepted that the majority of reperfusion induced cell death occurs in the first few minutes of reperfusion and is a result of sarcolemmal rupture (Piper *et al.*, 1998; Yoshida *et al.*, 1995) (see Figure 1.3).

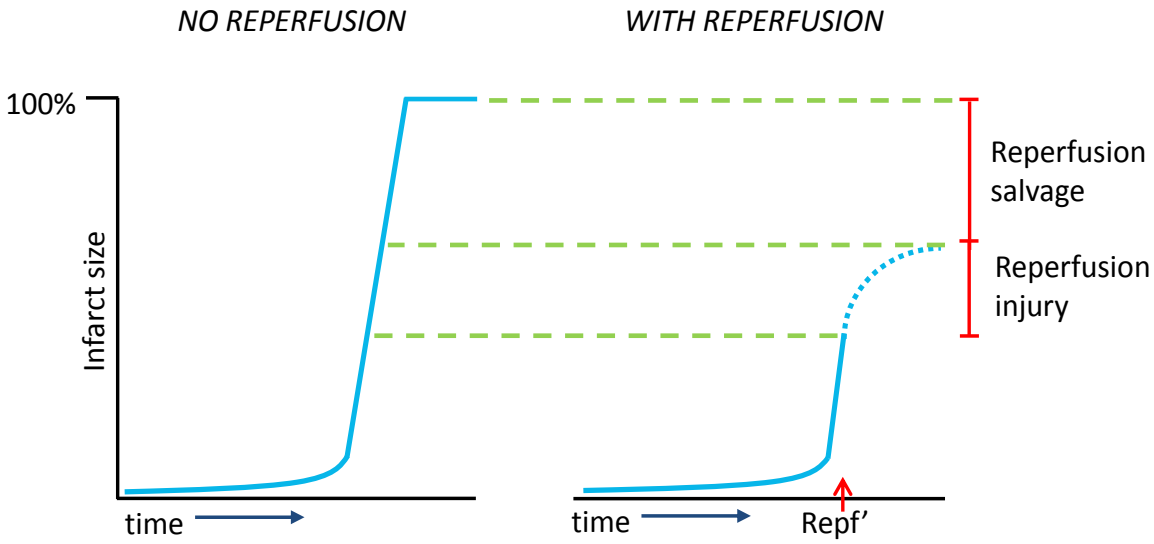

Figure 1.3 *Schematic representation of reperfusion injury. With no reperfusion infarct size will increase with time to 100% (left hand side). Reperfusion at time (red arrow, right hand side) limits infarct size (reperfusion salvage) compared to no reperfusion. However, reperfusion does cause further infarction (reperfusion injury) depicted by the blue dotted line.*

1.9 The role of the mitochondrial permeability transition pore

Although the mPTP has been investigated extensively since its identification more than thirty years ago, it remains a complex and controversial component of ischaemia-reperfusion (Halestrap, 2009). Haworth and Hunter (1979) and Crompton (1987) identified the mPTP as a non-specific channel of defined diameter spanning the mitochondrial membrane. Recent work by Halestrap and colleagues has made the association between reperfusion and formation of this pore. They observed that opening of the mPTP is enhanced by adenine nucleotide depletion, as well as elevated phosphate and oxidative stress, which are biochemical anomalies associated with ischaemia-reperfusion injury (Halestrap *et al.*, 1998; 2009). mPTP opening allows molecules up to 1.5kDa, including protons, into the mitochondria, resulting in uncoupling of oxidative phosphorylation, ATP depletion and necrotic cell death. Work by Crompton *et al.* (1988) and Griffiths *et al.* (1995; 1993) has shown direct evidence that mPTP opening does occur during reperfusion , but not during ischaemia. As yet, there is little evidence to suggest a role for the mPTP in healthy cells as a knockout (KO) model of mice lacking cyclophilin D (CYPD), a cyclosporin binding protein which is a component of the mPTP, appear normal but are protected against reperfusion injury (Baines *et al.*, 2005). The continued publication of literature supporting a role of mPTP in reperfusion injury has created a surge of investigation in targeting this pore in the hope of limiting lethal ischaemia-reperfusion injury.

1.10 Cardioprotection- ischaemic preconditioning

Early experiments that investigated the possibility of salvaging myocardial tissue after an ischaemic episode came to no avail. The models being used were inconsistent and no standardised approach was taken to allow data to be reliably compared. Any success in reducing infarct size was modest, in the order of 10 %, which was not sufficient to proceed to the clinic. It was Reimer and Jennings' group who led the way in tackling the cardioprotection paradigm. Charles Murry, working in their laboratory, undertook a seminal study which showed that performing 4 cycles of 5 minute coronary artery occlusion/5 minute reperfusion prior to 40 minutes of index ischaemia would substantially protect the heart (Murry *et al.*, 1986). They showed infarct limitation of up to 75 %, far greater protection than anyone had reported before and reproducible by all who subsequently applied it. The protection afforded was termed ischaemic preconditioning (IPC), later known as classic or early IPC, and was not dependent on collateral blood flow. As well as reduction in infarct size, it has been reported that IPC can afford cardioprotection in other ways. In dogs (Vegh *et al.*, 1990) and rats (Shiki & Hearse, 1987) IPC has been shown to reduce the incidence of ischaemia-reperfusion arrhythmias. Interestingly, the rat is the only species where improved recovery of mechanical function can be shown (Steenbergen *et al.*, 1993), and is reported to be as a result of altered adenosine metabolism in the rat heart (Gelpi *et al.*, 2002).

Murry *et al.* (1986), also reported data for a longer ischaemic model where hearts were not reperfused for three hours following IPC. The data from these experiments was in stark contrast to the acute ischaemic model. IPC was unable to protect hearts subjected to

three hours ischaemia, with infarct sizes comparable to controls, suggesting that prompt reperfusion after the ischaemic insult is a necessity. Later studies showed that the preconditioned state is very transient, lasting only 60-120 minutes and is completely lost between 2 and 4 hours in conscious rabbits (Burckhartt *et al.*, 1995). This means that if index ischaemia is not ensued within this time period, the IPC mediated reduction in infarct size will be lost.

1.11 Delayed preconditioning

Marber *et al* (1993) and Kuzuya *et al.* (1993) independently reported a new preconditioning observation: 24 hours after IPC, protection could be demonstrated in the rabbit and canine respectively. This "second window of protection" was termed delayed preconditioning and was later shown to last up to 72 hours after IPC (Baxter *et al.*, 1997; Imagawa *et al.*, 1999). This aspect of preconditioning goes beyond the scope of this thesis and is comprehensively reviewed by Hausenloy and Yellon (2010).

1.12 Mechanisms of classical ischaemic preconditioning

Many autacoids have been shown to trigger classical IPC, the first to be described being adenosine (Liu *et al.*, 1991). Downey's laboratory showed that the adenosine A_1 receptor activation triggered IPC and thus showed that IPC was receptor mediated. They showed that blocking of the adenosine A_1 receptor abolished the characteristic protection seen, suggesting the importance of endogenous G_i-protein-coupled adenosine A_1 receptor activation to afford IPC protection (Liu *et al.*, 1991). Further studies have characterised other receptors that can trigger IPC when activated by their ligands. Noradrenaline was shown to afford similar protection through activation of α-receptors in the rat heart

(Banerjee *et al.*, 1993). Bradykinin B_2 and opioid δ1 receptor activation have also been shown to trigger IPC mediated protection (Baxter & Ebrahim, 2002; Gross *et al.*, 2005). To date, it is suggested that any G_i-coupled receptor can trigger the preconditioned state through activation of G_i protein, although a limited number of endogenous autacoids participate naturally in the phenomenon.

1.13 Ischaemic preconditioning threshold

Downey's group identified that pharmacological inhibition of one autacoid receptor could only block the protection afforded by one cycle of IPC, but not from multiple cycles (Goto *et al.*, 1995). They introduced the idea of an IPC threshold, suggesting that any one receptor stimulus only contributed towards IPC and that increasing the stimulus via other receptors (pharmacologically) or increasing the number of IPC cycles (mechanically) could compensate and meet the threshold to initiate IPC. Similarly, other G_i receptors such as endothelin ET_1 (Wang *et al.*, 1996) and muscarinic M_2 receptors (Yao & Gross, 1993) have been shown to afford IPC when activated pharmacologically (meeting the IPC threshold), however their antagonism does not impair IPC as agonists to these receptors are not produced during IPC (Yellon & Downey, 2003).

The protection afforded by the stimulation of the above mentioned receptors has been reported to be mediated by protein kinase C (PKC). Inhibition of PKC will eliminate the protection from a preconditioned heart but has no effect on a nonprenconditioned heart (Ytrehus *et al.*, 1994). ATP sensitive potassium channels (K_{ATP}) have also been reported to play a crucial role in IPC. The K_{ATP} inhibitor glibenclamide abrogated the protective effects of IPC (Ferdinandy *et al.*, 1995), whereas the pharmacological activators, pinacidil

and chromakalin afford protection quantitatively similar to that of IPC (Grover *et al.*, 1989). In a rabbit model of IPC, Pain *et al.* (2000), suggested that the end effector of IPC was not opening of K_{ATP} channels. They demonstrated that this in fact triggered small bursts of ROS that trigger entrance into a preconditioned state that then activates downstream kinases, including PKC, and ultimately inhibition of mPTP formation. Other kinases that are involved in IPC signalling include activation of phosphatidylinositol 3-kinase (PI3K), and its substrate kinase Akt (Hausenloy *et al.*, 2005), p38 mitogen activated protein kinase (MAPK), p42/p44, the Janus kinase/ signal transduction activator of transcription (JAK/SAT) pathway (Dawn *et al.*, 2004) and more recently inhibition of glycogen synthase kinase 3β (GSK3β) (Gao *et al.*, 2009). Evidence that suggests these kinases play an important role in IPC relies on abolishing or abrogation of protection when their pharmacological inhibitors have been used in models of IPC (Hausenloy & Yellon, 2007).

1.14 Ischaemic preconditioning affords protection at reperfusion

Although many studies had been published documenting the kinases involved in IPC, it remained unclear how IPC afforded protection through K_{ATP} channel activation. It was well accepted that these channels played an important role in both pharmacological (Garlid *et al.*, 1997) and mechanical IPC (Hide & Thiemermann, 1996), yet whether they were triggers or mediators was not agreed. In 2002, Hausenloy and colleagues examined the role of the mPTP in the context of IPC. Using an isolated rat heart model, they reported that the infarct limitation afforded by both mechanical and pharmacological IPC was abolished if the mPTP was opened pharmacologically with atractyloside. They also

showed that inhibiting the opening of the mPTP at reperfusion with cyclosporine A, resulted in similarly protected hearts as those treated with IPC (Hausenloy *et al.*, 2002). Work by Halestrap's group the following year further investigated the role of the mPTP in IPC, using a more direct approach to assessing mPTP activity. They used the same technique that allowed them to show some years earlier that mPTP opening occurs during early reperfusion and not during index ischaemia (Griffiths & Halestrap, 1995). The method involved the mitochondrial entrapment of 2-deoxy[^{3}H]glucose ([^{3}H]DOG) which allowed them to measure mPTP opening. Their findings supported those of Hausenloy *et al. (2002)*, in that protection afforded by IPC is associated with indirect inhibition of the mPTP. They further concluded that in their hands, direct targeting of the mPTP was less effective than removing the conditions that are responsible for pore opening in the first place (Javadov *et al.*, 2003). As momentum gathered during the early 2000's, Yellon's group investigated the link between IPC and protection afforded in early reperfusion further. Two studies suggested that the kinases previously thought to be activated during ischaemia in response to IPC were in fact phosphorylated during early reperfusion in response to this stimulus (Hausenloy *et al.*, 2004). Furthermore, IPC increased phosphorylation of PI3K-Akt and MEK-1/2-ERK-1/2 pathways, but during reperfusion, after index ischaemia. They also suggested that these kinases are critical for IPC induced protection (Hausenloy *et al.*, 2005).

Work published by both Yellon's and Halestrap's laboratories shifted the focus of IPC mediated protection towards the first few minutes of reperfusion. The so-called survival kinase pathways had now been shown to be implicated in IPC mediated protection as well

as targeting reperfusion induced injury (Burley *et al.*, 2007; Yang *et al.*, 2006). Clinically these observations were appealing as it has long been a criticism of IPC mediated protection that it had limited clinical applicability. Clinical studies had been employed where high risk patients received K_{ATP} channel agonists or nitric oxide (NO) donors. However the protective effects were limited, and dosing was a challenge due to the generally unpredictable nature of AMI (Lee *et al.*, 2002; Rezkalla & Kloner, 2007). The focus had now shifted towards targeting ischaemia-reperfusion injury in the first few minutes of reperfusion, a strategy which is both clinically desirable and practical (Hausenloy *et al.*, 2005).

1.15 Postconditioning

At the time when the mechanisms of IPC protection were being investigated, Vinten-Johansen's group published a landmark study that again shifted attention away from pre-ischaemic targeting and focused on early reperfusion. They described a similar experimental protocol to that first reported by Murry *et al. (1986)*. However they applied the ischaemic conditioning after index ischaemia, and called this postconditioning. They showed that three intermittent 30 second periods of ischaemia in a canine model, following 60 minutes index ischaemia, resulted in a marked reduction in infarct size and identified the first moments of reperfusion to be a key therapeutic window (Zhao *et al.*, 2003). Similar to preconditioning, timing and duration of the cyclical ischaemic stimulus is crucial in determining the amount of protection afforded. It is also apparent that species variation plays a role in the timing and duration. In the rabbit model protection was afforded when the first re-occlusion was initiated after 30 seconds reperfusion but this

protection was lost if re-occlusion was delayed until 60 seconds (Yang *et al.*, 2004). In an *in vivo* rat model, protection was lost if re-occlusion was delayed from 10 to 30 seconds (Kin *et al.*, 2004). It is clear however that delaying the postconditioning stimulus to 5 minutes or more appears to be too late to stimulate the protective mechanisms (Ovize *et al.*, 2010).

Prior to the publication of Vinten-Johansen's seminal paper, Na *et al.* (1996), reported a reduction in the incidence of VF in a feline model of regional ischaemia following VPB-driven intermittent reperfusion. They demonstrated a significant reduction in VF following what they described as postconditioning, concluding that it was as good as, if not better than IPC. It could therefore be argued that the first report of postconditioning came from the Korean group, predating the formal description that is now widely accepted.

1.16 Mechanisms of postconditioning

The proposed mechanisms of postconditioning initiate signal transduction pathways by recruiting autacoids to act on cell surface receptors and the transport of ions through cell surface channels. In 2002, Yellon and colleagues proposed the idea that there is a pro-survival signalling cascade that is endogenously activated upon reperfusion. They used a urocortin-mediated model of cardioprotection to show that the ERK 1/2 MAPK-dependendent signalling pathway represented an important survival mechanism against reperfusion injury (Schulman *et al.*, 2002). Signalling through PI3K has also been shown to afford protection against early reperfusion injury via activation of the serine-threonine kinase, Akt (Fujio *et al.*, 2000; Matsui *et al.*, 1999). This work and further studies of kinase activation using G-protein coupled receptor agonists and

natriuretic peptides (NP) that promoted the idea that the heart possesses "Reperfusion Injury Salvage Kinase" (RISK) pathways (Hausenloy & Yellon, 2004) (see Figure 1.4). Further kinase interactions involved in the so called RISK pathway are discussed in Chapters 3, 4, 5 and 6.

It has recently been proposed that a second signalling cascade, the so called "Survivor Activating Factor Enhancement" (SAFE) pathway is activated endogenously upon reperfusion and has been demonstrated to afford cardioprotection (Boengler *et al.*, 2008b; Lecour, 2009). Activation of the JAK-STAT signalling cascade via interleukin type cytokines transports stress signals to the nucleus from cell surface receptors and its inhibition has been shown to abrogate cardioprotection at reperfusion (Boengler *et al.*, 2008a). Although this pathway has been investigated much less widely than the RISK pathway it is suggested that its end effector, like that proposed for the RISK pathway, is the mPTP (Heusch *et al.*, 2008).

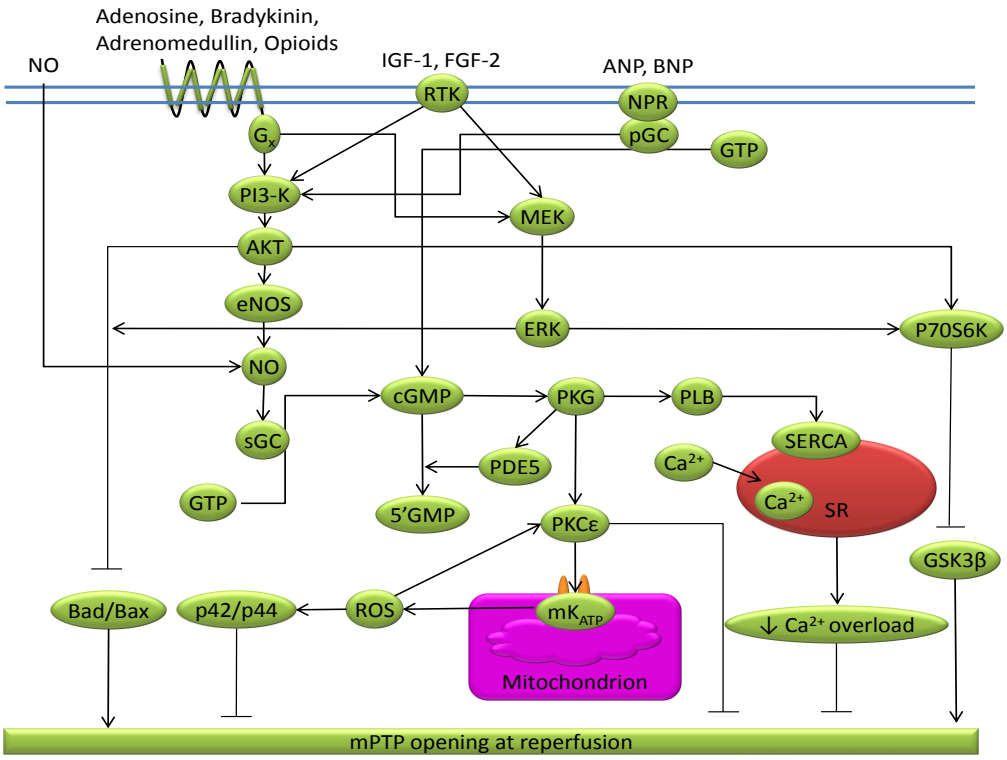

Figure 1.4 *Schematic representation of the reperfusion injury salvage kinase (RISK) pathway first proposed by Hausenloy & Yellon (2006). Schematic illustrates the dynamic signalling cascades that culminate in inhibition of the mitochondrial permeability transition pore (mPTP) opening and ultimately limiting cell death. Autacoid factors activate G proteins signalling through PI3-K/Akt/eNOS and ultimately sGC/cGMP/PKG. Similarly natriuretic peptides (ANP/BNP) stimulate pGC to convert GTP to cGMP and signalling through the same downstream targets as sGC. As depicted there are several proposed end mediators of mPTP inhibition.*

1.17 Natriuretic peptides

The majority of the investigative work that led to the discovery of the natriuretic factor was carried out during the 1950's. Kisch first reported anatomical differences in the Golgi networks of atrial and ventricular cells, commenting that in atrial cells they were similar to those of secretory cells (Kisch, 1956). In the same year Henry and colleagues reported that balloon distension of the atria correlated with increased urination in dogs (Henry *et al.*, 1956). By the late 1960's de Bold's laboratory were characterising the granules found in the atria first reported by Jamieson and Palade (de Bold *et al.*, 1981; Jamieson & Palade,

1964). de Bold *et al.* reported that the granules changed in response to alterations in electrolyte and water balance. It was then in their seminal paper in 1981 that they brought together the work of Kisch, Jamieson & Palade and Henry *et al.*, documenting that atrial but not ventricular extracts contain a potent blood pressure lowering factor, proposing that it worked by stimulating renal sodium and water secretion (de Bold *et al.*, 1981). This was the first description of atrial natriuretic peptide (ANP), originally atrial natriuretic factor (ANF). In 1988, the second family member was characterised and named brain natriuretic peptide (BNP) as it was isolated from the brain (Sudoh *et al.*, 1988). It was later reported that it was most highly concentrated in cardiac ventricles of patients with heart failure (Mukoyama *et al.*, 1990). Sudoh *et al.* then identified the third family member, C-type natriuretic peptide (CNP) from porcine brain extracts based on its ability to relax smooth muscle (Sudoh *et al.*, 1990).

All NPs are synthesised as prepro peptides. ANP undergoes cleavage from a 151 prepro form to a 126 amino acid proANP that is the predominant form stored in atrial granules. This allows rapid cleavage to the biologically active carboxyl-terminal 28 amino acid ANP upon secretion by the transmembrane cardiac serine protease, corin (Yan *et al.*, 2000). BNP undergoes similar cleavage events to yield a biologically active 32 amino acid peptide (Sudoh *et al.*, 1988). Unlike in the atria, BNP production is transcriptionally regulated by GATA4 and is released in response to cardiac wall stretch, resulting from volume overload (Lang *et al.*, 1985; Ruskoaho *et al.*, 1989). Plasma BNP concentrations in healthy individuals are approximately 1 fmol/mL which is elevated two-three hundred fold in patients with congestive heart failure, making it a good indicator of disease (Mukoyama *et*

al., 1991a). CNP is cleaved from its prepro peptide into two biologically active residues. An intracellular endoprotease cleaves proCNP into the 53 amino acid active form, but the 22 amino acid form is cleaved by an as yet unidentified enzyme (Wu *et al.*, 2003). The predominant active form found in the heart is the 53 amino acid peptide (Minamino *et al.*, 1991).

Most recently a new class of so-called "designer" NPs is being developed. Centeritide is the first in this class of designer NPs which co-activates both NPR1 and NPR2 (see 1.18). Initial clinical trials suggest a reduction in blood pressure in stable heart failure patients as well as reduced creatine levels. It is proposed that continuous infusion of centeritide through a subcutaneous pump will improve patient outcome and reduce the duration of hospital stay (Burnett, 2011).

1.18 Natriuretic peptide receptors

There are three natriuretic peptide membrane associated guanylyl cyclase receptors (NPRs). NPR1 and NPR2 mediate most physiological actions (Chinkers & Garbers, 1991), whereas the third receptor, NPR3 is described as a clearance receptor (Maack *et al.*, 1987). NPR1 and NPR2 can be found expressed in many tissues, particularly in the cardiovascular system, localised to the atria, ventricles, aorta and peripheral vasculature (Tsuchimochi *et al.*, 1988; Wilcox *et al.*, 1991). NPR1 is bound by both ANP and BNP although ANP is an order of magnitude more potent. CNP's ability to bind to NPR1 is negligible (Nakao *et al.*, 1991). In comparison CNP is the only peptide that can significantly increase cyclic GMP levels in cells expressing NPR2 (Suga *et al.*, 1992). All NPs bind the so-called clearance receptor, NPR3. Upon binding of the peptides to the

receptor, they undergo internalisation and lysosomal degradation. The peptides are also cleared to a lesser extent by proteolytic cleavage and renal excretion (Condra *et al.*, 1988).

From binding to the NPR, intracellular signalling occurs principally by means of cGMP elevation and activation of distal pathways via cGMP dependent proteins. The NP signalling cascade initiated during early reperfusion, and the cardioprotective mechanisms proposed are discussed in more detail in Chapter 6.

1.19 Nitric oxide

Over the last 30 years, interest in NO and our understanding of it as a biological mediator has grown at increasing pace. Originally described as a noxious gas, it was the seminal work of Furchgott and Zawadzki(1980) and Ignarro *et al.* (1982) who discovered an endothelium derived relaxing factor (EDRF) and later Palmer and colleagues (1987) who identified it as NO that created the foundation for this large body of research. In 1867, it was documented in the Lancet that organic nitrovasodilators (amyl nitrite) were efficacious in patients presenting with angina pectoris (Brunton, 1867). It was not until the latter part of the 20th century that pharmacology of these compounds was understood. Furchgott *et al.* (1980), noticed that there were inconsistencies in data reporting the vasoactive properties of acetylcholine (ACh) on blood vessels *in vitro*. They observed in thoracic aortic rings from rabbits that if the endothelium was damaged on dissection then the responses to ACh were different. This led them to conclude that the endothelium must be present for ACh mediated relaxation. Furthermore, they proposed that ACh acting on muscarinic receptors in the endothelial cells stimulates the release of a substance

that causes relaxation of vascular smooth muscle. It was then Palmer *et al.* (1987) who documented that relaxation induced by both EDRF and NO was inhibited by haemoglobin and enhanced by superoxide dismutase (SOD) to a similar degree. They deduced that NO released from endothelial cells was indistinguishable from EDRF in terms of biological activity, stability and susceptibility to an inhibitor. They concluded that EDRF and NO are identical.

NO is synthesised by a group of enzymes called nitric oxide synthases (NOS). To date there are three distinct NOS isoforms located in different tissues. They are distinguished by their dependence on calcium and calmodulin (Bredt & Snyder, 1990). Neuronal NOS (nNOS, NOS I), isolated from brain tissue, and inducible NOS (iNOS, NOS II) are mostly found in soluble portions (Stuehr *et al.*, 1991). Endothelial NOS (eNOS, NOS III) is mainly found in the particulate fraction in endothelial cells (Forstermann *et al.*, 1991). nNOS and eNOS are constitutive enzymes, constantly producing nanomolar amounts of NO. In contrast, iNOS is not present constitutively; it is induced by pro-inflammatory cells of the immune system (Griffith & Stuehr, 1995). NOS produce NO by catalysis of a 5-electron oxidation of one nitrogen atom of the guanidine group of L-arginine to form NO and L-citrulline. It is important to consider the distribution and expression of NOS isoforms when thinking about the actions NO has in different tissues. eNOS and nNOS are relatively low output enzymes and associated with basal physiological function. On the other hand, iNOS is a high output enzyme, generating 1000-fold more NO than eNOS (Singh & Evans, 1997). The sheer amount of NO that iNOS can produce can result in detrimental effects, not directly by NO, but by the ROS produced in an NO and super

oxide rich environment (Ferdinandy & Schulz, 2003). It is important to note that studies have also shown that eNOS can be regulated directly by PI3K/Akt (Fulton *et al.*, 1999), and more recently it has been reported that oestrogen receptor binding can lead to phosphorylation and activation of eNOS (Haynes *et al.*, 2000; Russell *et al.*, 2000).

NO's charge neutrality enables it to diffuse freely in aqueous solutions across cell membranes. The rate of diffusion is dependent on its half-life, which in turn is dependent on the rate of formation. Typically, in aqueous solution, the intracellular half-life of NO is in the millisecond range (Bolli, 2001; Hakim *et al.*, 1996). Biological breakdown of NO can occur in numerous ways. The most common intermediate breakdown product is nitrite (NO_2^-) (Kelm, 1999). Nitrite can then be taken up by red blood cells, where further oxidation by a haemoglobin dependent mechanism leads to production of nitrate (NO_3^-), which can be redistributed in plasma.

1.20 Nitric oxide and cardioprotection

For almost as long as research has been carried out exploring cardioprotective paradigms, particularly ischaemia-reperfusion injury, there has been controversy over the role that NO plays (Bolli, 2001; Ferdinandy & Schulz, 2003; Schulz *et al.*, 2004). It would appear that in recent years this controversy is dwindling and it is generally accepted that NO is cardioprotective in the ischaemia-reperfusion setting. It is however important to note when drawing conclusions from the literature that various end points and animal models have been used and there is certainly species variation in NO production and distribution (Jones & Bolli, 2006). The source of NO and the amount produced or the NO donor being used, will affect whether NO affords protective or deleterious actions.

NO was initially documented as the only endogenous ligand of soluble guanylyl cyclase (sGC), activating it in all tissues tested (Waldman & Murad, 1987). However, it was later demonstrated that carbon monoxide (CO), produced by haem oxygenase could mediate smooth muscle relaxation and platelet aggregation via sGC (Brüne & Ullrich, 1987). Most recently it has been demonstrated that nitroxyl could activate sGC, however it was noted that the nitroxyl donors used could not activate the oxidised form of sGC. The authors further reported that the activation observed was independent of NO (Miller *et al.*, 2009).

When acting as a signalling molecule, NO binds to the haem iron moiety, resulting in its activation and generation of guanosine-3',5'-cyclic monophosphate (cGMP) from guanosine triphosphate (GTP) (discussed in more detail in 1.25), and cGMP acts at distal targets. It is worthy to note that NO can act independently of sGC and cGMP to initiate other actions such as protein S-nitrosylation (Ziolo, 2008). It has also been demonstrated that NO can directly activate adenylate cyclase, thus increasing adenosine-3',5'-adenosine monophosphate (cAMP) levels and myocardial contractility (Burgoyne & Eaton, 2009).

The role NO plays differs depending on whether it is involved in the classical or late phase preconditioning phenomenon. In classical IPC it has been shown that inhibition of NOS by pharmacological intervention has no effect on IPC's ability to limit infarct size. However, exogenous delivery of NO, by NO donors such as SNAP, NOC-9 and diethylenetriamine NONOate (DETA-NO) prior to index ischaemia protect the heart comparably to IPC itself (Nakano *et al.*, 2000; Post *et al.*, 2000). More recently, du Toit *et al.* (2007), reported that mice over expressing eNOS demonstrated a maximally protective state, comparable to wild type littermates who had undergone IPC. In contrast to reports of

protection, others have reported deleterious actions of NOS inhibition during preconditioning. Vegh *et al*. (1993), showed in some of the earliest NO IPC studies, that inhibition of NO by the NOS inhibitor L-NAME, both pre and post IPC abolished the antiarrhythmic effects of IPC in an open chest dog model of coronary artery occlusion. Inhibition of NOS was also shown to increase post ischaemic contractile dysfunction (Lochner *et al.*, 2002).

Evidence suggests that NO plays an important part in both classical and delayed IPC. Although the exact mechanism that affords protection under the delayed IPC is unclear, pharmacological mimetics and triggers such as bradykinin and adenosine, NO and ROS have been identified. Several of these triggers initiate signalling that converges on NOS activation, demonstrating the importance of NO production in initiating delayed IPC. Bolli and colleagues, who have led the way in investigating NO's role in delayed IPC, propose that NO has a functional role both in initiating, but also mediating delayed IPC (Bolli *et al.*, 1998). It is suggested that stimulation of production of NO by eNOS triggers downstream signalling that results in upregulation of cardioprotective mediators, including iNOS. Bolli *et al.* (2001), propose that phosphorylation of PKC, recruitment of tyrosine kinases and activation of NF-κB, as well as ROS production play crucial roles. More recently, studies have shown that NO is a potent stimulator of mitochondrial biogenesis which is consistent with the NO hypothesis of IPC (McLeod *et al.*, 2005; Nisoli *et al.*, 2003). Although there is increasing evidence to support the notion that both eNOS and iNOS are important in IPC, the role of nNOS remains unclear (Murillo *et al.*, 2011). As with the other isoforms of NOS, the literature remains divided about nNOS. Recent

studies have shown that while inhibition of nNOS is protective in ischaemia-reperfusion, its function is required to appreciate the protective effects of delayed IPC (Barua *et al.*, 2010; Lu *et al.*, 2009).

1.21 Nitrite

Originally believed to be an inert by-product of NO metabolism, nitrite is now believed to be an "endocrine reservoir" of NO, which can be reduced to NO when demand requests it, usually under pathological conditions (Lundberg *et al.*, 2008). This paradigm shift in how we view nitrite stems from seminal work by Zweier and colleagues'. They showed that NO formation increased during an ischaemic episode independently of enzymatic activity (Zweier *et al.*, 1999). Pharmacological preconditioning with nitrite in an isolated rat heart model showed a dose dependent limitation of infarct size (Webb *et al.*, 2004). These findings are in support of previous work that suggests that IPC leads to an increase in iNOS expression and nitrite levels (Bolli *et al.*, 1998), which has led to the general hypothesis that nitrite may be an integral mediator of delayed IPC (Murillo *et al.*, 2011). The levels of nitrite generated in the heart during IPC reported to be in the order of nmol/mg corroborate with data that suggests that a cardiac nitrite concentration of pmol/mg protein mediates cytoprotection (Raat *et al.*, 2009).

Most recently, nitrite has been shown to afford cardioprotection when administered at early reperfusion. Duranski *et al.* (2005) administered sodium nitrite in an *in situ* mouse model where the left coronary artery was ligated for 30 min, followed by 24 h reperfusion. They reported a dose dependent protection, with highly significant infarct limitation at near physiological (48 nM) blood nitrite concentrations. A recent study by

Gladwin's group investigated the protective effects of nitrite perfusion as an adjunctive therapy at reperfusion (Gonzalez *et al.*, 2008). They found that independent of time or ischaemic severity, nitrite afforded both infarct limitation and antiapoptotic effects (Gonzalez *et al.*, 2008). The mechanism by which nitrite affords protection has been suggested to be similar to that of NO, supported by the abolition of nitrite protection when NO scavengers are present (Shiva *et al.*, 2007). Gladwin and colleagues have however postulated that reduction of nitrite to NO results in the inhibition of complex I of the mitochondrial transport chain by post-translational S-nitrosation. They further reported that this dampens electron transfer, reducing ROS generation during early reperfusion, ameliorating oxidative inactivation of complexes II-IV, hence preventing mPTP opening and cytochrome *c* release (Gonzalez *et al.*, 2008). In a renal model of ischaemia-reperfusion injury, it has been proposed that eNOS may play a role in the reduction of nitrite, a mechanism that is enhanced in low oxygen environments (Milsom *et al.*, 2010). Whether or not similar mechanisms are employed when myocardial ischaemia-reperfusion injury is limited by nitrites remains to be explored.

1. 22 Soluble guanylyl cyclase

Like many other areas of research, parallel studies were being carried out during the 1970's exploring the different aspects of cyclic nucleotide and NO physiology that, in later years, would forge connections that would stimulate our progression and understanding of the field enormously. In early studies by Sutherland's group, they reported that calcium infusions stimulate urinary excretion of cGMP and not cAMP (Kaminsky *et al.*, 1970). At the same time, it was shown that ACh could increase levels of cGMP indirectly in isolated

perfused rat hearts (George *et al.*, 1970), and later that calcium is important for cGMP regulation (Schulz *et al.*, 1973). The extensive work carried out during the 1970's and 80's exploring EDRF and NO eventually explained these early observations, particularly the work by Rapoport *et al.* (1983) and Rapoport & Murad (1983), which demonstrated that EDRF increases cGMP synthesis in isolated blood vessels and increases protein phosphorylation in smooth muscle cells. Price *et al.* (1967), first used the term guanyl (guanylyl) cyclase (GC), to describe the then unknown enzyme that catalysed the conversion of GTP to cGMP. It was some years later that a number of laboratories reported the presence of a prosthetic haem moiety which was shown to be required for stimulation by NO (Gerzer *et al.*, 1981; Ignarro *et al.*, 1982), and also required for enzyme purification (Kamisaki *et al.*, 1986a), that a structural understanding of the enzyme was developed.

sGC is a heterodimeric haem protein consisting of both an alpha and beta subunit. To date, two alpha and two beta isoforms have been identified. The $\alpha 1\beta 1$ protein has been most extensively researched and is found in most tissues, including the kidney, brain, heart and vascular organs. sGC mediates a wide range of physiological functions, including platelet aggregation, relaxation of smooth muscle, vasodilatation, neuronal signal transduction and modulation of the immune system (Collier & Vallance, 1989), but requires expression of both subunits for catalytic activity (Buechler *et al.*, 1991; Kamisaki *et al.*, 1986b). Russwurm and colleagues have shown that the $\alpha 2\beta 1$ heterodimer exhibits ligand-binding characteristics comparable to the $\alpha 1\beta 1$ protein, yet a splice variant of the $\alpha 2$ subunit, ($\alpha 2i$) forms a dimer with the $\beta 1$ subunit to form an inactive protein (Mergia *et*

al., 2003). In the rat, the α1 and β1 subunits are 690 and 619 amino acids in length, respectively. Both are made up of four domains, an N-terminal haem binding region, a predicted Per/Arnt/Sim (PAS) -like domain, a putative amphipathic helix and the C-terminal cyclase catalytic domain (Iyer *et al.*, 2003; Karow *et al.*, 2004; Wedel *et al.*, 1995). The identification of the haem binding domain on the β subunit required sGC truncations and site direct mutagenesis (Cary *et al.*, 2006). Mutating consecutive histidines to phenylalanine (Wedel *et al.*, 1994), expressing truncations in *E. Coli* (Zhao & Marletta, 1997), and deleting the N-terminal 64 amino acid residues of the β1 subunit (Wedel *et al.*, 1995), together demonstrated that the N-terminus, specifically histidine 105 was the proximal haem ligand. Initially thought to be an exclusive domain, conserved only in mammalian sGC for haem binding, it was later discovered that this region is part of a highly conserved family of proteins found in prokaryotes as well as eukaryotes (Iyer *et al.*, 2003).

1.23 NO activation of sGC

NO binds to sGC leading to a 200-fold increase in the synthesis of its second messenger, cGMP. The rate of reaction in physiological conditions is governed by diffusion; other diatomic gases such as dioxygen and CO, which do not bind, also activate sGC. Our understanding of the exact mechanisms by which NO stimulates sGC to act remains unclear; however, progression with structural cloning is helping this area advance. Although it is beyond the realms of this thesis to explain in detail the chemistry that has elucidated the current proposals of NO binding and stimulation of sGC, an overview of the process is described below.

Initially the model proposed for activation involved rapid binding of NO to the haem group, resulting in the breakage of the histidine 105 bond and a conformational change of the protein and increased catalytic activity. In this model, disassociation of NO from the haem group results in restoration of basal activity (Ignarro *et al.*, 1982; Wedel *et al.*, 1994). This mechanism however is refuted by *in vivo* studies which suggest that deactivation of sGC occurs rapidly when NO levels drop (Bellamy & Garthwaite, 2001). Most recently Cary *et al.(2005)*, and Russwurm & Koesling *(2004)*, reported that NO co-ordinate bonding to the haem group was not sufficient for full enzymatic activation. Both groups proposed alternative mechanisms of activation. Russwurm and Koesling (2004) proposed a two step binding of NO to the haem site, first to the proximal site and then the distal haem cofactor site, producing the high activity complex. Cary and colleagues suggested that NO binds to the haem pocket for low enzymatic activity, but a non-haem site needs to be occupied by further NO to create a fully active complex (Cary *et al.*, 2005). These proposals were tested by Derbyshire and Marletta (2007), using the haem ligand butyl isocyanide to block NO binding to sGC haem. They showed that NO activated the sGC-butyl isocyanide complex without coordinating to the haem cofactor, and further supports the notion that there is a non-haem NO binding site.

1.24 sGC isoform genetic knockouts

In recent years genetic KO mice have been developed for each of the three physiologically expressed sGC enzymes, creating mice deficient in either α1, α2 or β1 protein (Buys *et al.*, 2008; Friebe *et al.*, 2007; Mergia *et al.*, 2006). KO of either of the α subunits results in only one functional form of sGC being expressed, whereas deletion of the β subunit

results in a genotype expressing no functional sGC. In these severely compromised mice, phenotypes are similar, and are more pronounced than those of NOS or cGKI knockouts displaying poor gastrointestinal smooth muscle action (Ny *et al.*, 2000). Homozygous KO mice had only 20 % survival after 48 hours, with 90 % dead within 18 days. As with the NOS and cGKI KO's, gastrointestinal complications such as dysmotility and prolonged total gut transit time were reported (Friebe *et al.*, 2007).

Many cardiovascular specific phenotypes have been characterised using sGC KOs, including the surprising observation that the much less expressed (6 % cGMP produced in aortic smooth muscle) α2 isoform in aortic smooth muscle was able to completely relax aortic smooth muscle rings. This resulted in the characterisation of an unpredicted functional role of α2/β1 sGC producing cGMP sufficient to cause vascular relaxation (Mergia *et al.*, 2006). They also reported that male but not female KO mice developed hypertension, but there was no sex dependency in vascular relaxation experiments (Nimmegeers *et al.*, 2007). Blood pressure observations in two different α1 KO mice models report contrasting results. Considering that over 90 % of cGMP producing activity in this KO is lost, Mergia *et al.* (2006), only reported a 7 mmHg increase in systolic blood pressure, compared to a 26 mmHg increase in β1 subunit KO mice, with no sex difference in phenotype. Buys *et al.* (2008), however reported that in their α1 KO mice, males had significantly higher systolic blood pressure (147 vs. 118 mmHg) measured by a non-invasive tail cuff system. Hypertension was age and testosterone dependent, and was prevented by orchiectomy and/or treatment with an androgen receptor antagonist. The authors suggest that discrepancies in gender differences may be because of the different

genetic backgrounds of the KO models used. It has also been reported that both male and female α1 KO mice have increased cardiac contractility, arterial elastance as well as impaired ventricular relaxation, measured by invasive catheterisation of the carotid artery (Buys *et al.*, 2008).

1.25 Guanosine-3', 5'-cyclic monophosphate

cGMP is the second messenger produced in a reaction catalysed by the cytosolic sGC, or membrane associated pGC, from the purine nucleotide GTP (see Figure 1.5). It was after the discovery of cAMP by Sutherland and colleagues (1958), that it was proposed that another cyclic nucleotide may also be regulating physiological processes. Smith *et al.* were the first to synthesise cGMP, and it was shown by Drummond & Perrott-Yee (1961), that it was degraded in a similar fashion to cAMP, by enzymatic hydrolysis. Price's laboratory confirmed the identification of cGMP when they isolated it in rat urine (Ashman *et al.*, 1963). At this time, Sutherland's laboratory demonstrated that steroid, thyroid and pituitary hormones affected urine cGMP levels. Although at this time there was no proposal of direct production, it was suggested that thyroxine could not completely restore cGMP levels in hypophysectomised rats, demonstrating multiple stimulating factors (Hardman *et al.*, 1966). It was reported by Tsai *et al.* (1980), in sGC purified from rat liver that the sole products of catalysis of GTP are cGMP and pyrophosphate. Later analysis of pGC purified from sea urchin sperm showed that the α-phosphoanhydride bond is the site of cleavage during catalysis (Walseth *et al.*, 1981), and that it is a single displacement reaction (Senter *et al.*, 1983).

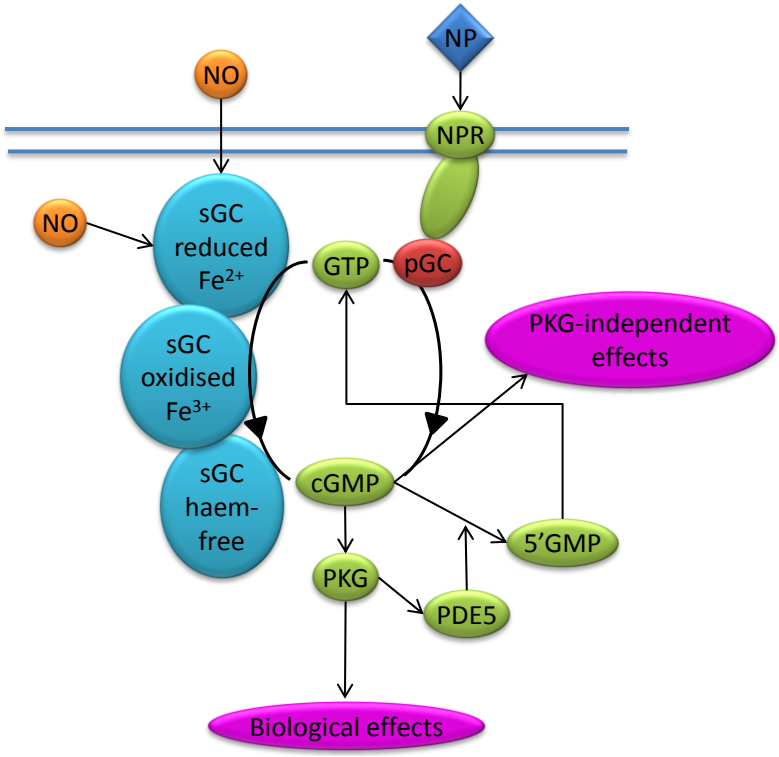

Figure 1.5 *Schematic representation of guanylyl cyclase mediators and effectors. Both pGC by NPR agonists and sGC by NO catalyse the conversion of GTP to cGMP, which can signal through PKG or via independent mechanisms. Note that it is proposed that sGC is present in three states, the reduced state which NO can stimulate to catalyse the conversion of GTP to cGMP, the oxidised and haem-free states which are insensitive to NO and presence is proposed to be increased under pathological conditions. PDE5 catalyses the breakdown of cGMP to 5'CMP, acting to regulate PKG cGMP/PKG signalling.*

1.26 cGMP localisation

Like much of our understanding of cGMP physiology, advances in our understanding of its cellular localisation developed based on experimental work investigating the same paradigm for cAMP. Compartmentation of cGMP was realised in the heart when Honda *et al.* (2001), and Sato *et al.* (2000), independently described genetically encoded, fluorescent cGMP indicators that could highlight cGMP intracellular localisation. Fischmeister and colleagues were employing a different technique to investigate diffusion of cAMP through frog ventricular myocytes using double-barrelled micro perfusion patch

clamping (Jurevicius & Fischmeister, 1996). This enabled them to stimulate one side of a cell with a receptor agonist, whilst monitoring its effects on the other side. They later employed the same technique for cGMP, documenting that application of a NO-donor to one side of a cardiac cell effectively blocked β-AR stimulation of L-type calcium channel current but had little effect on the other side of the cell. They interpreted these results to suggest that diffusion of cGMP signalling is limited (Dittrich *et al.*, 2001).

Localised actions of cGMP are not just as result of where an effector has acted. Whether cGMP is produced by pGC or sGC has been investigated and has been shown to play a role in spatiotemporal localisation of cGMP. Work by Zolle *et al.* (2000), and Hart *et al.* (2001), explored the responsiveness to congestive heart failure, examining the effects of calcium handling and β-adrenergic responsiveness respectively. BNP stimulation of pGC leads to inhibition of calcium efflux by the plasma membrane calcium ATPase pump and a reduction in cation influx (Zolle *et al.*, 2000). In contrast, activation of sGC did not alter calcium handling, but did cause increased uptake of calcium into the sarcoplasmic reticulum (Zolle *et al.*, 2000). In another model exploring calcium transients, Su *et al.* (2005), used mouse ventricular myocytes to investigate myocyte shortening after stimulation with either CNP or the NO donor SNAP. They reported that percentage shortening of myocytes was similar in both settings, however observed that CNP significantly reduced the amplitude of calcium transients, whereas SNAP had minimal effects. Further studies supporting the differential effects of cGMP produced by different GCs comes from work carried out in human umbilical vein endothelial cells (HUVEC). It was reported that in endothelial cells incubated with hydrogen peroxide, stimulation with

CNP had moderate relaxing effects, compared to NO which afforded much greater relaxation (Rivero-Vilches *et al.*, 2003).

1.27 cGMP dependent protein kinase

Evidence now supports differential effects of cGMP, determined by subcellular localisation and production. Like cAMP, cGMP also mediates its effects through a cyclase-dependent protein kinase. After initially identifying a partially purified protein kinase in lobster tail that was activated by both cAMP and cGMP (Kuo & Greengard, 1969), Kuo and Greengard later chromatographically separated the two activities and for the first time demonstrated that there is a separate cGMP-regulated protein kinase, cGMP-dependent protein kinase (PKG1/cGKI1) only weakly activated by cAMP (Kuo & Greengard, 1970). Following its identification, purification was necessary to understand and explore its actions. PKG activity was demonstrated in various tissues particularly rat brain cerebellum (Hofmann & Sold, 1972). The first full length sequence of PKG was reported by Takio *et al.* (1984), which facilitated the identification of two splice variants of PKG, 1α and 1β. They differ only at their N-termini (Wernet *et al.*, 1989; Wolfe *et al.*, 1989; Wolfe *et al.*, 1987) and are encoded in mammals by the *prkg 1* gene (Sandberg *et al.*, 1989; Wernet *et al.*, 1989). A second, novel, membrane associated kinase was proposed by de Jonge *et al.* (1981), and confirmed as cGMP-dependent protein kinase type II (PKGII/cGKII) by Uhler (1993), and Jarchau *et al. (1994)*, encoded by the *prkg 2* gene. PKG1 is found in high concentrations in many tissues, including smooth muscle, kidney, platelets and dorsal root ganglia and found in lower concentrations in cardiac muscle, vascular endothelium and osteoclasts (Feil *et al.*, 2005; Lochmann *et al.*, 1981). PKGIα is

found in lung, heart and cerebellum and Iβ is found in platelets and hippocampal neurons (Geiselhöringer *et al.*, 2004; Weber *et al.*, 2007). Both variants of PKG are composed of a regulatory domain and a catalytic domain, which is subdivided into the N-terminal, and cGMP biding domains. The binding domain contains the high affinity (cGMP I) and low affinity (cGMP II) binding pockets, which act allosterically. The catalytic domain contains the MgATP and peptide binding pockets. When cGMP binds to both cGMPI and cGMPII domains, inhibition of the catalytic centre is released allowing phosphorylation of serine/threonine residues in target proteins (Feil & Kemp-Harper, 2006; Francis & Corbin, 1999; Lucas *et al.*, 2000).

Determining which isozyme of PKG1 affords specific actions in the cardiovascular system has proven difficult and controversial. However recent transgenic mouse strains expressing either PKG1α or PKG1β only in all smooth muscles has suggested that previous *in vitro* studies may not translate into the *in vivo* setting. Weber *et al.* (2007), showed reconstitution of basic functions in both mouse lines. Smooth muscle function and early lethality were abolished when either isozyme was restored supported by calcium lowering and smooth muscle relaxation by both PKG1α and PKG1β.

1.28 cGMP dependent phosphodiesterases

Regulation of cGMP and its downstream effectors, as has already been elucidated in 1.25, is a complex if not sophisticated process. Phosphodiesterases (PDEs) are responsible for the hydrolysis of cGMP by insertion of a hydroxyl group into the phosphate ring, the product being an inactive 2nd messenger, 5'GMP. Conversely they can also act as downstream effectors of cGMP and to complicate things further, cGMP can modulate the

activity of several of the PDE family, by binding to a regulatory domain or phosphorylation which can both inhibit or stimulate the enzyme (Feil *et al.*, 2005). PDE activity was first reported in the heart. In the laboratory of Sutherland, they hypothesised that for cyclic nucleotides to have any real physiological purpose there must be a mechanism to remove them from the signalling cascade (Sutherland & Rall, 1958). In 1958, they reported that heart extract contained PDE activity that regulated cAMP action. They also reported that inhibitory action was blocked by methylxanthines such as caffeine (Butcher & Sutherland, 1962).

All mammals have at least 21 PDE genes encoding a superfamily of enzymes subdivided into 11 families (PDE1-PDE11) (Conti & Beavo, 2007). Although PDEs of different families contain reasonably high sequence homology at the catalytic region, several of the families are highly specific for cGMP and not cAMP and *vice versa*. PDEs 5 (Loughney *et al.*, 1998), 6 (Gillespie & Beavo, 1988), and 9 (Fisher *et al.*, 1998), are highly specific for cGMP, while 1 (Yan *et al.*, 1996), 2 (Martins *et al.*, 1982), 10 (Fujishige *et al.*, 1999), and 11 (Fawcett *et al.*, 2000), hydrolyse both cAMP and cGMP. All cell types contain PDEs, yet which families are expressed and when varies. Typically, different subcellular compartments will contain different subtypes depending on the developmental stage of the cell. Evidence suggests that the distribution and sub families of PDEs present in specific cell types changes during cell development, particularly during embryogenesis (Fischmeister *et al.*, 2005). The theory behind cGMP compartmentalisation stems from work investigating the distribution of different PDE subtypes.

1.29 Cyclic nucleotide-gated ion channels

Cyclic nucleotide-gated (CNG) ion channels are another cellular target of cGMP signalling. They are activated by both cAMP and cGMP binding to a cyclic nucleotide-binding domain (CNBD), and regulate the opening of several classes of cation channel. The passage of Na^+, K^+ and Ca^{2+} can be regulated by these channels (Armstrong & Bezanilla, 1973). They belong to the pore-loop cation channel family, being most extensively researched in the signal transduction of photoreceptors and olfactory neurons. There is evidence to suggest that the channels are present in other cell types such as brain, kidney and endocrine tissues, as well as the heart, however CNG KO experiments have provided little evidence to suggest a functional role of the channels outside of the sensory systems (Podda *et al.*, 2008).

1.30 sGC/pGC-cGMP-PKG signalling in cardiovascular disease

As has already been highlighted in 1.16 and 1.20, and later in Chapters 3, 4 and 5, signalling via cGMP and its downstream targets play crucial and diverse roles within the cardiovascular system. During ischaemia, production of nitric oxide is reported to increase, based on analysis of NOS activity. However during early reperfusion the reported burst in ROS is a target for NO and so availability of NO in the first few minutes is reduced (Zweier *et al.*, 2010). There has been extensive research carried out investigating cGMP production and hydrolysis during ischaemia-reperfusion, yet many of the reports are contradictory. Lochner *et al.* (1998), demonstrated increases in cGMP levels during index ischaemia in an isolated perfused rat heart model following IPC, compared to non IPC hearts. A less dramatic increase in cGMP levels was reported by

Depré *et al.* (1994), in a working rat heart model, where L-NAME abolished the cGMP elevation induced by ischaemia. Another group reported that there was no increase in cGMP levels during ischaemia, but there was an increase following perfusion of L-arginine 10 minutes prior to ischaemia (Maulik *et al.*, 1995). Further studies by Yamaguchi *et al.* (1997), reported a decrease in cGMP levels following ischaemia reperfusion. One explanation for the reported differences in cGMP levels during ischaemia and reperfusion in isolated heart models would be the specific timing of the measurements. To date there have been no reports of a time profile of cGMP production during an ischaemia-reperfusion protocol, therefore uniform production or hydrolysis cannot be assumed. Both Agulló *et al. (2003)*, and Geisbuhler *et al*. (1996), report that maintaing physiological cardiomyocyte cellular pH is crucial for the successful production of cGMP by NPR or sGC stimulation, however cardiomyocyte pH fluctuates during ischaemia and reperfusion which may lead to similar changes in intracellular cGMP concentrations.

1.31 Cardioprotection afforded by downstream targets of PKG

Cellular targets of PKG are both numerous and diverse. Within the cardiovascular system many substrates have been identified including, but not exhaustively, several L-type Ca^{2+} channel subunits, K_{ATP} channels, inositol triphosphate receptors, large conductance Ca^{2+}-activated K^+ channels, Na^+/Ca^{2+} ATPase, PDE5, phospholamban (PLB), ryanodine receptors, thromboxane A_2 receptor and vasodilator-stimulated phosphoprotein. It is generally accepted that signalling through cGMP and activation of PKG contributes to cell signalling cascades that afford cardioprotection in both IPC and ischaemia-reperfusion postconditioning paradigms. If one thinks of the mechanisms of cell death and the end

effectors involved in triggering this in cardiomyocytes, it is clear that PKG signalling plays an important role (Buja & Vela, 2008; Lucas *et al.*, 2000).

Hypercontracture occurs because of calcium overload and altered calcium handling, which, as mentioned previously, is a consequence of ischaemia-reperfusion injury (Inserte *et al.*, 2002). Calcium oscillations occur as a result of altered calcium handling when Ca^{2+} moves back and forwards between the cytosol and intracellular stores (Piper *et al.*, 2004). Schäfer *et al.* (2001), proposed that Ca^{2+} oscillations caused by reverse mode of the Na^{+}/Ca^{2+} exchanger during early reperfusion played a causal role in hypercontracture. It was later reported that cGMP-mediated stimuli enhance the clearing of Ca^{2+} during early reperfusion in the cytosol, reducing Ca^{2+} oscillations and hence reducing myocyte death (Abdallah *et al.*, 2005). They conclude that it is unlikely that calcium transients are due to efflux from the Na^{+}/ Ca^{2+} exchanger as they showed that cGMP signalling had no effect on the main Ca^{2+} extruder. They proposed instead that Ca^{2+} is sequestered to the SR via the Ca^{2+} ATPase. This Ca^{2+} uptake was abolished in the presence of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin during reoxygenation (Abdallah *et al.*, 2005).

Recent studies have suggested a role for PKG mediated protection during ischaemia-reperfusion by inhibiting the mPTP and cytochrome *c* release, limiting ischaemia-induced necrosis. Borutaite *et al.* (2009), showed that perfusion of an NO donor prior to index ischaemia preserved the structural integrity and respiratory function of mitochondria, preventing leakage of cytochrome *c,* which was abolished in the presence of PKG inhibition. In similar studies exploring PKG mediated cardioprotection, Costa *et*

al. (2006), reported that PKG mediates opening of mitochondrial K_{ATP} channels which has been demonstrated to afford cardioprotection in numerous models. Using purified PKG, they were able to elicit opening of the K_{ATP} channel comparably to the pharmacological channel openers, chromakalin and diazoxide. The PKG inhibitor KT-5823 and PKC inhibitor RO318220 blocked the channel opening. They concluded that a PKCε constitutively expressed in the mitochondrial inner membrane by indirect activation via PKG, opens the mitochondrial K_{ATP} channel. These observations were the basis of later work by Borutaite *et al*. (2009), who tested whether their previous observations relating to cytochrome *c* release via NO were related to mK_{ATP} channel opening. Using the K_{ATP} channel inhibitor 5-hydroxy decanoate (5-HD) for 15 minutes prior to perfusion of DETA-NO, they could not abrogate the NO-induced protection against ischaemia induced cytochrome *c* release. These results oppose those of Qin *et al.* (2004), who reported that inhibition of the K_{ATP} channel blocks NO-induced protection against ischaemia-reperfusion-induced necrosis.

Costa *et al.* (2006), proposed that there were two PKCε-subtypes involved in the distal signalling of cardioprotection. PKCε1 is proposed to regulate mK_{ATP} channel opening and PKCε2 is suggested to negatively regulate mPTP opening. They propose that the same signalling pathway mediates protection afforded by both mK_{ATP} channel opening and mPTP inhibition.

Opening of the mK_{ATP} via inotropic stimuli ensures efficient energy transfer from the mitochondria to the cytosol under conditions of stress such as ischaemia. The opening of the channel inhibits the onset of mPTP formation and contributes to inhibition of necrosis

(Bopassa *et al.*, 2006). It is prevention of mPTP formation that is believed to be one of the major end effectors of the cardioprotective paradigm. Although the mPTP is now widely accepted as being a major therapeutic target in cardioprotection, the molecular mechanism and composition of mPTP remains unclear. Several proteins have been implicated as components of the pore including adenine nucleotide translocase, the mitochondrial phosphate carrier and CYPD (Halestrap, 2009). CYPD's role has been explored extensively and following work by Crompton *et al.* (1988), who showed that CSA blocked mPTP opening, Halestrap *et al.* (1990), showed that the blockade was mediated by inhibition of a protein they later identified as CYPD. Yellon's group later went on to show that CYPD was a critical component of mPTP formation, by using a CYPD KO mouse model which was maximally protected against ischaemia-reperfusion injury (Lim *et al.*, 2007a; Lim *et al.*, 2007b).

Although there is no evidence to support direct inhibition of mPTP by PKG, recent cell based studies by Chanoit *et al.* (2011), suggest that inhibition of PDEs prevents mPTP opening by inactivating GSK3β through both PKA and PKG. This is in disagreement with Downey's group who suggest that, in an isolated rabbit heart model, the protection afforded by a GSK3β inhibitor was not abolished by L-NAME or sGC inhibitor ODQ, suggesting GSK3β mediated protection is downstream or independent of NO (Cohen *et al.*, 2010). Further evidence to support a major role for PKG mediated postconditioning is documented by Inserte *et al.* (2011), who report that PKG contributes to postconditioning protection in part by delaying normalisation of pHi during reperfusion; the authors propose PKG-mediated inhibition of the Na^+/H^+ exchanger as the probable mechanism.

1.32 PKG regulation of cell death

PKG's role in apoptotic cell death remains unclear. There are numerous studies that report both pro and anti apoptotic effects of PKG, triggered by NPs and NO. The difficulty in reviewing the literature and making any general statements favouring one mechanism or another is due to the diverse range of methodologies and cell types used to assess the actions of PKG. Both NO and ANP have been shown to induce apoptosis in rat vascular endothelial cells in a concentration dependent manner. In rat neonatal cardiomyocytes, Shimojo *et al.* (1999), demonstrated the pro-apoptotic effect of NO/cGMP/PKG pathway which was supported by other groups using rat cardiomyocytes (Rabkin & Kong, 2000; Taimor *et al.*, 2000; Uchiyama *et al.*, 2002). Using a model of simulated ischaemia, the laboratory of Kukreja demonstrated that PKG1α attenuates apoptosis following ischaemia/reoxygenation. Adult rat cardiomyocytes infected with adenoviral vectors containing hPKG1α were 66 % less likely to undergo apoptosis than control cells (Das *et al.*, 2006). This supports a previous study that demonstrated that SNAP inhibited cardiomyocytes apoptosis by regulating cyclin A-associated kinase activity (Maejima *et al.*, 2003). In an unrelated model investigating apoptosis in neuronal cells, Johlfs and Fiscus (2010), reported that the apoptosis regulating protein Bad is phosphorylated at serine 155 directly by PKG. The authors showed that inhibition of cGMP/PKG by ODQ decreased serine 155 phosphorylation of Bad in N1E-115 cells, resulting in increased apoptosis. From the above evidence, it is clear that the mechanisms by which PKG may potentiate or attenuate apoptosis are complex and tissue specific, yet all evidence is consistent in reporting that PKG plays a role in Bad/Bcl-2 expression.

1.33 Cardioprotection afforded by phosphodiesterase inhibitors

As described above, PDEs facilitate the major pathway of cGMP degradation, and so PDE inhibition is a target for cardioprotection. In the myocardium, PDE5 has been described as the most significant of the PDEs (Takimoto *et al.*, 2005). Early studies using the selective PDE5 inhibitor zaprinast suggested that PDE inhibition abrogated ischaemia-reperfusion induced ventricular fibrillation and contractile failure (Pabla *et al.*, 1995). Following the success of sildenafil as a specific PDE5 inhibitor, a surge in the literature for its use in cardiovascular disease and particularly ischaemia-reperfusion injury has demonstrated that it limits infarct size *in vivo* (Bremer *et al.*, 2005; Das *et al.*, 2008; Kukreja, 2006; Ockaili *et al.*, 2002; Rosanio *et al.*, 2006; Vidavalur *et al.*, 2009) and comparable results were obtained with tadalafil (Sesti *et al.*, 2006). The mechanism by which PDE5 inhibition reduces infarct size has been proposed by Kukreja's laboratory. They reported that sildenafil and vardenafil mediated infarct reduction in *ex vivo* rabbit hearts was abolished by the mK_{ATP} channel inhibitor 5-HD (Salloum *et al.*, 2007). In a more recent study, they report a novel pathway whereby PKG phosphorylates both ERK 1/2 and GSK3β in conjunction with an increase in the Bcl-2/Bax ratio that culminates in mK_{ATP} channel inhibition. The evidence proposing a PKG dependent phosphorylation of GSK3β is of interest as previous studies had suggested that cardioprotection through inhibition of GSK3β was mediated by an Akt dependent phosphorylation (Badorff *et al.*, 2002; Cross *et al.*, 1995).

1.34 Conclusions

Reviewing the evidence provided above strongly suggests that there is a need to further explore the endogenous and exogenous cardioprotective mechanisms and develop adjunct therapies to support thrombolysis and PPCI. Up until now, there is increasing evidence to suggest that cGMP is a key mediator in cardioprotective signalling. There is also evidence to suggest that this protection is in part afforded by distal targets of PKG. Elevation of cGMP via activation of pGC has been explored considerably in recent years. Cardioprotection afforded by NO mediated sGC targeting has also been well documented, as well as the undesirable tolerance associated with NO donor therapeutics. Experimental evidence discussed in this chapter along with the results of experiments documented in the following chapters strongly supports the notion that GC/cGMP plays a crucial role in survival signalling during ischaemia-reperfusion. Hence, pharmacological targeting of this pathway may have therapeutic potential.

1.35 Overview of experimental chapters

The scope of this thesis has been limited to pharmacological targeting of ischaemia-reperfusion injury in the myocardium, particularly targeting sGC with NO donors and NO-independent stimulators/activators. The method chosen to simulate ischaemia-reperfusion was the isolated perfused rat heart according to Langendorff, subjecting hearts to regional ischaemia by occluding the left descending coronary artery (LDCA) as described in Chapter 2. Two main pharmacological tools were chosen to manipulate sGC, BAY 41-2272, a sGC stimulator and BAY 60-2770, a sGC activator. To assess the ability of interventions to afford protection, infarct size was determined using the well-characterised tetrazolium staining technique again described in Chapter 2. Further methods used include radio-immunosorbent assay (RIA) to quantify the amount of cGMP present in myocardial tissue samples and to determine whether pharmacological intervention modified the amount of cGMP produced. Ozone based chemiluminescence (OBC) was employed to measure NOx in samples to ascertain efficacy of pharmacological inhibition of NO. SDS-polyacylamide gel electrophoresis was used to investigate the expression of proteins proposed to be downstream of the NPR in cardioprotective signalling.

1.36 Questions addressed in this thesis

i. Can cardioprotection be afforded by exogenous stimulation/activation of sGC?

ii. Can sGC stimulation afford protection independently of NO?

iii. Can greater protection be afforded by targeting different redox states of sGC?

1.37 General hypothesis

This thesis tests the hypothesis that pharmacological stimulation/activation of sGC limits myocardial reperfusion injury (see Figure 1.6).

1.38 Rationale for choice of pharmacological agents used in this thesis (Figure 1.6)

BAY 41-2272 was chosen as the pharmacological stimulator of sGC as it has been well characterised in rat tissues and was commercially available. It specifically targets the reduced (Fe^{2+}) state of sGC and its action is independent of NO.

ODQ is a selective inhibitor of sGC, oxidising the haem group of the protein rendering sGC insensitive to NO. ODQ was chosen as an inhibitor of cGMP production.

L-NAME was selected as an inhibitor of NOS.L-NAME was therefore used to prevent the production of NO from NOS and so limiting concentrations of the endogenous ligand of sGC.

C-PTIO is a NO scavenger and was chosen to investigate the NO independent action of BAY 41-2272. C-PTIO's ability to scavenge NO ensured that NO produced independently of NOS was not able to bind to sGC.

NOC-9 is an NO donor, which was used to investigate the infarct limiting properties of concomitant sGC stimulator and NO donor perfusion. NOC-9 is a member of the diazeniumdiolates (NONOates).

BAY 60-2770 is a sGC activator, which when bound to sGC increases cGMP production. It specifically targets sGC in the oxidised (Fe^{3+}) and haem-free states. BAY 60-2770 was

chosen to target these so called pathological states of sGC, proposed to be increased during ischaemia.

BNP was used to activate the membrane bound (particulate) guanylyl cyclase receptor. BNP was chosen as we have previously extensively investigated BNP in the ischaemia-reperfusion setting determining that 10 nM BNP limits infarct size when perfused during early reperfusion.

Wortmannin is a specific inhibitor of PI3K/Akt signalling and was used to investigate the downstream signalling of BNP during early reperfusion.

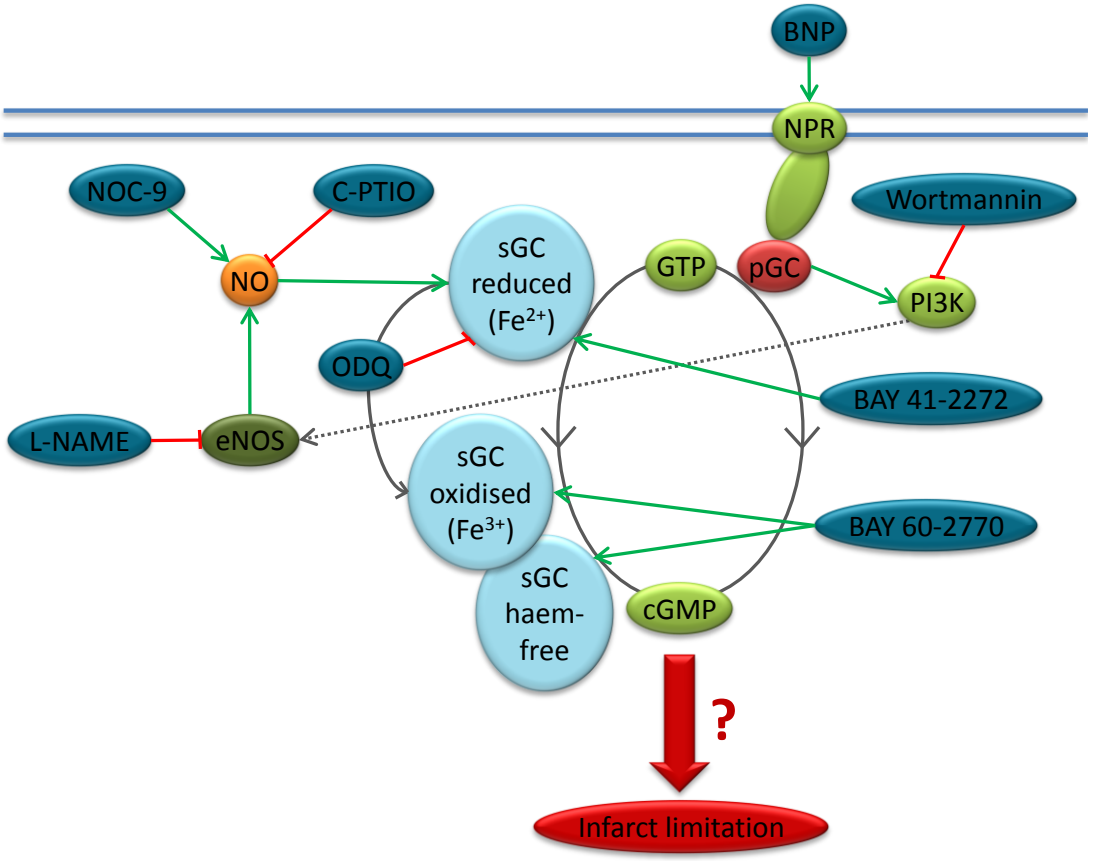

Figure 1.6 *Illustrates the pharmacological tools used in this thesis. Green arrows highlight agonistic action and red lines highlight antagonistic action or inhibition. NO action was manipulated by inhibiting its production with L-NAME, scavenging it using C-PTIO or producing it using NOC-9, an NO donor. ODQ was used to oxidise sGC from its Fe^{2+} NO-sensitive state to the Fe^{3+} NO insensitive state. The NPR ligand BNP was used to stimulate production of pGC mediated cGMP and Wortmannin was used to block signalling through the proposed PI3K/eNOS pathway (dotted grey line). BAY 41-2272 stimulated cGMP production through reduced sGC and BAY 60-2770 activated the oxidised and haem-free sGC to produce cGMP. All pharmacological interventions are proposed to act upstream of cGMP and ultimately mediate infarct limitation.*

Chapter 2
General Methods

2 Materials and Methods

2.1 The isolated perfused heart according to Oscar Langendorff

Isolated heart perfusion has been a highly valued technique for studying numerous physiological and pathophysiological aspects of the myocardium for more than a century. Oscar Langendorff first reported his method of studying the mechanics of the completely isolated mammalian heart in 1895 (Langendorff, 1895). The basic principles of the method were to create a preparation whereby the heart could be studied in an *ex* vivo setting, whilst perfusing the organ and maintaining basic cardiodynamic function. Perfusate, whether it be whole blood or other physiological fluid such as Krebs' buffer is forced towards the heart through a cannula inserted in the ascending aorta. The pressure of the perfusate flowing through the ascending aorta in a retrograde direction (reverse to that *in vivo*) causes the aortic valve to close, diverting the perfusate through the coronary arteries. The direction of flow then follows that of blood *in vivo*, through the arterioles, capillaries, the venous system and finally through the coronary sinus into the right atrium. Unlike the *in vivo* heart the cardiac cavities remain basically empty throughout the experiment (Doring & Dehnert, 1988) (Figure 2.1).

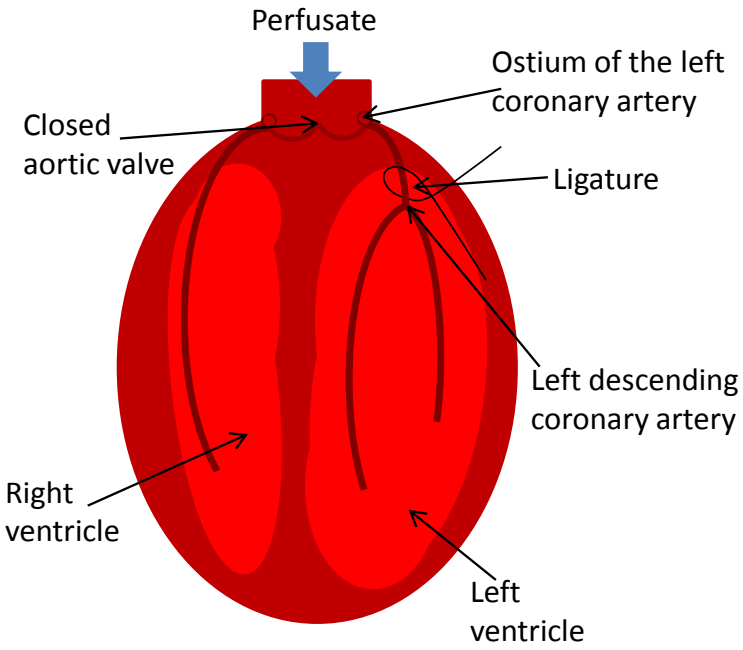

Figure 2.1 *Diagram of the myocardium highlighting the retrograde perfusion of Krebs Henseleit buffer through the coronary ostia. Also shown is the placement of a ligature to occlude the LDCA.*

The fact that the Langendorff perfused heart preparation has been employed as the method of choice when studying many diverse myocardial parameters is a testament to its simplicity and utility. With the increasing number of genetic knock-out and knock-in animals available, the Langendorff method allows for the characterisation of phenotype, investigation of complex signalling processes, pharmacological intervention and isolation of myocytes for primary culture. It does, however, have to be acknowledged that the isolated perfused heart is a deteriorating preparation; work by Sutherland and Hearse (2000) using a mouse model and others since have reported a decrease in contractile and chronotropic function of between 5 and 10 % per hour under control conditions. Whilst important to note, suitable experimental protocols can be designed within these parameters.

There are two methods widely described to perfuse the isolated heart in the Langendorff model. Both constant pressure and constant flow systems have been used; each offers advantages and disadvantages. Constant pressure perfusion allows the accommodation of the heart's natural regulation of coronary tone and can be achieved by maintaining a constant hydrostatic pressure between the meniscus of the buffered perfusate and the tip of the cannula attached to the ascending aorta (Doring & Dehnert, 1988). Constant pressure can be achieved reasonably inexpensively using standard laboratory glassware and tubing or using a pump system with pressure feedback control. The latter is typically the set up of the commercially available perfusion systems which have the advantage over the hydrostatic systems of reducing dead space which, when perfusing pharmacological agents, can be an important cost consideration. The constant flow system allows the perfusion apparatus to be much more compact, again removing dead space and is the method of choice if vasoactive parameters and resistance are of interest (Bell *et al.*). However, a constant pressure system overrides the auto-regulatory functions of the heart and therefore there is no feedback on the flow demands of the heart when vascular interventions are performed, such as occlusion of a major vessel in an ischaemia-reperfusion protocol. This can cause shear stress and damage to the vasculature in this setting (Sutherland & Hearse, 2000).

The longevity of the Langendorff perfused heart method may also be attributed to the wide variety of species that can be used. For the most part, the rat is the species of choice. The isolated rat heart has been experimentally characterised, especially with respect to ischaemia-reperfusion studies. However for experiments where genetically modified

animals are needed, then the mouse heart has been extensively used. In early work, the canine heart was reported as well as the rabbit. Practical considerations need to be reviewed when selecting animal models, particularly the economics of perfusing larger animal hearts, especially when pharmacological agents are to be used. The anatomy of the heart must also be considered. The guinea-pig heart has been Langendorff perfused but for regional ischaemia models is not suitable because of its extensive native collateral circulation; it is more suited for global ischaemia studies. The pig isolated heart is still used in some laboratories, for transplant and stem cell studies due to the continuing exploration of the similarities between the swine and human hearts.

2.2 Animals

Male Sprague Dawley rats sourced from B&K Universal Ltd. (Bristol, UK), Harlan UK Ltd (Oxfordshire, UK) and Charles River Laboratories Inc. (Maidenhead, UK) were selected for use in this thesis. Rats were housed in the institutional animal house and allowed to acclimatise for 7 days. The care and use of animals was in accordance with the Animals (Scientific Procedures) Act 1986 (The Stationery Office, London, UK). Both water and food were available *ad libitum*. Food pellets contained 4 % fat and 18 % protein. Animals were exposed to 12 h on, 12 h off light cycles.

2.3 Langendorff heart perfusion

Group sizes were determined based on historical data from our laboratory and others that suggests that n=5-6 is sufficient to resolve statistical differences between groups. Animals were used at 300-400g body weight. The animals were placed under surgical anaesthesia using pentobarbital sodium (175mg/kg) with heparin (200 units) given concomitantly by

intraperitoneal injection. Once animals were unconscious and surgical plane anaesthesia had been reached, (characterised in part by a lack of pedal reflex), the animal was placed in the supine position, a sagittal incision was made ventrally exposing the xiphoid process and diaphragm. The thoracic cavity was then entered by bilateral caudal-cranial dissection of the rib-cage. The ribcage was then reflected cranially to expose the thoracic cavity containing the heart. Excision of the heart was then performed by cradling the heart gently between the thumb and fore-finger and cutting above the heart ensuring a sufficient length of ascending aorta was also removed. The heart was immediately placed in ice cold modified Krebs Henseleit (KH) buffer, NaCl 118.5 mM, $NaHCO_3$ 24.8 mM, d-Glucose 11 mM, KCl 4.7 mM, $MgSO_4.7H_2O$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2.2H_2O$ 1.3 mM aerated with 95 % O_2/5 % CO_2 (pH 7.3-7.5 at 37.0 °C).

The ascending aorta of the rat heart was cannulated with a metal cannula (Fisher Scientific) using mini surgical forceps, and initially held with a small metal clip. The heart was rotated so that the anterior surface of the heart was facing forward. At this point the heart was perfused only partially (half opened tap) with modified KH buffer to ensure that there was no leaking of perfusate whilst positioning the heart. Once the heart was positioned correctly it was secured with two braded silk ligatures, the metal clip was removed and the heart was perfused fully at a constant hydrostatic pressure of 74 mmHg (100 cm H_2O). Warming of KH buffer was achieved by warming the jacketed pre-warmers and Baker coil by a thermo-regulated circulator (see Figure 2.2). Actual heart temperature was monitored by a thermocouple probe positioned under the right atrium and maintained between 36.8-37.4 °C.

The left atrial appendage was removed to expose the atrial chamber. An 80 mm length of 3-0 suture material (Ethicon, UK) was placed through the myocardium to sit posterior to the LDCA at its origin, ensuring a margin of approximately 2 mm either side of the anterior LDCA was achieved on entrance and exit of the heart tissue.

A latex balloon (Harvard Apparatus, UK) attached to a polypropylene cannula was then inserted through the bicuspid valve into the left ventricle (LV) and held just above the apex (see Figure 2.3). The balloon was then filled with distilled water and the cannula was attached to a pressure transducer and Powerlab data acquisition system apparatus (AD instruments, Abington, UK). The left ventricular end diastolic pressure (LVEDP) was set between 5 and 10 mmHg. This allowed isovolumetric pressure measurements to be recorded continuously. Coronary flow rate (CFR) was measured by collecting the coronary effluent from the apex of the heart for 30 seconds and expressed in mL/min.

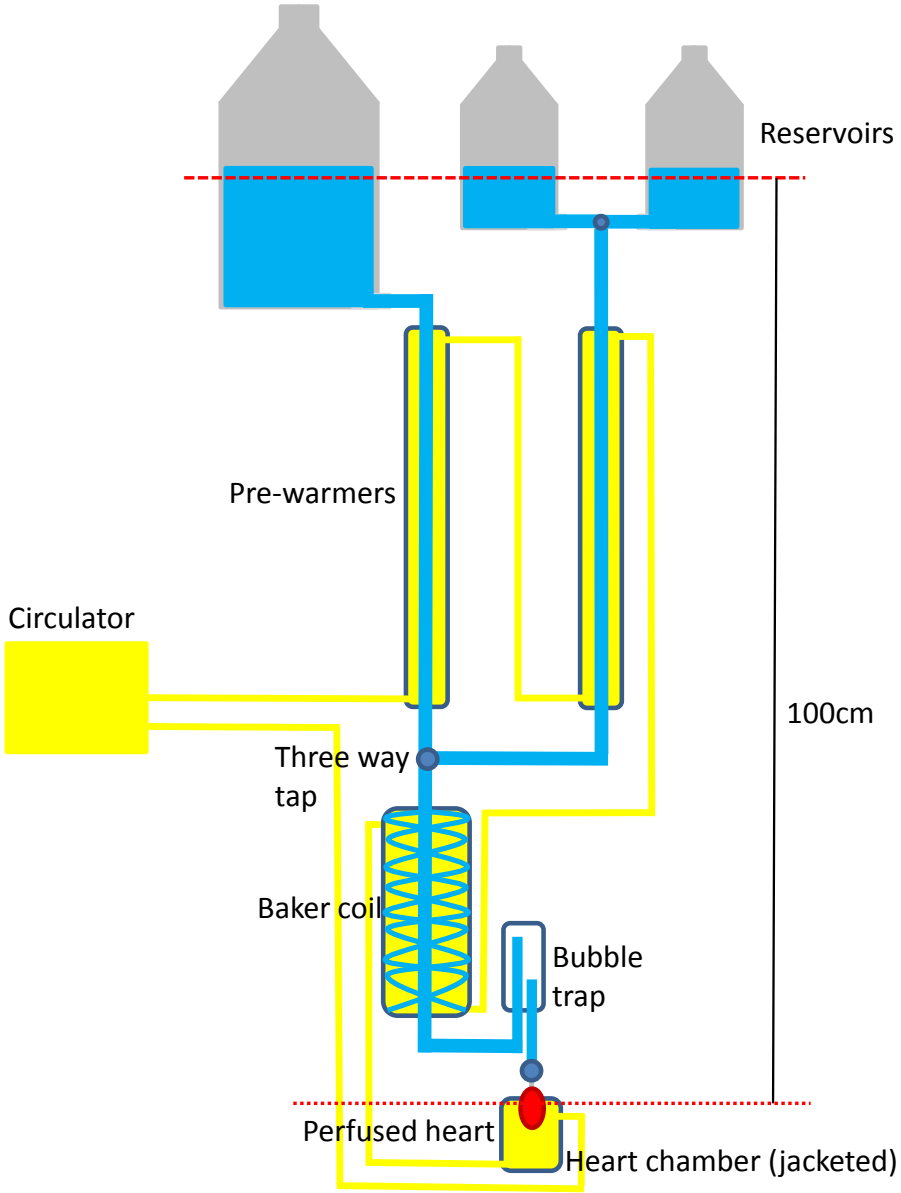

Figure 2.2 *Diagram representing the apparatus used for Isolated Heart perfusion experiments. Of importance is the difference in height between the perfused heart and the KH level in the reservoir. Yellow indicates glass wear that is jacketed and warmed to 37 ˚C by the heated circulator. The bubble trap ensures that any air in the system is captured and does not pass into the myocardium through the aorta.*

2.4 Stabilisation

A period of stabilisation was initiated to ensure that the haemodynamic functions of the heart met the inclusion criteria. These included a LVEDP of between 5 and 10 mmHg, a left ventricular developed pressure (LVDP) of at least 50 mmHg, a CFR between 10 and 24 mL/min, heart rate (HR) of 200-350 beats per minute (BPM) and a maintained temperature of 36.8-37.4 °C. During stabilisation these haemodynamic measurements were monitored.

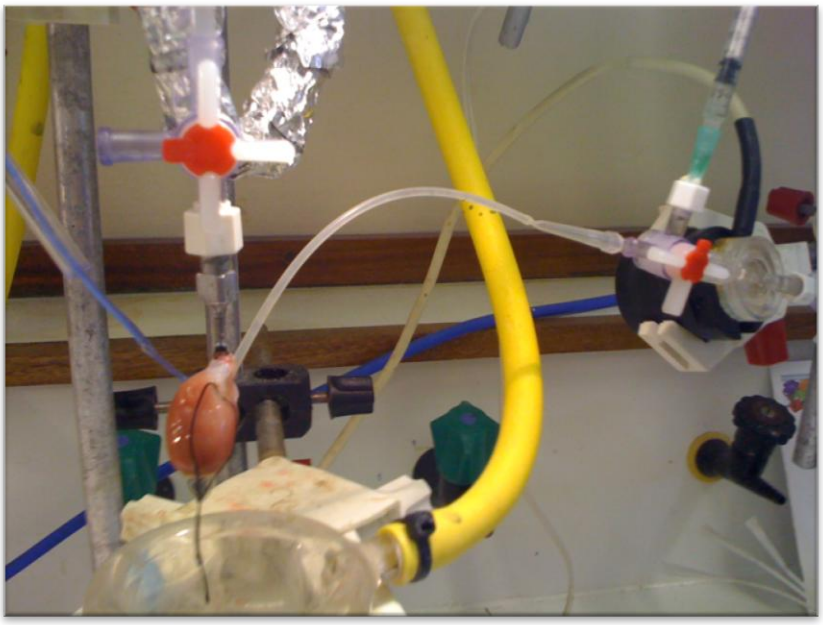

Figure 2.3 *Isolated rat heart with balloon inserted into the left ventricle and connected to a pressure transducer via a sealed water filled cannula.*

2.5 Induction of ischaemia

Regional ischaemia was induced following the stabilisation period by occluding the anterior LDCA by threading both ends of the silk suture though a shortened 200 µL

pipette tip. A second 200 µL pipette tip was placed inside the first creating a snare that could be tightened, occluding the LDCA with the surrounding tissue. Confirmation that the artery was sufficiently occluded was gained by a >30 % decrease in CFR. The heart was subjected to 35 min regional ischaemia and then reperfused for 120 min. Reperfusion was achieved by removing the pipette tip snare, ensuring that the silk suture was still in place, but not occluding the artery, confirmed by an increase in CFR towards baseline. 120 min of reperfusion allowed washout of reducing enzymes and co-factors from irreversibly damaged cells that would otherwise have interfered with staining the heart in the next part of the protocol.

- *2.6 Re-occluding and staining*

Following 120 min of reperfusion the LDCA was ligated with a surgeon's knot using the silk ligature that was already in place. Between 0.5 and 1.0 mL of Evans Blue (0.4 %), dye was then perfused through a side arm in the cannula tap into the heart, staining the non-risk zone (see Figure 2.4). The heart was then removed from the cannula and frozen at -20°C for 3-24 hours.

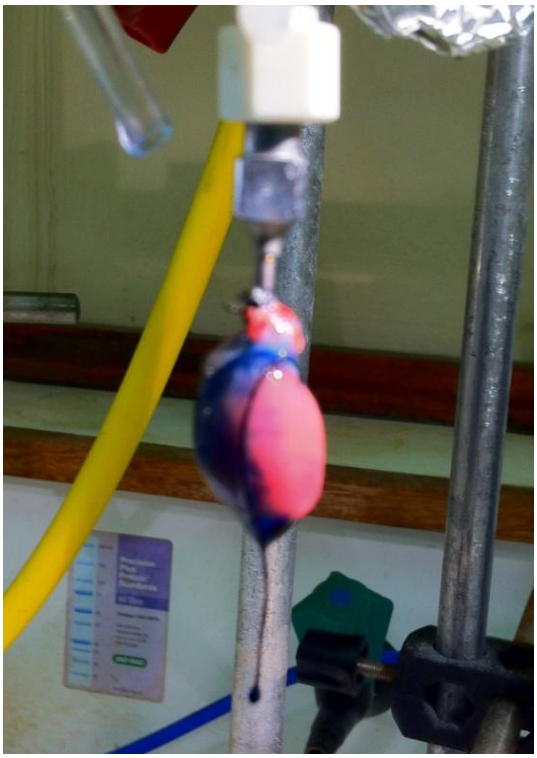

Figure 2.4 *Isolated rat heart, which has had the LDCA permanently occluded by tying the ligature with a surgeon's knot. The heart has been perfused with 0.4 % Evans Blue, which delineates non risk (blue) and risk zone (pink) tissue.*

The frozen heart was allowed to partially thaw for 2-3 min and then sectioned transversely from the apex into 2 mm thick sections. These sections were then thawed completely before being incubated in 1 % triphenyltetrazolium chloride (Sigma-Aldrich, UK) at 37.0 °C for 20 min, with frequent agitation. The red formazan pigment observed in the non-blue stained regions was produced by the reduction of the triphenyltetrazolium chloride by enzymes and the nicotinamide adenine dinucleotide phosphate (NADPH) cofactors in viable tissue. The stained sections were then fixed in 10 % formalin for 48 hours before being imaged from both sides of the cross section using computer imagery software ImageJ version 1.45q (National Institute of Health, USA) (Figure 2.5).

Planimetry was then performed on each image using a graphics tablet (Trust, USA), measuring the total area, risk zone and infarct size of each section. Planimetry was performed blindly and the recorded values were converted into volumes and combined to give total heart area at risk and infarct size as a percentage of the risk zone.

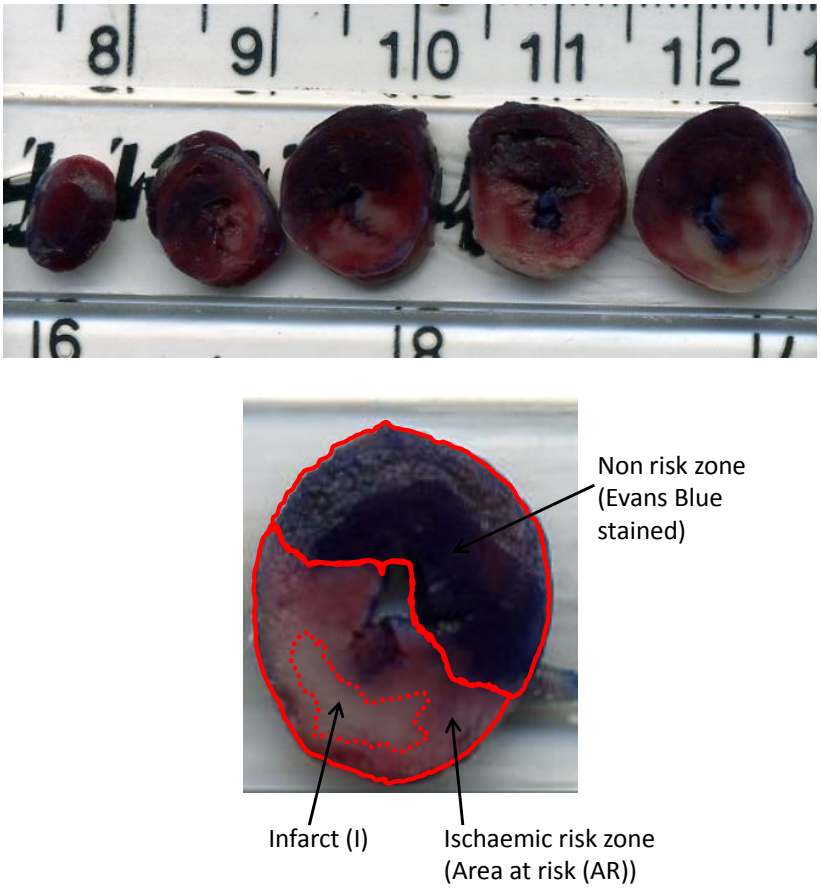

Figure 2.5 *Scanned image of heart sections following tetrazolium staining and 48 hours fixation in formalin. Blue colouring delineates tissue not subjected to ischaemia. Red coloured tissue delineates tissue subjected to ischaemia but not infarcted. White colouring highlights infarcted tissue. The sum of red and white tissue equals the risk zone.*

2.7 Statistical analysis

Experimental data were analysed using Prism 5.0. Data are expressed as mean ± standard error of the mean (SEM). Normality testing was performed prior to subsequent analysis. All data sets were found to be normally distributed, confirmed by the Kolmogorov-Smirnov test. ONE-way ANOVA was used when stated to analyse the arithmetic means followed by Newman-Keuls *post-hoc* test when significance was reported. This was used to compare arithmetic means for raw data corresponding to specific treatment groups. Cardiodynamic data including HR, LVDP and rate pressure product (RPP, heart rate multiplied by LVDP) were analysed using repeated measures ANOVA followed by Newman-Keuls *post-hoc* test. Values were considered statistically significant if $p<0.05$.

Chapter 3
NO-independent stimulation of sGC in the reperfused myocardium

3.1 Introduction

3.1.1 Direct targeting of sGC/cGMP/PKG

As described previously in Chapter 1, a series of kinases was identified to play a crucial role in postconditioning, later described as the RISK pathway (Hausenloy & Yellon, 2004). Targeting this pathway became the focus of many research groups. Agullo *et al. (1999)* reported that administration of L-arginine, a precursor of NO production, was protective, because it limited infarct size in the pig isolated heart; this suggests an NO mediated protection.

Extensive focused research on the components of the RISK pathway and how it can be targeted to modify reperfusion and ultimately limit infarct size have led to specific interest in cGMP and its downstream kinase PKG. cGMP has been shown to reduce contractility during reperfusion by inhibition of Na^+/Ca^{2+} exchange and activation of SERCA via PKG mediated phosphorylation of PLB. PKG has also been shown to activate BK_{Ca} channels which when inhibited at reperfusion abolish protection afforded by upstream targets of cGMP (Burley & Baxter, 2007).

D'Souza *et al.* (2003) reported that low concentrations of 8-Br-cGMP, a synthetic cGMP analogue given just prior to ischaemia through to early reperfusion limited infarct size in a isolated rat heart model. It was later shown that 8-Br-cGMP also showed infarct limiting properties when given at reperfusion, reducing infarct size by 40 % compared to control hearts (Giricz *et al.*, 2009). These data demonstrated that targeting cGMP/PKG directly could afford infarct limitation mediated by elevating cGMP.

The long history of the use of nitrates in the treatment of cardiovascular pathologies has been well documented (Ferdinandy & Schulz, 2003; Furchgott, 1995), with many of their protective effects being mediated through sGC-cGMP-PKG signalling. However, tolerance is a major problem when NO donors are used for any length of time (Csont *et al.*, 1998; Gori & Parker, 2002). Infarct limitation afforded by NO donors is discussed in detail in Chapter 4. The potential for targeting the RISK pathway downstream of NO is desirable, attempting to eliminate tolerance issues from future treatment protocols. Compounds that target sGC/cGMP/PKG independently of NO have become an attractive option. The rationale is that the beneficial actions of NO such as platelet aggregation and vasodilatation are almost exclusively mediated through sGC/cGMP, whilst the undesirable effects such as tyrosine nitration and DNA damage are predominantly cGMP-independent.

3.1.2 NO-independent haem-dependent stimulators of sGC

Following the discovery of several light enhanced compounds reported to stimulate production of cGMP, Ko *et al.* (1994) described a structurally related indazole derivative, 5-[1-(phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol (YC-1), which unlike the earlier compounds has light-independent pharmacokinetics. YC-1 was later described as the first NO-independent, haem-dependent stimulator of sGC (Friebe *et al.*, 1996). This discovery prompted scientists at Bayer Pharma AG (Wuppertal, Germany) to run a screening programme in the late 1990's with the intention of further developing NO-independent, haem-dependent stimulators of sGC (Straub *et al.*, 2001). Using porcine endothelial cells, they screened over 20,000 potential compounds that could stimulate production of

cGMP, independently of NO. As a result, structurally related compounds were discovered that stimulate sGC by similar mechanisms, including 3-(4-amino-5-cyclopropylpyrimidine-2-yl)-1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-b]pyridine (BAY 41-2272) (see Figure 3.1) and Methyl N-[4,6-diamino-2-[1-[(2-fluorophenyl)methyl]-1H-pyrazolo[3,4-b]pyridin-3-yl]-5-pyrimidinyl]-N-methyl-carbaminate (BAY 63-2521/Riociguat) (Stasch *et al.*, 2001). The synthesis of these compounds was based on YC-1 as a lead structure and resulted in compounds that were more than two orders of magnitude more potent (Stasch *et al.*, 2001). The mechanisms by which these compounds stimulate the activation of sGC are still debated. It is known that, like NO, these stimulators require the presence of the prosthetic haem moiety (Friebe *et al.*, 1996). Koglin *et al.* (2002) have identified specific areas of the beta subunit that are required for enzymatic activation of BAY 41-2272. Stasch *et al.* (2002a) used photo affinity labelling to identify two cysteine residues on the alpha subunit were also needed for enzymatic activation. The lack of crystal structure of either of the sub-units has made it difficult to confirm any activation theories, with further studies involving the mutation of specific residues on the alpha sub-unit failing to inhibit enzymatic activity (Schmidt *et al.*, 2004). Our understanding of the NO-sGC-cGMP signalling pathway and the ability of these compounds to stimulate sGC led to experimental studies that report that they may be suitable compounds for the treatment of pulmonary hypertension. Evgenov *et al.* (2006) and Dumitrascu *et al.* (2006) reported that BAY 41-2272 caused a significant decrease in pulmonary arterial pressure and reversal of right ventricular hypertrophy in a mouse model. A pharmacologically-related compound BAY 63-2521 (Riociguat) is now being used in phase III clinical trials for the treatment of two major forms of pulmonary

hypertension, chronic thromboembolic pulmonary hypertension (CTEPH) and primary pulmonary arterial hypertension (PAH) (Mittendorf *et al.*, 2009). Other family members are still being used as pharmacological tools to further elucidate the complexity of the sGC/cGMP signal transduction pathway.

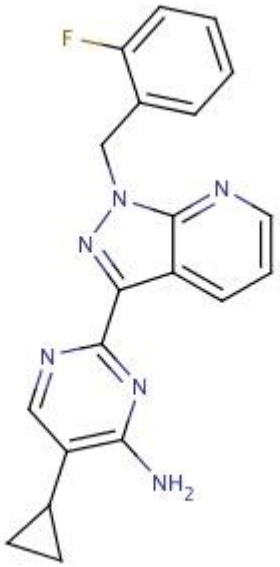

Figure 3.1 *Chemical structure of 3-(4-Amino-5-cyclopropylpyrimidine-2-yl)-1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-b]pyridine (BAY 41-2272) (Schmidt, P.M., Schmidt, H.H.H.W., Hofmann, F., Stasch, J.-P. (2009) Handbook of Experimental Pharmacology: cGMP: Generators, Effectors and Therapeutic Implications Vol. 191, pp 281 Springer Berlin Heidelberg).*

3.1.3 Hypotheses

The aim of this study was to investigate the effect of sGC stimulation at reperfusion by means of an NO-independent stimulator. The commercially available compound BAY 41-2272 was selected for these experiments.

Based on the work of others it was hypothesised that

i. Exogenous stimulation of sGC by BAY 41-2272 at reperfusion in the isolated rat heart would limit infarct size compared to controls;
ii. Oxidation of the haem site of sGC by ODQ would abrogate the protection afforded by BAY 41-2272;
iii. The infarct limitation would be at least in part as a result of elevated cGMP levels at reperfusion in the left ventricle of the myocardium.

The specific objectives were to:

1. Undertake a concentration response for BAY 41-2272 during early reperfusion in a rat isolated heart model

2. Concomitantly perfuse the haem site oxidiser ODQ with BAY 41-2272 during early reperfusion

3. Measure cGMP levels in LV and RV tissue samples from hearts perfused with BAY 41-2272

3.2 Materials and Methods

3.2.1 Pharmacological compounds

All salts used to make modified KH solution were sourced from Fisher Scientific LTD (UK) and were of analytical or ultrapure quality. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (selective sGC inhibitor) was sourced from Tocris Bioscience (UK) and BAY 41-2272 (NO-independent, haem-dependent sGC stimulator) from Sigma-Aldrich (UK). ODQ and BAY 41-2272 were dissolved in dimethyl sulphoxide (DMSO), the final concentrations of DMSO in the KH was 0.04 % v/v and 0.05 % v/v respectively.

3.2.2 Rat isolated heart perfusion

Animals used in this chapter were sourced from B&K Universal Ltd. (Bristol, UK). Rat isolated hearts were retrograde perfused as described in Chapter 2. Once a period of 20 min haemodynamic stabilisation had been established, rat hearts were randomised and assigned to one of the groups described in 3.2.3 and 3.2.4. All hearts were subjected to 35 min LDCA occlusion followed by 120 min of reperfusion.

3.2.3 Concentration response to BAY 41-2272: Study 1

This study was undertaken to assess the infarct limiting properties of the sGC stimulator BAY 41-2272. A concentration response to BAY 41-2272 was carried out by perfusing hearts with BAY 41-2272 100 nM - 3 μM at reperfusion (see Figure 3.2).

Group 1, Control, (n=17). This group includes some hearts that were perfused with the vehicle DMSO 0.05 % v/v from 30 min ischaemia until 10 min reperfusion. There was no statistical significant difference between control hearts perfused with or without DMSO vehicle and so all hearts were pooled for statistical analysis.

Group 2, BAY 41-2272 3 µM, (n=7). BAY 41-2272 3 µM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 3, BAY 41-2272 1 µM, (n=6). BAY 41-2272 1 µM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 4, BAY 41-2272 300 nM, (n=6). BAY 41-2272 300 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 5, BAY 41-2272 100 nM, (n=6). BAY 41-2272 100 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 6, ODQ 2 µM (n=6). ODQ 2 µM was perfused from 28 min ischaemia through until 10 min reperfusion.

Group 7, ODQ 2 µM + BAY 41-2272 3 µM, (n=7). ODQ 2 µM was perfused from 28 min ischaemia and BAY 41-2272 3 µM from 30 min ischaemia, both until 10 min reperfusion.

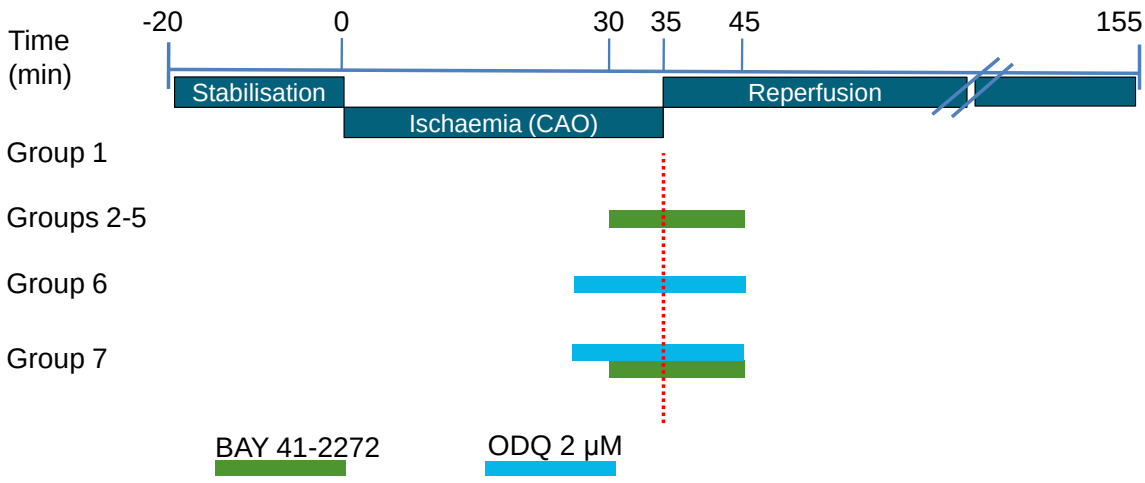

Figure 3.2 *Experimental protocol for groups 1-7 in Study 1. All hearts were stabilised for 20 min followed by 35 min regional ischaemia and then reperfused for 120 min. BAY 41-2272 was perfused at 1 µM, 3 µM, 300 or 100 nM.*

3.2.4 cGMP Radio immuno-sorbent assay: Study 2

RIA was performed to investigate further the pharmacological action of BAY 41-2272. A commercially available radioimmunoassay kit (IBL-Transatlantic Corp., Toronto, Canada) was used. The assay is based upon competition binding of the cyclic nucleotide with radiolabelled cyclic nucleotide derivatives for sites on antibody specific for the cyclic nucleotide (Steiner *et al.*, 1972). Tissue samples were prepared from rat hearts randomly assigned to one of 4 groups shown in Figure 3.4. Hearts that were subjected to regional ischaemia were treated as described for Study 1.

Hearts were removed from the cannula and rapidly sliced. Hearts were sectioned as described below to ensure that cGMP measurements were made on consistent samples that contained both infarcted and non-infarcted tissue as specifically targeting infarcted tissue is not possible without carrying out staining delineation. The apex was removed along with the base and the left and right ventricles were separated. The left ventricle was then cut caudo-cranially and each of these sections was then cut in the same way again. Without moving the sections around, pieces one and three (LV1) and two and four (LV2) were grouped. Each piece was further cut into 3-4 smaller pieces, blotted dry and immediately frozen in liquid nitrogen and stored at -80 °C until required for analysis (see Figure 3.3).

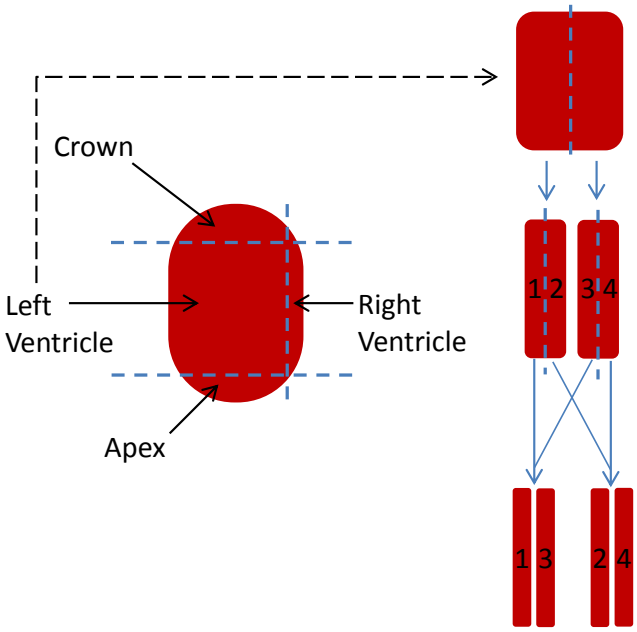

Figure 3.3 *Schematic illustrating preparation of myocardial tissue for cGMP RIA. The crown was removed and discarded. The apex was removed, cut into 3-4 pieces and snap frozen in liquid N_2 for later use. Similarly, the right ventricle was removed, cut into 3-4 pieces and snap frozen. The remaining left ventricle was cut in half and each half cut again as shown above. The four pieces were separated into two groups (pieces 1&3 and 2&4) and further cut in to 3-4 small pieces and snap frozen. Left ventricle RIA was performed using tissue from pieces 1&3.*

The following was performed by Yvonne Keim at Bayer Pharma AG, (Wuppertal, Germany). Samples were powdered under liquid N_2 using a mortar and pestle and were homogenised in lysis buffer with 0.1 M 3,7-Dihydro-1-methyl-3-(2-methylpropyl)1H-purine-2,6-dione (IBMX). Samples were then sonicated for 40 s and centrifuged for 60 min at 10,000 rpm. The supernatant was then transferred into new tubes with the addition of 400 µL trichloroacetic acid (TCA) 10 % and left for 30 min at 37 °C. Samples were then spun further for 10 min at 5,000 rpm. The supernatant was solvent cleaned 3 times with 700 µL diethyl ether, saturated in water. The aqueous phase was then dried in a speed vacuum. The pellet was then dissolved in 300 µL cGMP-RIA-buffer.

Standards (100 µL) and samples (20 µL) were pipette in duplicate into their respective tubes. Assay buffer (100 µL) was added to the non-specific binding (NSB), B_0 and sample tubes followed by 200 µL of NSB solution to the NSB tubes. ^{125}I-Tracer (100 µL) was added into each tube including two tubes for total activity.

Antiserum (200 µL) was pipette into all but the NSB and total activity tubes. All tubes were then vortexed and incubated for 24 h at 4 °C. After incubation, 1 mL of cool separation reagent was added to all but the total activity tubes and vortexed. All tubes were then centrifuged for 15 min at 2,500 rpm at 4 °C. Each of the tubes except those measuring total activity were then carefully decanted and drained onto blotting paper. Each of the tubes was then counted in a Gamma counter for 1 min. Using a standard curve plotted using the standards, cGMP values were directly calculated for each sample expressed as fmol/mg tissue. The following groups were analysed.

Group 1, Naive, (n=6). Hearts were excised and washed in KH to remove any blood and then sectioned.

Group 2, Stabilisation, (n=6). Hearts were excised and perfused under normoxic conditions for 20 min.

Group 3, 10 min reperfusion, (n=6). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min.

Group 4, 10 min reperfusion + BAY 41-2272 3 µM, (n=6). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BAY 41-2272 3 µM was perfused as described for Group 2 in 3.2.3.

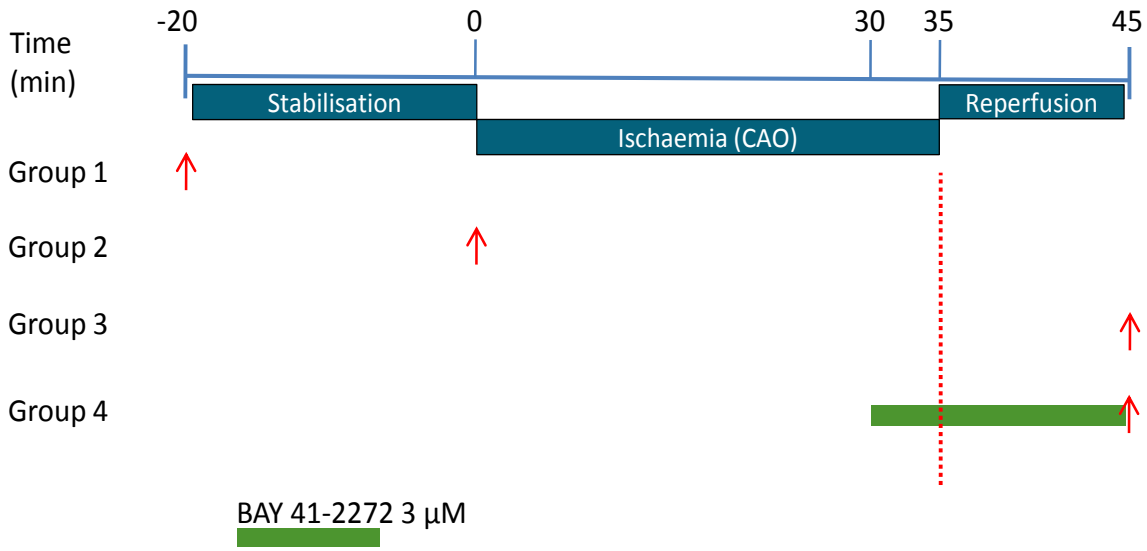

Figure 3.4 *Experimental protocol for hearts prepared for RIA Study 2. Red arrows indicate time points at which hearts were sampled.*

3.3 Results

3.3.1 Summary of experiments

In Study 1, 63 rats were used. Two animals died before their hearts were excised and five hearts were excluded from the study due to technical error, thus 56 completed experiments are reported. In Study 2, 24 rats were used for RIA, there were no exclusions and so data for 24 hearts are reported. The period of stabilisation before the onset of ischaemia was carried out to allow the hearts to stabilise and reach pre determined criteria (see below). For a heart to be included and subjected to ischaemia it had to achieve the following baseline cardiodynamic criteria:

CFR between 10 and 24 mL/min, LVEDP 5-10 mmHg, HR 200-350 BPM, LVDP greater than 50 mmHg and a steady sinus rhythm. Hearts were also excluded during analysis if there was inadequate delineation of the risk zone and infarcted tissue (poor staining).

3.3.2 Infarct size data: Study 1

Study 1 contains Langendorff perfusion experiments for the initial dose response to BAY 41-2272 and pharmacological inhibitor of sGC, ODQ. The area at risk for all hearts in all groups was 45 to 55 % of the combined left and right ventricular tissue. There were no statistical differences between groups for risk zone sizes. Infarct size was expressed as a percentage of the risk zone, calculated as described in Chapter 2 and reported in Figures 3.5 & 3.6. Under control conditions (35 min ischaemia followed by 120 min reperfusion) hearts had infarct sizes of 31.5 ± 2.8 % (n=17) compared to 17.0 ± 2.1 % (n=7) ($p<0.05$) for hearts treated with the highest concentration of BAY 41-2272 (3 μM).

Treatment with 1 μM, 300 nM and 100 nM BAY 41-2272 showed a concentration dependent reduction in infarct size compared to control (20.7 % ± 2.8 (n=6), 26.1 % ± 2.2 (n=6) and 28.1 % ± 3.8 (n=7) respectively). The highest concentration of BAY 41-2272 showed a 46 % relative reduction in mean infarct size compared to control hearts demonstrating that at the highest concentration BAY 41-2272 is cardioprotective (Figure 3.5).

Concomitant perfusion of the haem oxidising agent ODQ 2 μM with BAY 41-2272 at the highest concentration was carried out to explore the requirement of the prosthetic haem group on the beta subunit of sGC for catalytic activity and cGMP production. It was decided that co-treatments would be perfused alone for 2 minutes prior to BAY 41-2272 to allow them to reach the heart tissue and cause their inhibitory action, prior to BAY 41-2272 reaching the heart. Treatment with ODQ alone had no statistically significant effect on infarct size (32.5 ± 4.2 % (n=6)) ($p<0.05$) compared to controls. However, ODQ abrogated the effect of BAY 41-2272 (29.6 ± 1.7 % (n=7)) (Figure 3.6).

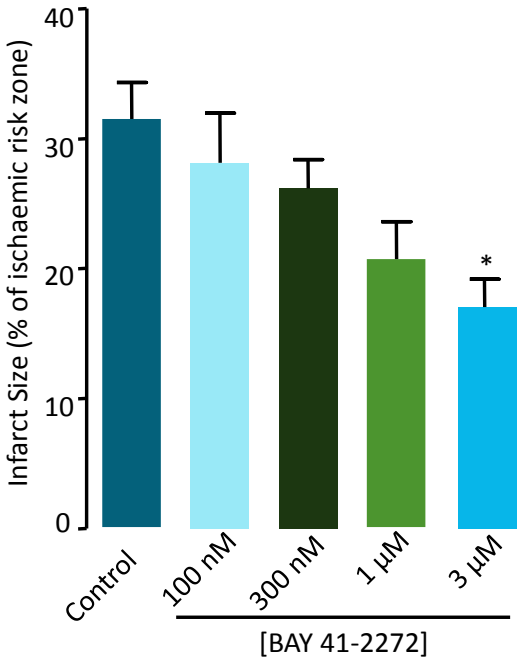

Figure 3.5 *Infarct size expressed as percentage of ischaemic risk zone for BAY 41-2272 concentration response (* $p<0.05$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-17).*

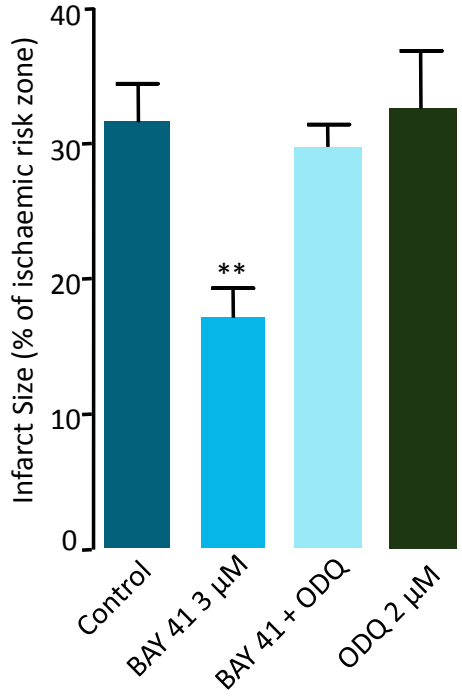

Figure 3.6 *Infarct size expressed as percentage of ischaemic risk zone (** $p<0.01$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-17).*

3.3.3 Cardiodynamic data: Study 1

Baseline cardiodynamic data are presented in Table 3.1. There was no statistical difference in any of the parameters between groups. RPP dropped by as much as 95 % upon induction of ischaemia in all treatment groups, recovering partially throughout ischaemia and then dropping again upon reperfusion due to the tendency for the hearts to fibrillate. This recovered before a gradual decline throughout reperfusion (Figures 3.7A & 3.8A).

Upon induction of ischaemia, CFR dropped in all experiments by at least 30 % (Figures 3.7B & 3.8B). CFR increased towards baseline once the ligature had been removed from the LDCA, confirming successful reperfusion and then decreased gradually during reperfusion.

Baseline cardiodynamic data. Study 1

Treatment Group	n	CFR (mL/min)	HR (BPM)	LVDP (mmHg)	RPP (mmHg/min x 10^3)	Vol. LV and RV (cm^3)	Risk Zone Vol. (cm^3)	Risk Zone (% Vol. LV and RV)
Control	17	14.6 ± 1.0	272 ± 8	69.3 ± 4.3	18.9 ± 1.4	0.95 ± 0.03	0.39 ± 0.03	44.7 ± 2.4
BAY 3 µM	7	17.1 ± 1.2	296 ± 18	69.7 ± 4.7	21.0 ± 2.6	0.95 ± 0.03	0.48 ± 0.04	50.1 ± 3.7
BAY 1 µM	6	18.1 ± 1.3	293 ± 11	69.2 ± 4.5	20.3 ± 1.5	0.91 ± 0.04	0.46 ± 0.08	49.2 ± 6.3
BAY 300 nM	6	15.3 ± 0.7	295 ± 10	59.7 ± 3.9	17.6 ± 1.1	0.87 ± 0.05	0.40 ± 0.06	45.7 ± 4.7
BAY 100 nM	7	17.3 ± 1.0	296 ± 12	62.7 ± 4.6	18.7 ± 1.7	0.87 ± 0.03	0.42 ± 0.04	47.9 ± 3.6
ODQ	6	14.7 ± 1.2	315 ± 21	70.7 ± 10.5	21.7 ± 2.3	0.98 ± 0.02	0.44 ± 0.04	45.4 ± 3.3
ODQ + BAY	7	15.7 ± 0.8	311 ± 10	80.0 ± 8.7	24.4 ± 2.0	1.01 ± 0.04	0.46 ± 0.03	46.4 ± 2.1
Total	56							

Table 3.1 *Baseline cardiodynamic data. (BAY = BAY 41-2272). No statistical differences between groups (ONE-way ANOVA + Newman-Keuls* post-hoc.

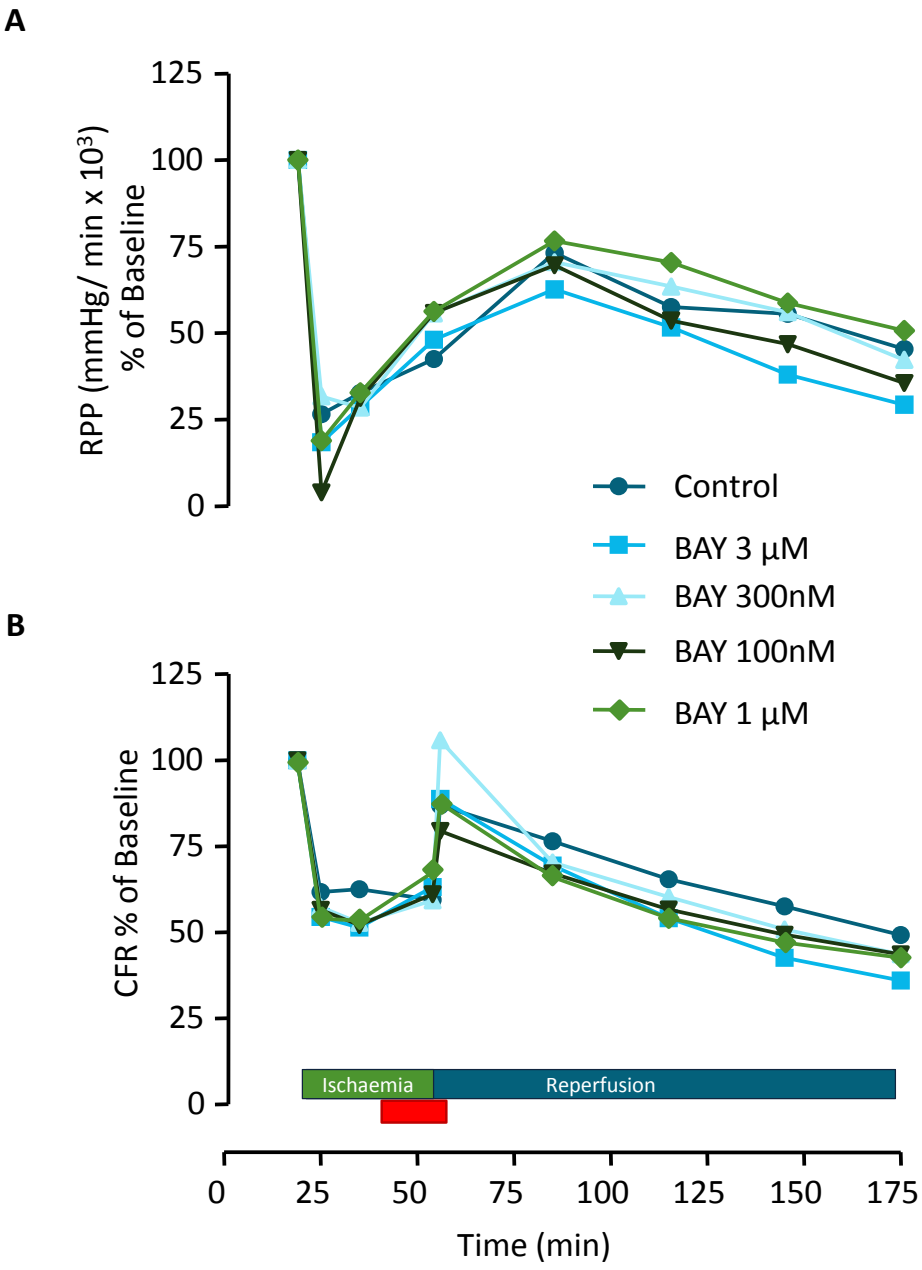

Figure 3.7 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 3.5. SEM bars have been removed for clarity. There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

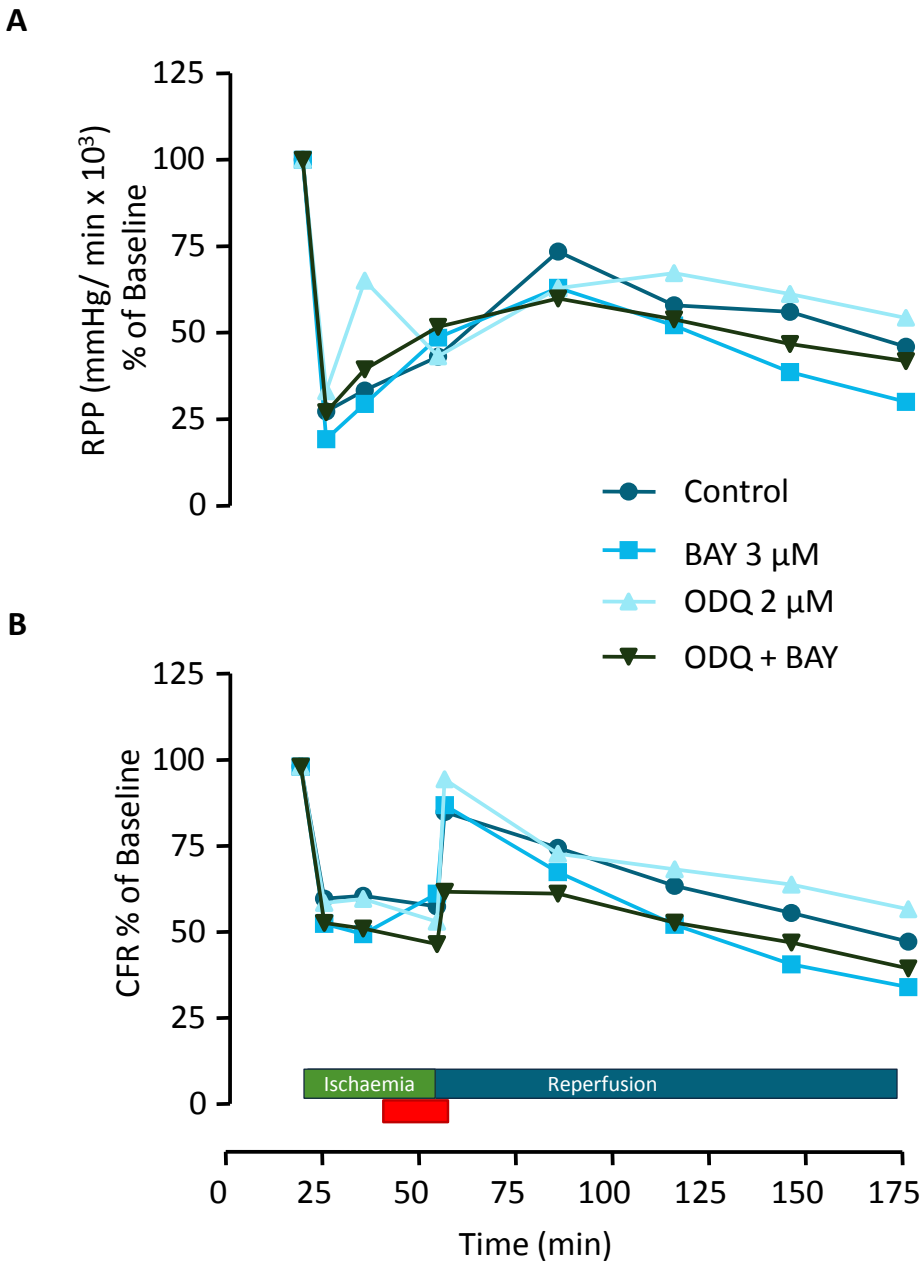

Figure 3.8 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 3.6. SEM bars have been removed for clarity. There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

3.3.4 RIA cGMP measurements: Study 2

Tissue levels of cGMP were measured in hearts that had been treated with or without BAY 41-2272 and subjected to 35 min regional ischaemia by occlusion of the LDCA. In both left and right ventricular samples, cGMP levels were significantly elevated in tissue that had been perfused with BAY 41-2272 compared to control tissue. Tissue samples from BAY 41-2272 perfused left ventricle had cGMP levels 88 % greater than those not perfused with BAY 41-2272 (17.76 ± 1.87 (n=6) vs. 9.44 ± 0.61 (n=6) fmol/mg tissue, $p<0.01$) and 57 % greater than naive samples. RV samples that were not subjected to LDCA occlusion had cGMP levels 126 % greater when perfused with BAY 41-2272 at reperfusion compared to control samples. Interestingly, cGMP levels were significantly higher in naive right ventricle compared to the adjacent left ventricle samples (11.28 ± 0.54 (n=6) vs. 17.87 ± 2.56 (n=6) fmol/mg tissue, $p<0.01$) (Figure 3.9 A and B).

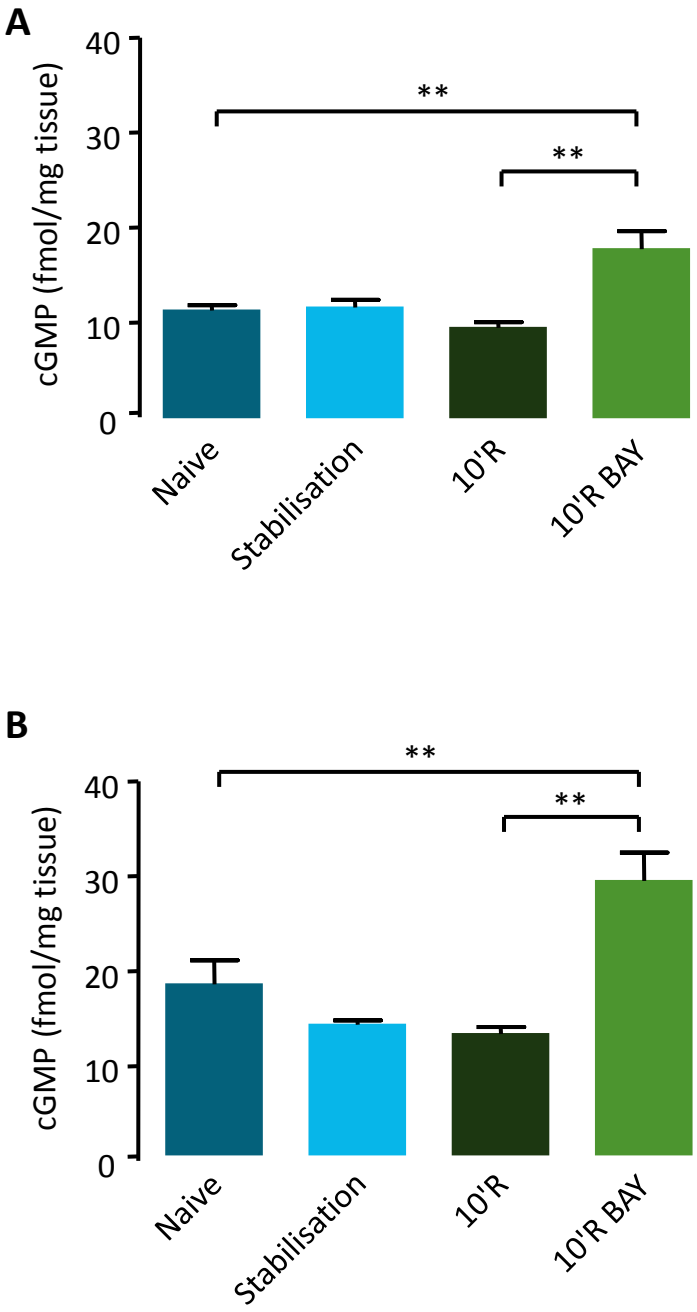

Figure 3.9 *cGMP levels in LV (A) and RV (B) heart samples perfused with or without BAY 41-2272 3 µM (** $p<0.01$) ONE-way ANOVA + Newman-Keuls post-hoc (n=6).*

3.4 Discussion

3.4.1 Summary of findings

The major findings of the studies in this chapter are:

i) BAY 41-2272 limited infarct size when given at reperfusion in a concentration-dependent manner

ii) The haem moiety on the beta subunit of sGC was required in its reduced state for BAY 41-2272 to afford protection

iii) The sGC stimulator, when given at reperfusion, elevated tissue cGMP levels up to 126 % compared to naive tissue levels

The findings in these studies support and add to our understanding of the GC/cGMP/PKG pathway and its importance as a cardioprotective signalling cascade for reperfusion salvage.

3.4.2 Infarct limitation

Previous work by Giricz and colleagues (2009) and D'Souza *et al.,* (2003) has shown that administration of 8Br-cGMP, a cGMP analogue at early reperfusion limits infarct size. It was from these studies that our initial hypothesis was derived. That is, that administration of BAY 41-2272 at early reperfusion would limit infarct size in the isolated perfused rat heart. At the highest concentrations of BAY 41-2272, infarct size was significantly reduced. These results are reproducible and demonstrated again in Chapters 4 and 5. These results support the work of many others that have reported the crucial role of the sGC-cGMP pathway in post conditioning (Cohen *et al.*, 2010; Hamid *et al.*; Penna *et al.*, 2006; Tsang *et al.*, 2004).

The extensive literature characterising the effects of BAY 41-2272 in a number of different models documents its ability to stimulate sGC to produce cGMP independently of NO. In a purified sGC model, Stasch *et al.* (2001) reported that BAY 41-2272 could stimulate sGC activity 20 fold above baseline. It was also reported that, unlike YC-1, BAY 41-2272 does not have PDE5 inhibitory actions. Koesling's group refuted this in an *in vitro* model that suggests that although the sGC stimulator increases the activity of basal sGC, at high cGMP levels there was an inhibitory component of BAY 41-2272 on PDE5 activity (Mullershausen *et al.*, 2004). Further literature that supports the consensus view that BAY 41-2272 does not inhibit PDE5 activity includes work by Evgenov *et al.*, (2004). They reported that infusion of BAY 41-2272 in lambs with acute pulmonary hypertension caused strong pulmonary vasodilatation but did not inhibit PDE5. They also showed the pulmonary vasodilator effects of BAY 41-2272 were not suppressed by NOS inhibition with L-NAME, yet Weimann *et al.*, (2000) reported that pre-treatment with L-NAME completely blocked the vasodilatation afforded by sildenafil.

3.4.3 cGMP levels in the heart

Previous work in our laboratory showed that the PKG inhibitor KT5823 abolished protection seen by cGMP elevation (Burley & Baxter, 2007). Results from experiments in this chapter support the notion that the protection afforded by BAY 41-2272 is associated with cGMP elevation. Tissue levels of cGMP in LV heart samples that had undergone ischaemia but received treatment with BAY 41-2272 3 μM at reperfusion were 88 % higher than those not treated and 56 % higher than naive LV tissue. Interestingly, cGMP levels were significantly higher in the RV compared to LV in naive tissue samples. It has

been reported that PDE distribution is altered throughout embryogenesis in the heart, which suggests there may be differences in cyclic nucleotide regulation also. Whether this is transferred into the adult heart is unclear (Fischmeister *et al.*, 2005). Further exploration into cGMP distribution within the LV and RV could be of benefit for specific therapeutic targeting.

3.4.4 Inhibition of sGC and use of ODQ

Garthwaite and colleagues (1995) first described ODQ as a selective inhibitor of sGC, following tests in slices of cerebellum. They reported that ODQ was a selective, potent and reversible inhibitor of NO-stimulated sGC. ODQ has since been used as the drug of choice when investigating sGC-cGMP signalling. However, it must be acknowledged that several publications have documented non-specific effects. Feelisch *et al.* (1999) investigated the functional and biochemical inhibitory actions of ODQ on isolated rat aortic rings, concluding that ODQ may have non-specific effects on other haem containing proteins such as cytochrome c oxidase. These observations were however, concentration dependent, with effects being documented with concentrations of ODQ 10 fold higher than those used in experiments in this thesis. Work by Weisbrod *et al.* (1998) and Onoue *et al.* (1998) suggest that non-specific activity of ODQ depends heavily on concentration and whether ODQ is pre-incubated for any length of time. The concentration of ODQ used in this study was based on previous work reporting successful inhibition of sGC-mediated effects (Hamid *et al.*, 2010). Other sGC inhibitors available were considered. Methylene blue was discounted as it has a strong blue colour in its hydrated form that would interfere with infarct delineation. NS 2028, a compound

structurally-related to ODQ was also discounted as previous pharmacological studies had used ODQ to confirm mechanistic actions of BAY 41-2272 (Koglin *et al.*, 2002; Stasch *et al.*, 2002a; Stasch *et al.*, 2001).

The use of ODQ as an inhibitor of sGC by oxidation of the prosthetic haem group supports the work of others, particularly Stasch *et al.* (2002a), that BAY 41-2272 is haem-dependent in its sGC stimulation. Infarct size was comparable to controls in hearts treated with ODQ and those treated concomitantly with BAY 41-2272 showed no significant infarct limitation. This is unsurprising as the documented mechanism of action for BAY 41-2272 reports that the haem moiety in its oxidised state is imperative for enzymatic activation and cGMP elevation (Stasch *et al.*, 2001). It is acknowledged that cGMP measurements from LV tissue samples perfused with ODQ would add support to the conclusion; however, priority was made for BAY 41-2272 treated hearts.

3.5 Conclusions

Results from these studies support previous work that demonstrates that BAY 41-2272 stimulates sGC (Bischoff & Stasch, 2004; 2001; Zhou *et al.*, 2008). They further contribute by showing that administration of the highest concentrations of the drug at early reperfusion limits infarct size in regionally ischaemic isolated rat hearts. RIA confirms that BAY 41-2272 elevates cGMP levels in LV and RV when given at reperfusion suggesting that the protection afforded could be associated with cGMP elevation. Further studies need to be performed to investigate the interaction of exogenous NO and BAY 41-2272 and whether concomitant perfusion of both the endogenous ligand and stimulator is a better therapeutic target.

Chapter 4
The role of endogenous and exogenous NO in BAY 41-2272 mediated infarct limitation

4.1 Introduction

4.1.1 Endogenous nitric oxide and postconditioning

Nitric oxide has been identified as an important mediator in the postconditioning paradigm. Tsang *et al.* (2004), first reported that ischaemic postconditioning requires activation of the prosurvival kinases PI3K-Akt and eNOS in an isolated perfused rat heart model. They demonstrated that mechanical postconditioning causes a significant increase in phosphorylation of these kinases and pharmacological inhibition of PI3K-Akt abrogated protection afforded by post conditioning. Further evidence that supports the need for NOS and therefore NO in postconditioning came from Downey's group who demonstrated that postconditioning was abolished by L-NAME and ODQ, suggesting that NO and cGMP produced by sGC are required to afford protection (Yang *et al.*, 2005; Yang *et al.*, 2004). It was further demonstrated that eNOS activation was dependent on the upstream phosphorylation of Akt, as the PI3K/Akt inhibitors Wortmannin and LY294002 attenuated eNOS phosphorylation (Cohen *et al.*, 2006; Hausenloy *et al.*, 2004; Hausenloy & Yellon, 2004; Tsang *et al.*, 2004) (see Figure 1.4).

As well as the evidence for the importance of eNOS during early reperfusion, studies by Bolli's laboratory demonstrated the importance of iNOS during reperfusion to afford cardioprotection by means of decreased reperfusion induced oxygen radicals and inhibition of both mitochondrial swelling and mitochondrial pore formation (West *et al.*, 2008). Using a genetic mouse model that had cardiomyocyte specific over expression of iNOS, they reported a decrease of over 50 % in infarct size compared to their wild type littermates. Furthermore, they proposed that this protection was mediated by limiting

reperfusion-induced oxygen free radical generation and formation of the mPTP (West *et al.*, 2008).

4.1.2 Autacoids and protection

Earlier work investigating autacoid action during early reperfusion helps to substantiate the salvage kinase targeted experiments. Bradykinin and adrenomedullin have been shown to afford protection when given at reperfusion and their mechanisms of action have been documented to involve the PI3K/Akt/eNOS pathway. The infarct limiting effects of exogenous bradykinin at reperfusion were associated with phosphorylation of both Akt and eNOS in an isolated mouse heart model (Bell & Yellon, 2003). Similar effects were recorded when adrenomedullin was administered at reperfusion in an isolated rat heart model whereby Akt phosphorylation was increased (Hamid & Baxter, 2005). It was also shown that L-NAME blunted the protective effects of the peptide. Further support of this mechanism was demonstrated in a mouse model, where the protection was abrogated by ODQ suggesting the protective signalling of adrenomedullin extends to sGC/cGMP (Hamid & Baxter, 2005). A comprehensive review of autacoid mediated protection can be found in Burley and Baxter (2009).

4.1.3 Gaseous anaesthesia mediated protection

It has been known for some time that gaseous anaesthetics can afford protection in models of ischaemia-reperfusion (Pagel, 2008), and their protective effects have been implicated to be mediated through the PI3K/Akt pro survival pathway (Feng *et al.*, 2005). Inamura *et al.* (2009) reported that protection afforded by sevoflurane in a global ischaemia guinea pig model was abrogated by aprotinin, an antifibrinolytic serine protease inhibitor used to

prevent peri-operative bleeding. What makes this observation interesting in this setting is that aprotinin has been shown to inhibit eNOS in rat coronary endothelial cells (Ulker *et al.*, 2002). This suggests the need for eNOS phosphorylation in sevoflurane mediated infarct limitation. Similarly, Ge *et al.* (2010), demonstrated in an *ex vivo* mouse heart model that isoflurane induced infarct limitation was abrogated in $eNOS^{-/-}$ mice. Furthermore, they reported that isoflurane-triggered mPTP inhibition was abolished in $eNOS^{-/-}$ mice, supplying evidence that NO mediated protection at reperfusion is in part afforded by inhibition of mPTP formation.

4.1.4 Statin mediated NO protection

In recent years, it has been shown that the cholesterol lowering drugs, hydroxymethylglutarate coenzyme A reductase inhibitors (statins), limit infarct size when given during early reperfusion. Yellon's laboratory provided evidence that the survival kinases and endogenous NO mediate the protection afforded. They reported that the PI3K/Akt inhibitor Wortmannin abolished the protective effects of atorvastatin and showed similar results in $eNOS^{-/-}$ mice. This is in agreement with Wolfrum *et al.* who demonstrated that simvastatin reduced infarct size in an *in vivo* rat model, which was inhibited by L-NAME (Wolfrum *et al.*, 2004). In contrast, Ferdinandy's laboratory reported that acute and chronic administration of another statin, lovastatin, interferes with the survival kinases in myocardial tissue samples. They demonstrated that both chronic and acute administration of lovastatin attenuated phosphorylation of Akt without effecting total Akt. They further reported that only acute lovastatin potentiated p42 MAPK/ERK phosphorylation; again total kinase levels were unaffected (Kocsis *et al.*, 2008). In

addition, it was shown that chronic lovastatin treatment reduced infarct size in nonconditioned rats and did not abrogate the infarct limitation afforded by IPC. However, it did reduce the protection afforded by postconditioning. Conversely, acute treatment of lovastatin did not abrogate the infarct limitation afforded by postconditioning, but did abolish the protection afforded by IPC and did not limit infarct size in nonconditioned animals as was shown with chronic treatment of lovastatin (Kocsis *et al.*, 2008). Assuming the infarct size results are as a result of the survival kinase pathways highlighted in other statin studies, it would be fair to speculate that eNOS and NO levels would be similarly affected as their upstream kinases; however, these experiments have not been reported.

4.1.5 Exogenously administered nitric oxide

As mentioned in Chapter 1, NO has been used clinically in the form of organic nitrate or nitrite for over a century mainly for the treatment of angina pectoris. In the setting of pharmacological postconditioning, data thus far are inconsistent. Early evidence from Liu *et al.* (1998), reported that the NO donor SP/W-5186 given just before reperfusion in an *in vivo* rabbit model reduced infarct size compared to non-treated animals. They went on to show in the same experimental model that S-nitrosoglutathione limited infarct size comparably (Ma *et al.*, 1999). Yellon's group subsequently reported that the NO donor SNAP could restore protection that was abrogated in bradykinin perfused $eNOS^{-/-}$ mice, suggesting that bradykinin afforded protection during early reperfusion through eNOS mediated production of NO (Bell & Yellon, 2003). In contrast to evidence supporting a protective effect of exogenous NO, previous work by our laboratory demonstrated that SNAP could not afford significant protection when given during early reperfusion over a

range of concentrations (1, 2, 5 and 10 μM) (Burley & Baxter, 2007). It is worthy of note, that these differences in results could be attributable to a number of factors. Differing NO donors used and their mechanism of action could be one reason for the differences. In addition, the speed at which the donor produces or releases NO and the logistics of perfusing the drug at the right time will play a part. Interestingly, Downey's group have most recently published data that shows concentration dependent protection afforded by SNAP during early reperfusion in an isolated rabbit model. They also reported that this protection was not abrogated in the presence of L-NAME at the highest doses of SNAP (4 and 6 μM) (Cohen *et al.*, 2010).

4.1.6 NO-independent stimulation of sGC

The sGC stimulator BAY 41-2272, described in the previous chapter, was identified as a NO-independent, haem-dependent stimulator of sGC. Stasch *et al.* (2001) demonstrated the ability of BAY 41-2272 to stimulate purified sGC to a level that would be expected to cause biologically important increases in cGMP at concentrations as low as 10 nM. They also describe the ability of sGC to be stimulated/activated by concomitant NO and BAY 41-2272, suggesting synergistic effects (Stasch *et al.*, 2001). It is suggested that BAY 41-2272, like YC-1, binds allosterically to NO adjacent to the histidine 105 bond of the haem group stabilising it for NO binding. It has been proposed that binding at a sixth coordinate of the haem confers ligand specificity for NO activation. Cys 238 and Cys 243 of the α1-subunit have been speculated to be important as the NO-independent regulatory site (Becker *et al.*, 2001; Stasch *et al.*, 2001). Schmidt *et al.* (2003) investigated the mechanism further using BAY 41-8543, a structurally similar sGC stimulator. Their data

support the previous idea of an allosteric binding site that when occupied sensitises the enzyme to NO, increasing the catalytic rate but not the affinity of the substrate GTP. The lack of crystal structures of any of the sGC subunit isoforms prevents certainty concerning the exact regions involved.

4.1.4 Hypotheses

The aim of this study was to investigate the effects of endogenous and exogenous NO on the infarct limiting properties of the NO-independent sGC stimulator BAY 41-2272. NOC-9 was selected as the NO donor for these experiments as it has rapid NO release characteristics.

It was hypothesised that;

i. Inhibition of endogenous NO would not abrogate the infarct limiting properties of BAY 41-2272
ii. Concomitant perfusion of an exogenous NO donor and BAY 41-2272 would afford greater protection than either individual component

The specific experimental objectives were to:

1. Concomitantly perfuse the NOS inhibitor L-NAME, or NO scavenger C-PTIO with BAY 41-2272 during early reperfusion
2. Undertake a concentration response for the NO donor NOC-9 during early reperfusion in a rat isolated heart model
3. Concomitantly perfuse submaximal concentrations of NOC-9 with BAY 41-2272 during early reperfusion
4. Measure cGMP levels in LV and RV tissue samples from hearts perfused with C-PTIO, L-NAME or NOC-9
5. Measure NOx levels in coronary effluent of hearts perfused with C-PTIO, L-NAME or NOC-9

4.2 Materials and Methods

4.2.1 Pharmacological compounds

All salts used to make modified KH solution were sourced from Fisher Scientific LTD (UK) and were of analytical or ultrapure quality. L-NAME (NOS inhibitor) and C-PTIO (NO scavenger) were sourced from Tocris Bioscience (UK) and NOC-9 (NO donor) and BAY 41-2272 (NO-independent, haem-dependent sGC stimulator) from Sigma-Aldrich (UK). BAY 41-2272 was dissolved in DMSO, the final concentration of DMSO in the KH was 0.05 % v/v.

4.2.2 Rat isolated heart perfusion

Rat isolated hearts were excised and retrograde perfused as described in Chapter 2. Once a period of 20 min haemodynamic stabilisation had been established, rat hearts were randomised and assigned to one of the groups mentioned in 4.2.3 and 4.2.4. All hearts were subjected to 35 min LDCA occlusion followed by 120 min of reperfusion unless otherwise stated.

4.2.3 Inhibition of endogenous NO: Study 1

Animals used in this Study were sourced from B&K Universal Ltd. (Bristol, UK). This study was undertaken to explore the role of endogenous NO on BAY 41-2272 mediated infarct limitation. Hearts were perfused as described above and either NOS was inhibited by L-NAME or NO was scavenged at reperfusion by C-PTIO alone and or in the presence of BAY 41-2272 3 μM (see figure 4.1).

Group 1, Control, (n=17). This group includes some hearts (n=5) that were perfused with the vehicle DMSO 0.05 % v/v from 30 min ischaemia until 10 min reperfusion.

There was no statistical significant difference between control hearts perfused with or without DMSO vehicle and so all hearts were pooled for statistical analysis.

Group 2, BAY 41-2272 3 μM, (n=7). BAY 41-2272 3 μM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 3, L-NAME 100 μM, (n=6). L-NAME 100 μM was perfused from 28 min ischaemia until 10 min reperfusion.

Group 4, L-NAME 100 μM + BAY 41-2272 3 μM, (n=8). L-NAME 100 μM was perfused from 28 min ischaemia and BAY 41-2272 3 μM from 30 min ischaemia, both until 10 min reperfusion.

Group 5, C-PTIO 30 μM (n=6). C-PTIO 30 μM was perfused from 28 min ischaemia until 10 min reperfusion.

Group 6, C-PTIO 30 μM + BAY 41-2272 3 μM, (n=6).C-PTIO 30 μM was perfused from 28 min ischaemia and BAY 41-2272 3 μM from 30 min ischaemia, both until 10 min reperfusion.

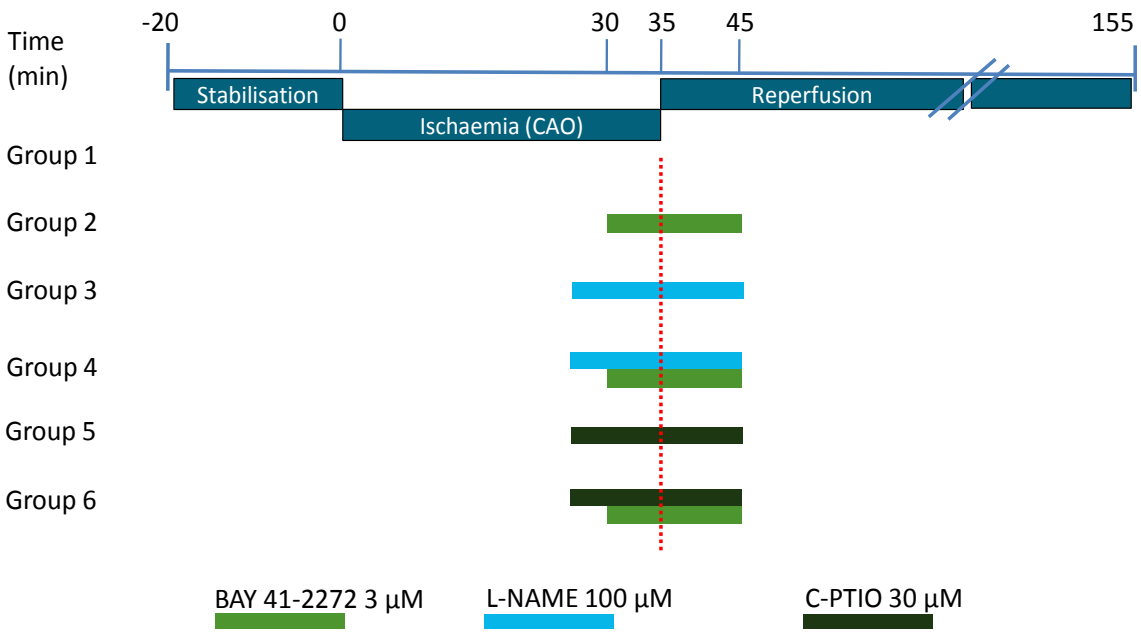

Figure 4.1 *Experimental protocol for groups 1-6 in Study 1. All hearts were stabilised for 20 min followed by 35 min regional ischaemia and then reperfused for 120 min.*

4.2.4 Exogenous NO and BAY 41-2272: Study 2

This study was undertaken to investigate the effect that exogenous NO has on BAY 41-2272 mediated infarct limitation and whether they act synergistically to afford protection. Hearts were perfused as described in Chapter 2, with NOC-9 1 nM - 1 μM, or BAY 41-2272 1 or 3 μM (see figure 4.2)

Group 1, Control, (n=12). This group includes some hearts that were perfused with the vehicle DMSO 0.05 % v/v from 30 min ischaemia until 10 min reperfusion. There was no statistical significant difference between control hearts perfused with or without DMSO vehicle and so all hearts were pooled for statistical analysis.

Group 2, BAY 41-2272 3 μM, (n=6). BAY 41-2272 3 μM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 3, BAY 41-2272 1 μM, (n=5). BAY 41-2272 1 μM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 4, NOC-9 1 μM, (n=9). NOC-9 1 μM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 5, NOC-9 100 nM, (n=6). NOC-9 100 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 6, NOC-9 10 nM, (n=6). NOC-9 10 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 7, NOC-9 1 nM, (n=6). NOC-9 1 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 8, BAY 41-2272 1 µM + NOC-9 1 nM, (n=6). BAY 41-2272 1 µM and NOC-9 1 nM were perfused from 30 min ischaemia until 10 min reperfusion.

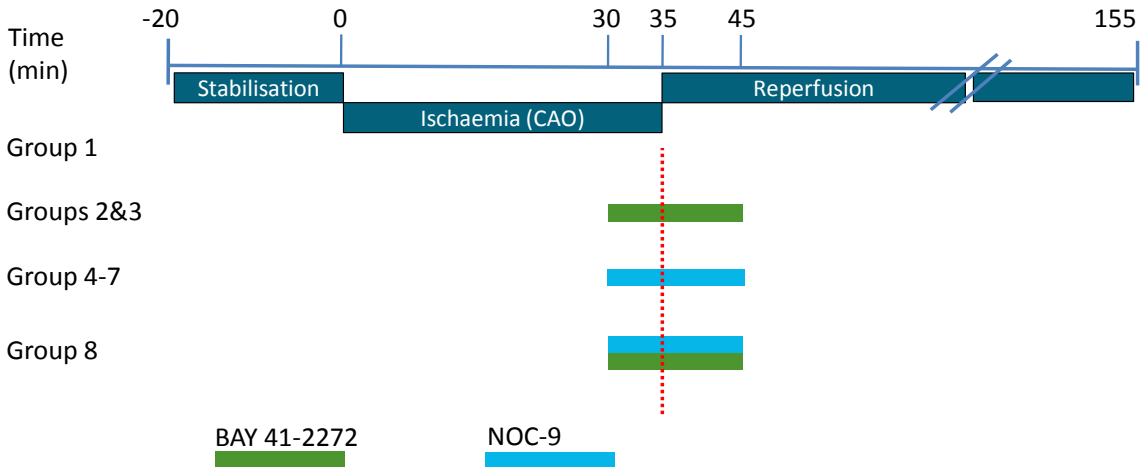

Figure 4.2 *Experimental protocol for groups 1-8 in Study 2. All hearts were stabilised for 20 min followed by 35 min regional ischaemia and then reperfused for 120 min. BAY 41-2272 1 and 3 µM perfused alone, NOC-9 1 µM, 100, 10 and 1 nM perfused alone. Concomitant perfusion of BAY 41-2272 1 µM and NOC-9 1 nM respectively.*

4.2.5 cGMP Radio immuno-sorbent assay: Study 3

To examine the effects of BAY 41-2272 and endogenous and exogenous NO on cGMP production, RIA was performed as described in Chapter 3.2.4. Tissue samples were prepared from rat hearts randomly assigned to one of 5 groups shown in figure 4.3. Hearts that were subjected to regional ischaemia were treated as described for Study 1. Animals used for RIA in this study were supplied by Charles River Laboratories Inc. (Maidenhead, UK).

Group 1, Naive, (n=6). Hearts were excised and washed in KH to remove any blood and then sectioned.

Group 2, 10 min reperfusion, (n=6). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min.

Group 3, 10 min reperfusion + BAY 41-2272 3 µM, (n=6). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BAY 41-2272 3 µM was perfused as described for Group 2 in 3.2.3.

Group 4, 10 min reperfusion, BAY 41-2272 3µM + L-NAME 100 µM, (n=5). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BAY 41-2272 3µM and L-NAME 100 µM were perfused as described for Group 4 in 4.2.3.

Group 5, 10 min reperfusion, BAY 41-2272 3µM + C-PTIO 30 µM, (n=5). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BAY 41-2272 3µM and C-PTIO 30 µM were perfused as described for Group 6 in 4.2.3.

Group 6, 10 min reperfusion, BAY 41-2272 1µM + NOC-9 1 nM, (n=5). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BAY 41-2272 1µM and NOC-9 1 nM were perfused as described for Group 8 in 4.2.4.

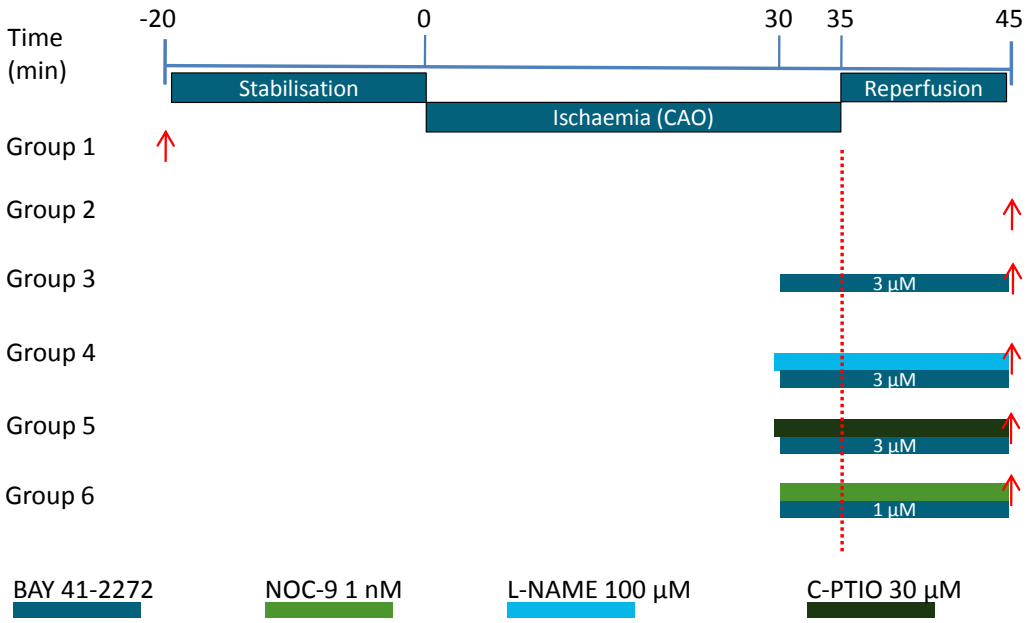

Figure 4.3 *Experimental protocol for hearts prepared for RIA in Study 3. Red arrows indicate time points at which hearts were sampled. (N.B. L-NAME and C-PTIO perfused from 28 min, whilst NOC-9 perfused from 30 min).*

4.2.6 NO_2^- measurement by ozone based chemiluminescence: Study 4

OBC was used to determine total NO_2^- levels in coronary effluent collected after ischaemia-reperfusion and perfusion with an NO donor (NOC-9), NOS inhibitor (L-NAME) or NO scavenger (C-PTIO). This method utilises the luminescent properties of an electrically excited state of NO_2, which is formed in the nitric oxide analyser (NOA). For NO levels to be quantified in this way it must be cleaved from its parent compound or reduced back to its radical state. This can be achieved using a tri-iodide cleavage reagent and NO is carried in an argon gas stream to the NOA at constant flow. A supply of oxygen in the NOA generates O_3, which reacts with NO to form NO_2 and O_2. A small proportion of the NO_2 produced is formed in an electrically excited state. The unstable electrons return to their original ground state releasing energy in the form of photons. The released photons are focused through a low pass filter (<900 nm wavelength) which amplifies the signal enabling an electrical (millivolt) signal to be recorded.

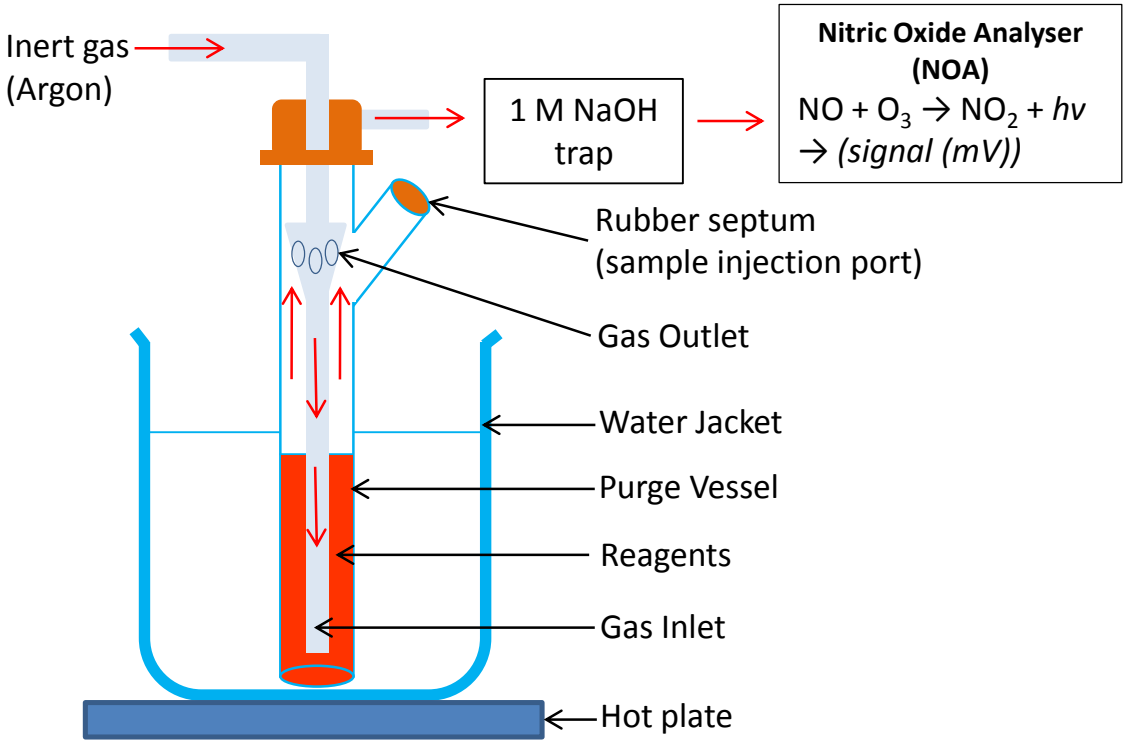

Figure 4.4 *Experimental apparatus for OBC. Purge vessel suspended in water bath (50 °C). Samples were injected through the rubber septum on the side of the purge vessel. Reaction mixture then passed through NaOH trap and into the NOA where emitted light (hv) was converted into an electrical signal (Adapted from Pinder et al. 2009).*

1 mL of coronary effluent was collected from each heart as described in Figure 4.5 and immediately snap frozen in liquid N_2 to be used in the NOA. The cleavage reagent was made by dissolving 1 g KI in 20 mL high performance liquid chromatography (HPLC) water and added to 70 mL acetic acid. This solution was stirred for 30 min ensuring the I_2 was dissolved. 5 mL of cleavage reagent was then loaded into the purge vessel and sealed with a rubber bung connected to the argon flow and a 25 mL 1 M NaOH trap and solvent filter (Figure 4.4). The alkali trap and solvent filter are used to protect the NOA from hot acid vapour produced by the cleavage reagent. The purge vessel was held by a boss and clamp and submerged in a heated water jacket (50 °C). The NOA (Sievers NOA 280i (Analytix, UK)) was calibrated by injecting 100 μM of known S-nitrosoglutathione (GSNO) standards (62.5, 125, 250, 500, 1000 nM) and HPLC grade ultra pure water into the purge vessel using a Hamilton syringe. A standard curve was produced by plotting peak area under the curve (AUC) against NO concentration. A straight line was achieved by subtracting the peak AUC for HPLC ultra pure water from the GSNO standards, which accounts for impurities in the water.

The frozen samples were thawed and 100 μL of each sample was injected into the purge vessel. Subsequent samples were added once the peak on the live trace returned to baseline. The cleavage reagent was replaced after 10 samples had been injected. Using the standard curve, total NO_2^- levels were calculated for each sample and were then standardised to CFR for each respective heart.

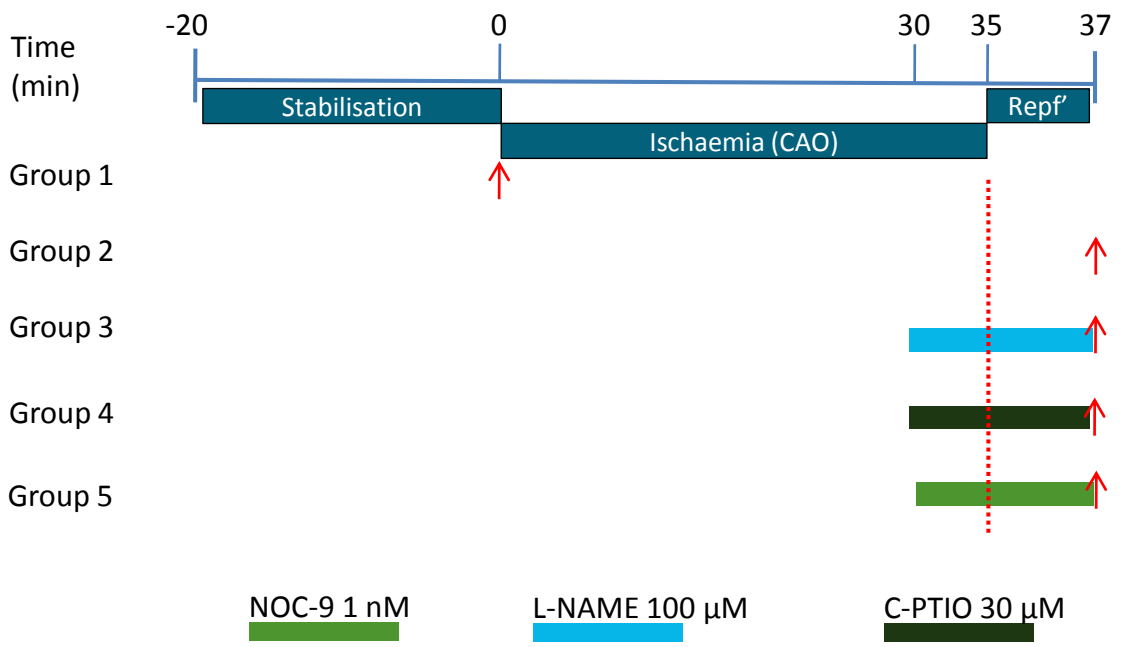

Figure 4.5 *Experimental protocol for OBC supplementary Study 4. (N.B. L-NAME and C-PTIO perfused from 28 min, whilst NOC-9 perfused from 30 min). Blue arrows indicate time points at which coronary effluents was collected for OBC.*

4.3 Results

4.3.1 Summary of experiments

In Study 1, 30 rats were used in addition to the shared control group (n=17) and BAY 41-2272 3 μM (n=7) from Chapter 3, Study 1. Four hearts were excluded from the study due to technical error; thus, a total of 26 (+24) completed experiments are reported. In Study 2, 62 rats were used, six hearts were excluded due to technical error; thus a total of 56 completed experiments are reported. In Study 3, 27 rats were used for RIA in addition to the previously reported BAY 41-2272 3 μM group (n=6) from Chapter 3, Study 2. There were no exclusions and so data from 27 (+6) hearts are reported. In Study 4, 20 rats were used for OBC NO_2^- measurements. There were no technical exclusions and so data from 20 hearts are reported. The period of stabilisation before the onset of ischaemia was carried out to allow the hearts to stabilise and reach pre-determined criteria (see below). For a heart to be included and subjected to ischaemia, it had to achieve the following baseline cardiodynamic criteria:

CFR between 10 and 24 mL/min, LVEDP 5-10 mmHg, HR 200-350 BPM, LVDP greater than 50 mmHg and a steady sinus rhythm. Hearts were also excluded during analysis if there was inadequate delineation of the risk zone and infarcted tissue (poor staining).

4.3.2 Infarct size data: Study 1

Study 1 contains Langendorff perfusion experiments for the pharmacological inhibition of NO, either with the NOS inhibitor L-NAME or NO scavenger C-PTIO. The area at risk for all hearts in all groups was between 44.7 and 53.4 % of the combined left and right

ventricular tissue. Statistical analysis of the risk zone sizes for each of the treatment groups showed that there were no statistical differences between groups. Infarct size was expressed as a percentage of the risk zone, calculated as described in Chapter 2 and reported in Figures 4.6 & 4.7. Under control conditions (35 min ischaemia followed by 120 min reperfusion) hearts had infarct sizes of 31.5 ± 2.8 % (n=17).

Infarct size for hearts treated with 100 μM L-NAME at reperfusion was 29.1 ± 1.9 % (n=6) with no statistical difference compared to control hearts, while treatment with both 100 μM L-NAME and BAY 41-2272 resulted in mean infarct sizes of 20.5 ± 2.5 % (n=8), ($p<0.05$ compared to control) (Figure 4.6). Perfusion with the NO scavenger C-PTIO resulted in a mean infarct size of 30.7 ± 2.3 % (n=6), while co-administration with BAY 41-2272 caused a reduction in infarct size to 23.6 ± 0.9 % (n=6), ($p<0.05$ compared to control) (Figure 4.7).

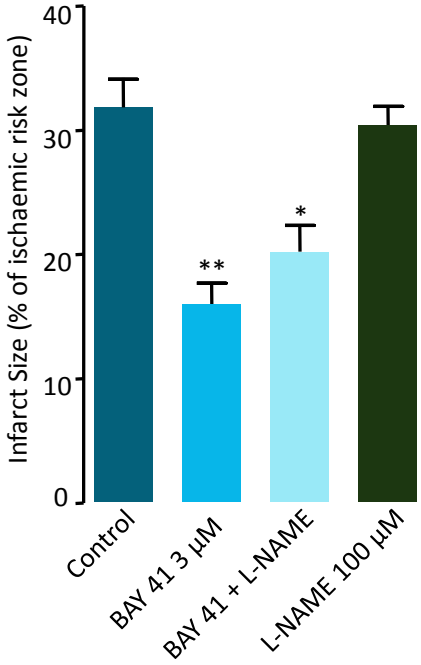

Figure 4.6 *Infarct size expressed as percentage of ischaemic risk zone for L-NAME and L-NAME + BAY 41-2272 treated hearts (* $p<0.05$, ** $p<0.01$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-17).*

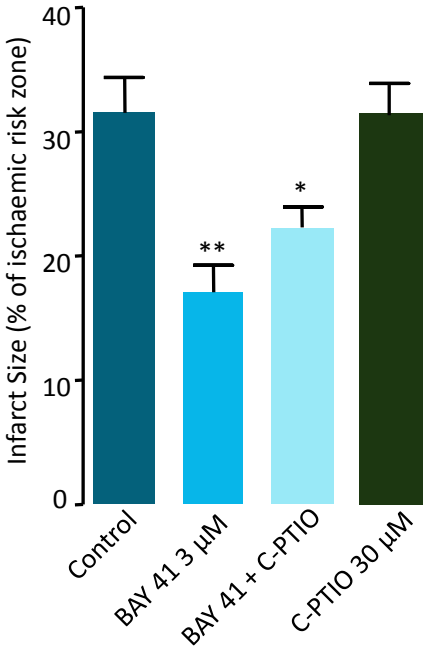

Figure 4.7 *Infarct size expressed as percentage of ischaemic risk zone for C-PTIO and C-PTIO + BAY 41-2272 treated hearts (* $p<0.05$, ** $p<0.01$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-17).*

4.3.3 Cardiodynamic data: Study 1

Baseline cardiodynamic data are presented in Table 4.1. There was no statistical difference between any of the parameters between treatment groups. RPP dropped by as much as 95 % upon induction of ischaemia in all treatment groups, recovering partially throughout ischaemia and then dropping upon reperfusion due to the tendency for the hearts to fibrillate; RPP recovered before gradually decreasing throughout reperfusion (Figures 4.8A & 4.9 A).

Upon induction of ischaemia, CFR dropped in all experiments by at least 30 % (Figures 4.8B & 4.9B). CFR increased towards baseline once the ligature had been removed from the LDCA confirming successful reperfusion, and then decreased gradually during reperfusion.

Baseline cardiodynamic data. Study 1

Treatment Group	n	CFR (mL/min)	HR (BPM)	LVDP (mmHg)	RPP (mmHg/min x 10^3)	Vol. LV and RV (cm^3)	Risk Zone Vol. (cm^3)	Risk Zone (% Vol. LV and RV)
Control	17	14.6 ± 1.0	272 ± 8	69.3 ± 4.3	18.9 ± 1.4	0.95 ± 0.03	0.39 ± 0.03	44.7 ± 2.4
BAY 3 µM	7	17.1 ± 1.2	296 ± 18	69.7 ± 4.7	21.0 ± 2.6	0.95 ± 0.03	0.48 ± 0.04	50.1 ± 3.7
L-NAME	6	13.8 ± 0.7	309 ±17	61.2 ± 1.8	18.3 ± 1.0	0.89 ± 0.27	0.44 ± 0.03	50.6 ± 3.8
L-NAME + BAY	8	15.3 ± 0.6	290 ± 9	68.4 ± 6.3	19.5 ± 1.3	0.96 ± 0.05	0.47 ± 0.03	49.9 ± 2.0
C-PTIO	6	15.7 ± 0.7	313 ± 6	64.5 ± 4.8	20.2 ± 1.5	1.03 ± 0.05	0.54 ± 0.04	53.4 ±4.3
C-PTIO + BAY	6	16.8 ± 0.7	298 ± 6	63.1 ± 3.6	18.8 ± 1.0	1.07 ± 0.03	0.52 ± 0.03	49.7 ± 4.0
Total	50							

Table 4.1 *Baseline cardiodynamic data. (BAY = BAY 41-2272). No statistical differences between groups (ONE-way ANOVA + Newman-Keuls* post-hoc.

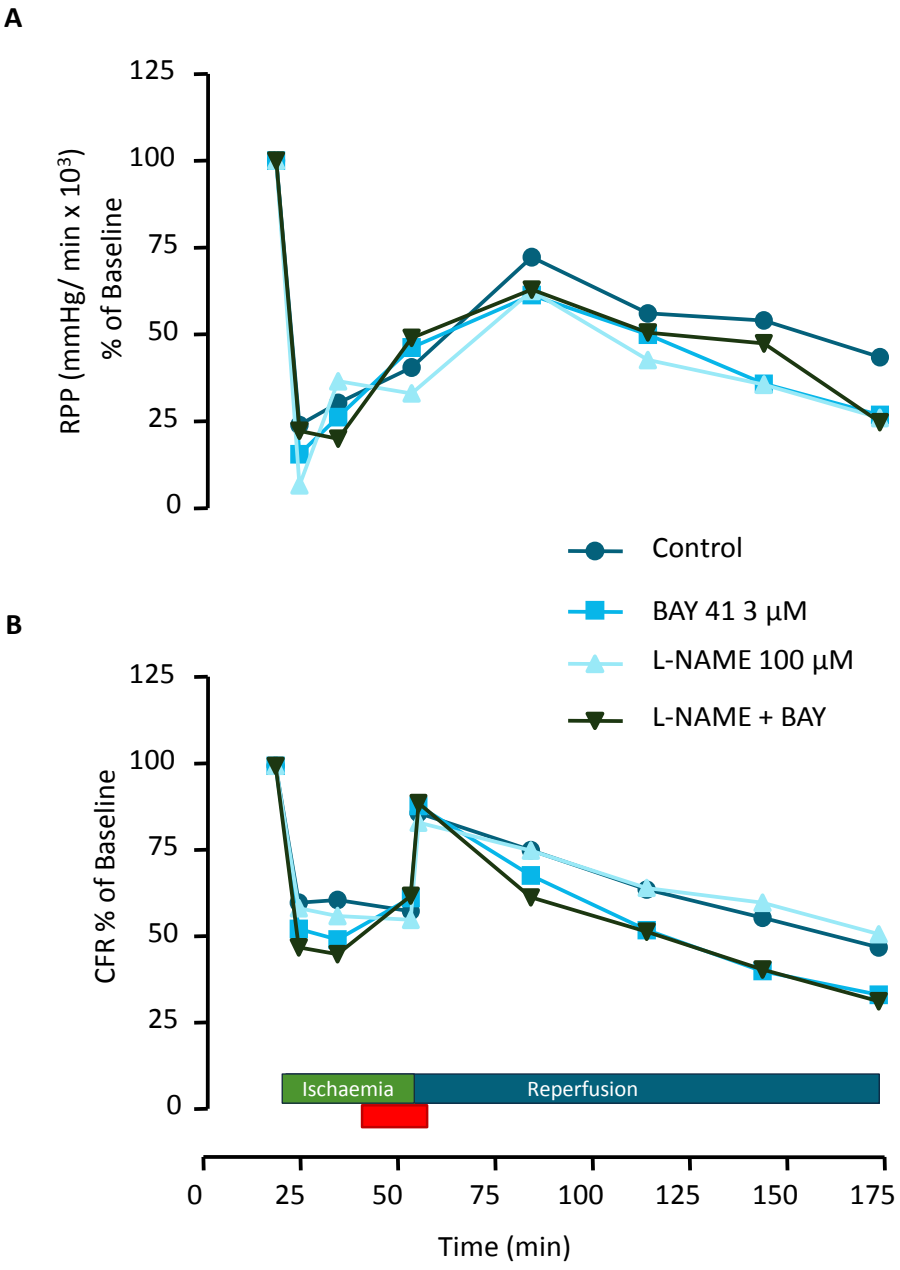

Figure 4.8 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 4.6. SEM bars have been removed for clarity. There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

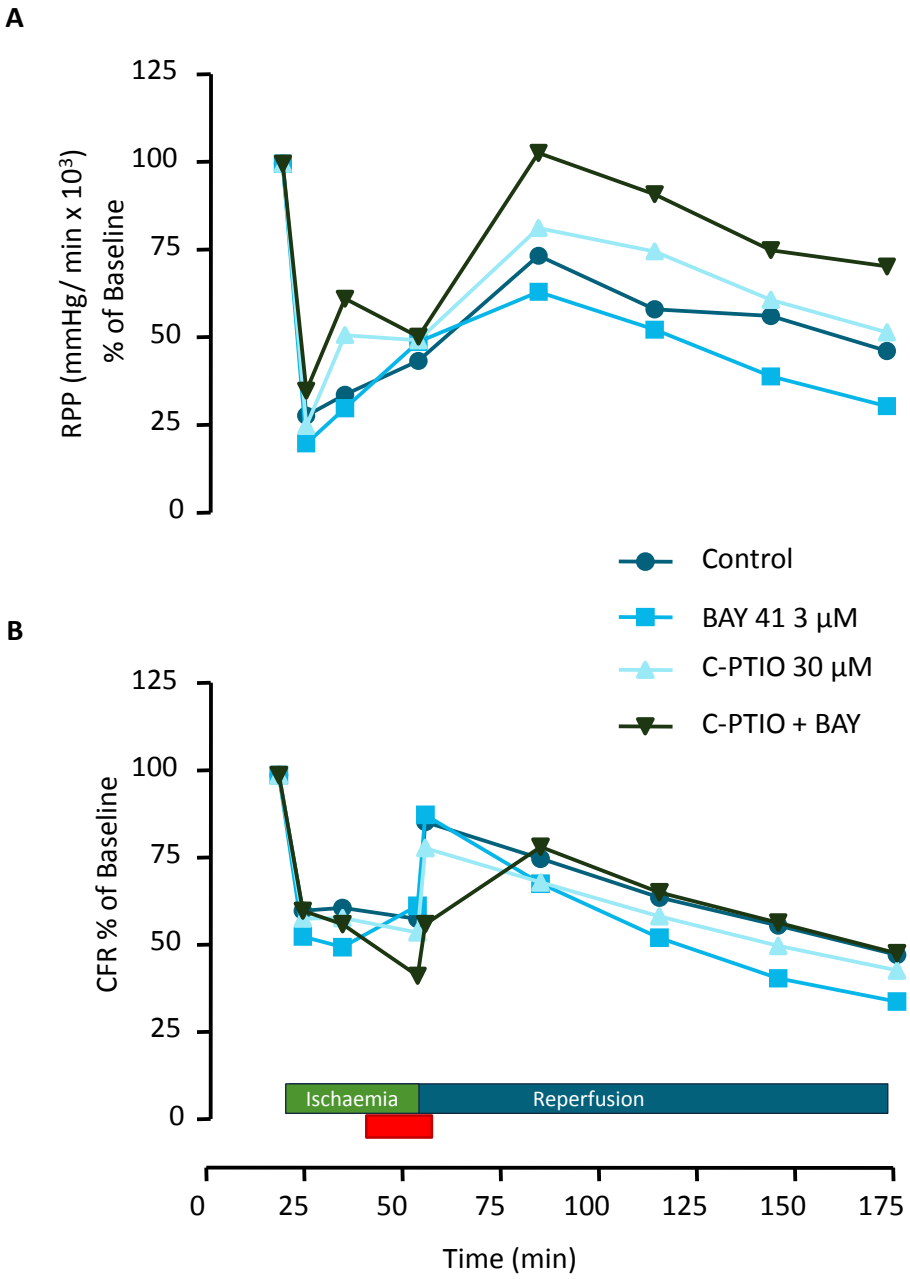

Figure 4.9 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 4.7. SEM bars have been removed for clarity. There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

4.3.4 Infarct size data: Study 2

Study 2 contains Langendorff perfusion experiments for the initial dose response to the NO donor NOC-9. The study also contains experiments where BAY 41-2272 and NOC-9 have been perfused concomitantly. The area at risk for all hearts in all groups was between 44 and 60 % of the combined left and right ventricular tissue. There were no statistical differences between the risk zone sizes for each of the treatment groups. Infarct size was expressed as a percentage of the risk zone, calculated as described in Chapter 2 and reported in Figures 4.10 & 4.11. Under control conditions (35 min ischaemia followed by 120 min reperfusion) hearts had infarct sizes of 34.7 ± 1.6 % (n=12) compared to 20.5 ± 1.3 % (n=9) ($p<0.001$) for hearts treated with the highest concentration of NOC-9 (1 μM). Treatment with 100 nM, 10 nM and 1 nM NOC-9 showed a concentration dependent reduction in infarct size (22.6 ± 0.8 ($p<0.001$, n=6), 25.5 ± 2.6 ($p<0.01$, n=6) and 28.2 ± 3.1 ($p<0.05$, n=6) respectively). The highest concentration of NOC-9 showed a 41 % relative reduction in infarct size compared to control hearts, and the lowest concentration affording an 18 % reduction, suggesting that at all concentrations examined, NOC-9 was cardioprotective (Figure 4.10).

Perfusion of BAY 41-2272 at 3 μM limited infarct size to 16.9 ± 2.0 % ($p<0.001$, n=6) and at a submaximal concentration (1 μM) to 29.3 ± 2.9 % (n=5). Concomitant perfusion of the submaximal concentration of BAY 41-2272 and the lowest concentration of NOC-9 limited infarct size to 21.5 ± 2.2 % (n=6) ($p<0.05$ vs. BAY 41-2272 1 μM and NOC-9 1 nM) *(*Figure 4.11*)*.

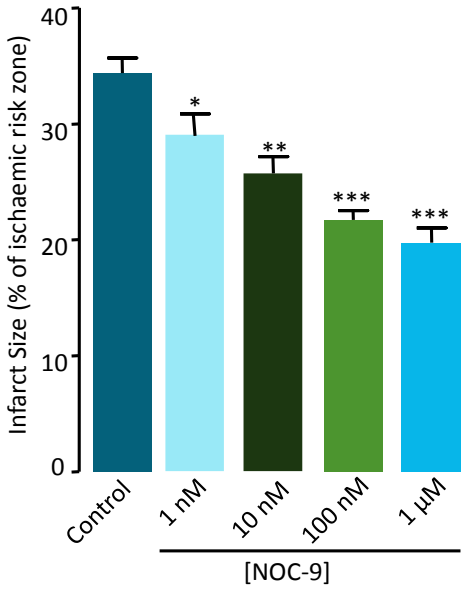

Figure 4.10 *Infarct size expressed as percentage of ischaemic risk zone for NOC-9 concentration response (* p<0.05, ** p<0.01, *** p<0.001 vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-12).*

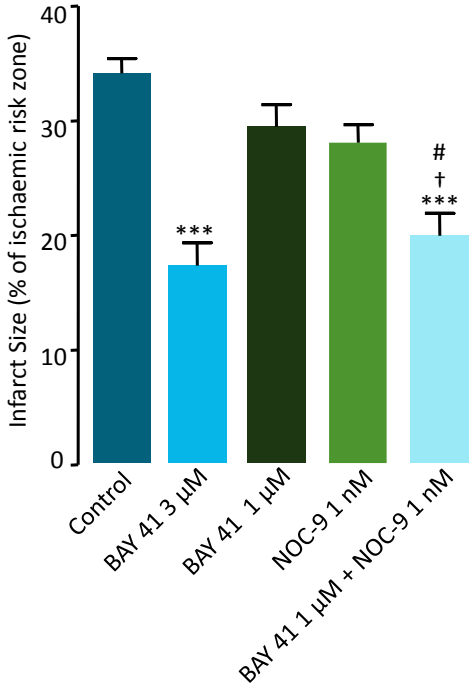

Figure 4.11 *Infarct size expressed as percentage of ischaemic risk zone for concomitant perfusion of NOC-9 and BAY 41-2272 (*** p<0.001 vs. control, # p<0.05 vs.BAY 41-2272 1 µM, † p<0.05 vs. NOC-9 1 nM) ONE-way ANOVA + Newman-Keuls post-hoc (n=5-12).*

4.3.5 Cardiodynamic data: Study 2

Baseline cardiodynamic data are presented in *Table 4.2*. There was no statistical difference between any of the parameters between groups. RPP dropped by as much as 80 % upon induction of ischaemia in all treatment groups, recovering partially throughout ischaemia and then dropping upon reperfusion due to the tendency for the hearts to fibrillate; RPP recovered before gradually decreasing throughout reperfusion (Figures 4.12 & 4.13 A).

Upon induction of ischaemia, CFR dropped in all experiments by at least 30 % (Figures 4.12B & 4.13B). CFR increased towards baseline once the ligature had been removed from the LDCA confirming successful reperfusion, and then decreased gradually during reperfusion.

Baseline cardiodynamic data. Study 2.

Treatment Group	n	CFR (mL/min)	HR (BPM)	LVDP (mmHg)	RPP (mmHg/min x 10^3)	Vol. LV and RV (cm^3)	Risk Zone Vol. (cm^3)	Risk Zone (% Vol. LV and RV)
Control	12	14.6 ± 0.6	313 ± 10	66.0 ± 6.6	20.4 ± 1.7	0.81 ± 0.04	0.35 ± 0.03	42.6 ± 2.9
BAY 3 µM	6	15.5 ± 1.1	322 ± 17	54.9 ± 4.2	19.3 ± 0.4	0.86 ± 0.02	0.46 ± 0.04	53.5 ± 4.7
BAY 1 µM	5	15.3 ± 1.1	354 ± 19	59.0 ± 7.8	20.8 ± 0.9	0.87 ± 0.06	0.36 ± 0.03	41.9 ± 2.5
NOC-9 1 µM	9	15.9 ± 0.8	341 ± 4	69.0 ± 4.9	22.9 ± 1.8	0.79 ± 0.02	0.40 ± 0.02	50.6 ± 3.3
NOC-9 100 nM	6	14.4 ± 0.2	329 ±14	63.9 ± 8.7	20.5± 1.9	0.82 ± 0.03	0.45 ± 0.02	54.6 ± 3.7
NOC-9 10 nM	6	15.2 ± 0.8	322 ± 8	70.1 ± 6.1	21.9 ± 1.2	0.83 ± 0.04	0.41 ± 0.02	49.4 ± 3.6
NOC-9 1 nM	6	14.9 ± 1.1	327 ± 11	69.9 ± 4.8	22.4 ± 1.4	0.81 ± 0.06	0.39 ± 0.03	48.2 ± 2.1
BAY 1 µM +NOC-9 1 nM	6	15.5 ± 1.0	318 ± 8	68.0 ± 8.7	20.2 ± 2.0	0.82 ± 0.05	0.38 ± 0.03	46.7 ± 3.3
Total	56							

Table 4.2 *Baseline cardiodynamic data. (BAY = BAY 41-2272). No statistical differences between groups (ONE-way ANOVA + Newman-Keuls* post-hoc.

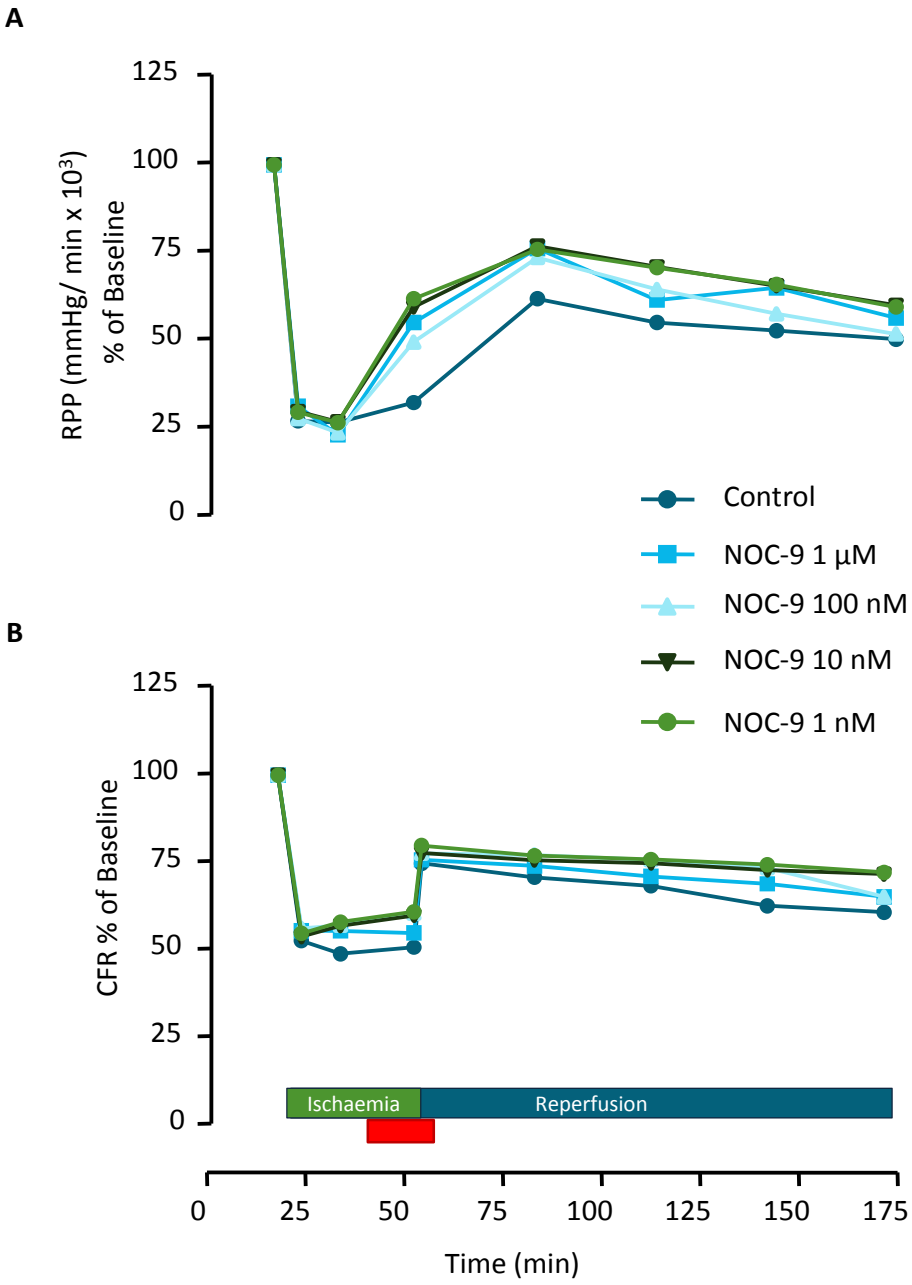

Figure 4.12 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 4.10. SEM bars have been removed for clarity. There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

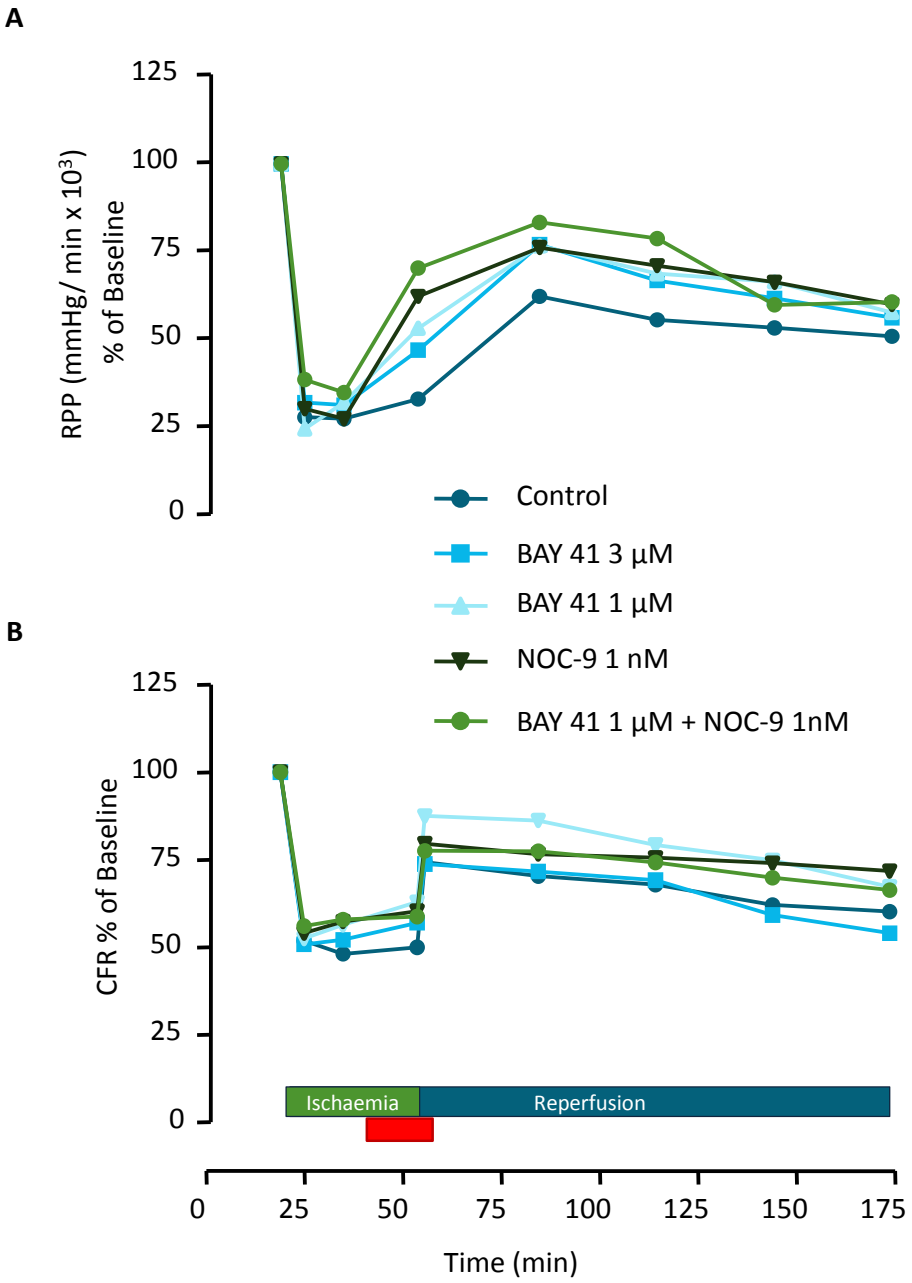

Figure 4.13 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 4.11. SEM bars have been removed for clarity. There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

4.3.6 RIA cGMP measurements: Study 3

Tissue levels of cGMP were measured in hearts that had been treated with or without BAY 41-2272 and subjected to 35 min regional ischaemia by occlusion of the LDCA. Measurements were also made in hearts perfused with BAY 41-2272 concomitantly with L-NAME, C-PTIO or NOC-9. In both left and right ventricular samples, cGMP levels were significantly elevated in tissue that had been perfused with BAY 41-2272 compared to samples that had not. Tissue samples from concomitant BAY 41-2272 and L-NAME perfused LV had cGMP levels 48 % lower than those perfused with BAY 41-2272 alone (11.15 ± 0.91 (n=5) vs. 17.76 ± 1.87 (n=6) fmol/mg tissue, $p<0.05$). Similarly, cGMP levels in LV tissue perfused with both C-PTIO and BAY 41-2272 were 54 % lower than BAY 41-2272 alone (8.29 ± 0.52 (n=5) vs. 17.76 ± 1.87 (n=6) fmol/mg tissue, $p<0.001$). LV tissue samples from hearts perfused with both NOC-9 1 nM and a submaximal concentration of BAY 41-2272 (1 μM) contained cGMP levels 46 % higher than reperfusion only hearts (13.44 ± 1.01 (n=5) vs. 9.44 ± 0.61 (n=6) fmol/mg tissue). Of interest, RV samples, which were not subjected to LDCA occlusion, had cGMP levels greater than their adjacent LV in all groups. Like cGMP measurements in Chapter 3, levels were 2-fold higher in naive RV compared to the adjacent LV samples (Figure 4.14 A and B).

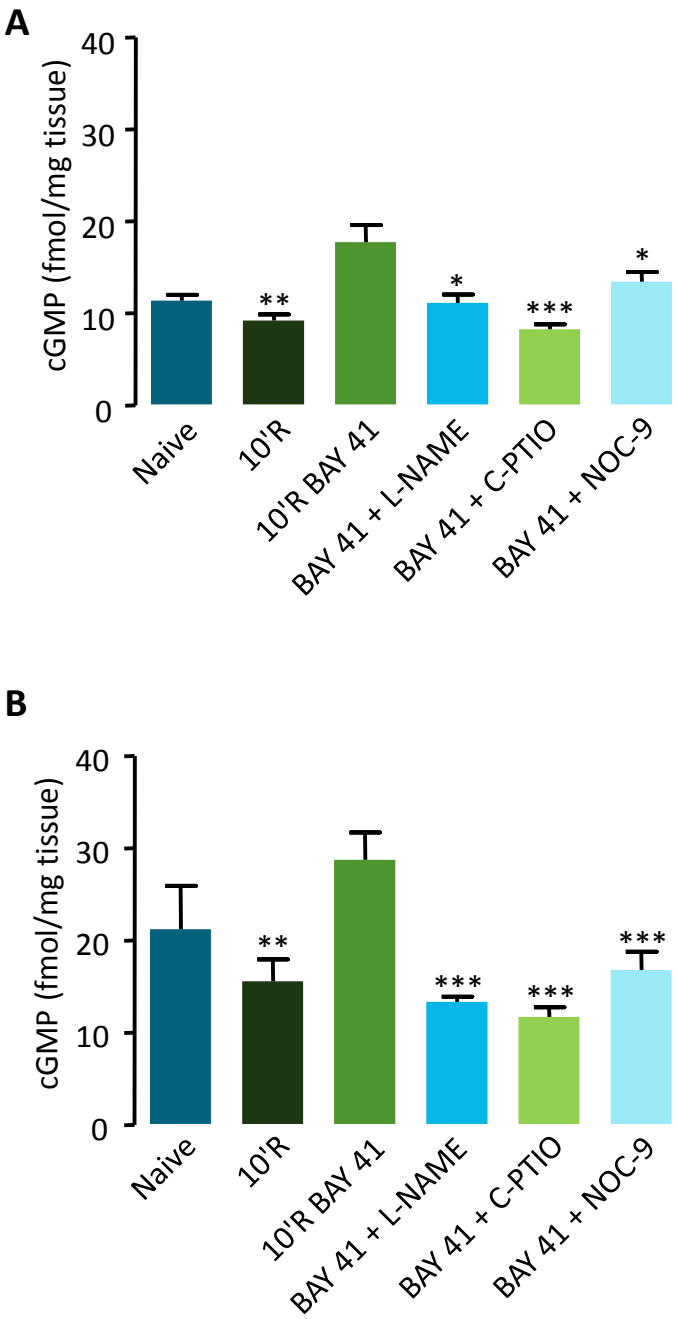

Figure 4.14 *cGMP levels in LV (A) and RV (B) heart samples perfused with or without BAY 41-2272 (3 μM) at reperfusion or concomitantly with L-NAME (100 μM), C-PTIO (30 μM) or NOC-9 (1 nM) (* p<0.05, **p<0.01, *** p<0.001 vs. 10'R BAY 41) ONE-way ANOVA + Newman-Keuls post-hoc (n=5-6).*

4.3.7 NO_2^- levels measured by ozone based chemiluminescence: Study 4

Nitrite levels were measured in coronary effluent collected from Langendorff perfused rat hearts 2 min after reperfusion. Because the estimation of NO_2^- was measured at a specific time point and CFR varied between hearts, data are represented as NO_2^- produced per minute in coronary effluent. Control samples demonstrated levels of 2582 ± 486 (n=5) pmoles/min compared to 4815 ± 634 pmoles/min (n=5) in L-NAME treated hearts. Hearts perfused with C-PTIO and NOC-9 recorded levels of 3380 ± 483 (n=5) and 3421 ± 174 pmoles/min (n=5) respectively (Figure 4.15).

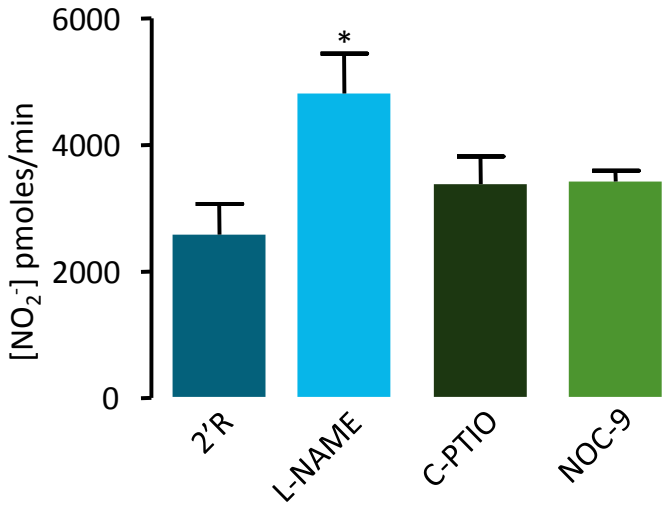

Figure 4.15 *NO_2^- levels in coronary effluent samples of hearts subjected to 35 min regional ischaemia followed by 2 min reperfusion, perfused with L-NAME (100 μM), C-PTIO (30 μM) or NOC-9 (1 nM) from 28 min ischaemia (30 min for NOC-9) until sampling at 2 min reperfusion measured by OBC (* $p<0.05$ vs. 2'R) ONE-way ANOVA + Newman-Keuls post-hoc.*

4.4 Discussion

4.4.1 Summary of findings

The major findings of the studies in this chapter are:

i) BAY 41-2272 afforded infarct limitation independently of endogenous NO

ii) The NO donor, NOC-9 limited infarct size when given at reperfusion in a concentration dependent manner

iii) NOC-9 and BAY 41-2272 acted synergistically to afford infarct limitation when submaximal concentrations of both drug were given concomitantly

iv) cGMP levels were significantly lower at reperfusion in hearts perfused with BAY 41-2272 and a NOS inhibitor or NO scavenger compared to BAY 41-2272 alone

The findings in these studies support and add to our understanding of the NO/GC/cGMP pathway and its importance as a cardioprotective signalling cascade for reperfusion salvage.

4.4.2 Infarct limitation

The NOS inhibitor L-NAME was given with or without BAY 41-2272 3 μM at reperfusion to investigate the dependency of endogenous NO on BAY 41-2272 induced infarct limitation. Concomitant perfusion of L-NAME did not abrogate the protection afforded by the highest concentration of BAY 41-2272 suggesting that this protection is independent of NOS function. Administration of the NOS inhibitor alone resulted in an infarct size similar to that of the control experiments; this observation is consistently seen in many laboratories (Burley & Baxter, 2007; Krieg *et al.*, 2009; Ren *et al.*, 2007). This suggests that inhibition of NO production by NOS at reperfusion does not increase infarct size

beyond control levels. It remains to be elucidated whether compensatory mechanisms are activated or increased to protect the heart from further damage. Pre-ischaemic treatment of the myocardium with L-NAME has however been shown to improve contractile recovery (Andelova *et al.*, 2005). It has been suggested that this may be of benefit as less NO produced during early reperfusion will limit the amount of deleterious ROS species produced (Zweier & Talukder, 2006). There is no evidence to suggest that this translates to infarct limiting protection in a model of reperfusion.

Infarct sizes in hearts treated with the NO scavenger C-PTIO also resulted in infarct sizes similar to those of control experiments in agreement with work conducted by Jung *et al.* (2010), who report control level infarct sizes in C-PTIO treated rats in a global model of ischaemia-reperfusion. The rationale for using an NO scavenger as well as an NO inhibitor was to eliminate any NO present at the time of administration of the sGC activator, not possible with L-NAME, which only inhibits the production of NO by inhibiting NOS. The half-life of endogenously produced NO in the vasculature is of the order of 5 seconds (Archer, 1993; Bates *et al.*, 1991). It has to be acknowledged that during ischaemia NO is produced by other means such as reduction from nitrite which further supports the need for an NO scavenger and not just a NOS inhibitor (Ferdinandy & Schulz, 2003). Concomitant perfusion of the NO scavenger and BAY 41-2272 abrogated some of the infarct limitation afforded by BAY 41-2272 alone. Stasch *et al.* (2001), reported that high concentrations of PTIO (65 μM), a structurally similar NO scavenger could not block BAY 41-2272 stimulation of purified sGC; however no data were shown and no numerical values were reported. The results of our study suggest that the NO scavenger was more

effective at limiting NO in the tissue at reperfusion and suggests that there is a component of endogenous NO dependency in BAY 41-2272 induced infarct limitation.

Boerrigter & Burnett (2007) suggested the theoretical possibility of a "coronary steal" phenomenon when using BAY 41-2272 and other sGC activators. They argue that because direct haem-dependent stimulators of sGC act synergistically with NO there is the possibility that non-diseased vessels will produce more NO than stenotic vessels do and so relax more in response to BAY 41-2272 with blood flow, or in the Langendorff preparation, perfusate being directed away from the stenotic vessels thereby aggravating ischaemia. Treatment with BAY 41-2272 and L-NAME or C-PTIO suggest that the possible steal phenomenon does not contribute to infarct size, similarly supported by the unremarkable vasodilatation as indicated by CFR seen in BAY 41-2272 only treated hearts where infarct limitation was greatest.

In Study 2, results support those of Yang *et al.* (2005), who demonstrated that exogenous NO limits infarct size when given at reperfusion. It further corroborates with studies that highlight the importance of NO/GC/cGMP signalling during early reperfusion (Cohen *et al.*, 2010; Hausenloy & Yellon, 2004; Tsang *et al.*, 2004). NOC-9 was chosen as the NO donor based on its rapid NO production and active concentration range of between 1 nM and 100 μM. A secondary reason for its selection was based on previous work by our laboratory that reported no protective effects of SNP when given at reperfusion in a model of ischaemia-reperfusion (Burley & Baxter, 2007). NOC-9 is a member of the diazeniumdiolates (NONOates) which have the attractive property, unlike other NO donors, that their decomposition is not catalysed by thiols or in biological tissue (Keefer *et*

al., 1996). The results of Study 2 show that at all concentrations, NOC-9 was able to afford significant infarct limitation.

BAY 41-2272 3 μM was again able to afford significant infarct limitation when given at reperfusion, supporting the findings of Study 1 in Chapter 3. This is in comparison to BAY 41-2272 1 μM which only demonstrated a 16 % reduction in infarct size compared to 34 % in Study 1, Chapter 3. A sub-threshold concentration of BAY 41-2272 was needed for analysis of concomitant NO donor and BAY 41-2272 treated hearts to detect a synergistic effect so this concentration was used for the remainder of the study. To investigate the proposed pharmacology of BAY 41-2272 and its ability to sensitize sGC to NO, submaximal BAY 41-2272 (1 μM) was perfused concomitantly with the lowest concentration of NOC-9 (1 nM). The results of these experiments support the mechanistic actions of BAY 41-2272 in the presence of NO as described by Stasch *et al.* (2001), and Schmidt *et al.* (2003). It is however the first demonstration that a combination of submaximal sGC stimulator and NO donor can afford greater protection than each dose independently. It also suggests that NO and BAY 41-2272 are not competing for the same active site and so not saturating the enzyme.

4.4.3 cGMP levels in the heart

cGMP levels were measured in LV and RV tissue samples similarly to Chapter 3. Convincingly, cGMP measured in both naïve and reperfusion only LV samples were very similar to the same groups in the previous chapter. cGMP levels in the adjacent RV for each group were also reproducible in the current study. To explore the relationship between NO inhibition and BAY 41-2272 mediated protection further, cGMP levels were

measured in LV and RV samples from hearts perfused in the same way as infarct Study 1. Concomitant perfusion of L-NAME and BAY 41-2272 resulted in a decrease of 37 % in cGMP compared to BAY 41-2272 perfused alone; however, cGMP levels were still 20 % higher than control (10'R) LV samples. These results suggest that only modest increases in cGMP at reperfusion are needed to limit infarct size. Similarly, cGMP levels in LV samples perfused with both BAY 41-2272 and C-PTIO at reperfusion resulted in a 53 % reduction in cGMP. These results support the infarct data reported in 4.3.3, suggesting that the ability of C-PTIO to scavenge NO has greater deleterious action than NOS inhibition by L-NAME. cGMP levels in LV control tissue were comparable to those treated with BAY 41-2272 and C-PTIO, yet the corresponding infarct study reported a reduction in infarct size of 25 %. This highlights the need for more specific investigation of intracellular cGMP production. Concomitant perfusion of submaximal NOC-9 and BAY 41-2272 increased LV tissue cGMP levels by 45 % compared to controls, supporting the infarct limitation observed in 4.3.4. These results suggest that there is not a linear relationship between cGMP levels and infarct size, but do suggest that a more extensive profile of cGMP levels for a given protective state would be valuable.

4.4.4 NO_2^- levels in coronary perfusate

Measurement of NO in biological samples has been carried out for many years, and there are many ways in which it can be done. The difficulty is measuring NO *in situ*, as apparatus and reagents make this technically challenging. Collecting samples and later analysing them is the way in which this can be overcome. However NO has a very short half-life and so measurements are usually made using NO metabolites or by trapping NO in a spin trap

for electroparamagnetic resonance (EPR). In an attempt to quantify NO levels during early reperfusion in the presence of the pharmacological NO manipulating drugs, OBC was used.

NO_2^- levels measured in coronary effluent samples following 2 min reperfusion were approximately 8 times higher in the current study, compared with previous work carried out in our laboratory (Hamid *et al.*, 2010), in an isolated mouse heart model. In the previous study, coronary effluent was collected over 60-120 seconds whereas samples in the current study were collected over 5 seconds. Apart from species differences and differing flow rates, this may in part explain the difference in NO_2^- levels as time point samples in the current study would be diluted less and preserved much quicker because of the smaller quantity of coronary effluent collected. Surprisingly, NO_2^- levels measured in coronary effluent samples that had been perfused with L-NAME during early reperfusion were significantly higher than untreated controls ($p<0.05$). It would have been expected that inhibition of NOS by L-NAME would result in a reduction in NO_2^- levels (Hamid *et al.*, 2010). Also unexpected were the results of perfusion with the NO donor NOC-9, which resulted in an insignificant increase in NO_2^-, comparable to the results for C-PTIO treated hearts. I would speculate that the concentration of NOC-9 used was not high enough to sustain a sufficient production of NO that could be measured in the coronary effluent. However, this concentration was sufficient to elicit a biological response in the previous infarct study.

4.5 Conclusions

Results from these studies support previous work that demonstrates that exogenous NO is cardioprotective when given at reperfusion. They also demonstrate that endogenous NO is not required to afford BAY 41-2272 mediated protection. However, NO can act synergistically with BAY 41-2272 to afford infarct limitation, when perfused at submaximal concentrations. These data also suggest that only small elevations in total tissue cGMP content are required to mediate infarct limitation.

Chapter 5
NO-independent, haem-independent sGC activation and protection of reperfused myocardium

5.1 Introduction

5.1.1 Three states of sGC

Understanding of NO/GC/cGMP signalling as a potential cardioprotective pathway has gained momentum in recent years. A potential limitation was identified in reduced NO generation under pathological conditions (see Chapter 1). However, it has recently been demonstrated that sGC expression and function can also be modified in pathological environments (Ruetten *et al.*, 1999; Schermuly *et al.*, 2008). Exploration of these concepts was helped by the discovery of a second distinct class of sGC modulating compounds, described as sGC activators. Similarly to the sGC stimulators described in Chapter 3, this unique class of compound was developed through a high throughput screening of around 250,000 compounds using a Chinese hamster ovary (CHO) cell line expressing a cGMP sensitive cation channel (Stasch *et al.*, 2002b). The result of the screening programme was a new class of aminodicarboxylic acids, which after optimisation led to the development of 4-(4-carboxybutyl)(2-((4-phenethylbenzol) oxy)phenethyl)amino)methyl)benzoic acid (BAY 58-2667). Described as the first and most potent NO-independent, haem-independent sGC activator, Stasch *et al.* (2002b) demonstrated that BAY 58-2667 activated sGC even after it had been oxidised by the sGC inhibitor ODQ, or even after losing its haem group.

5.1.2 Oxidised and haem-free sGC

Investigation of the pharmacology of this class of compound and the chemistry underlying its action highlighted that sGC activation goes beyond NO binding to the haem site on the β subunit. Stasch *et al.* (2006) demonstrated for the first time that oxidised sGC exists *in*

vivo and increases under conditions of oxidative stress associated with cardiovascular disease (see Figure 5.1). Up until this point, it was assumed that the haem-free and oxidised forms of sGC were biological artefacts and there was no evidence to suggest any clinical relevance to their presence. Using endothelial cells, Stasch *et al.* (2006), demonstrated that BAY 58-2667 could significantly increase cGMP levels in tissue incubated with the peroxynitrite donor SIN-1, yet an NO donor reduced cGMP production in this environment. They also demonstrated that BAY 58-2667 caused a greater increase in cGMP concentration in aortic rings from spontaneously hypertensive rats compared to the same treatment in normotensive Wistar Kyoto preparations. Furthermore, in the presence of the haem oxidising agent ODQ, cGMP levels were elevated between two and three fold. Using the structurally unrelated sGC activator HMR 1066, Schindler *et al.* (2006), demonstrated both with purified sGC and rat aortic rings that this compound preferentially activates the oxidised or haem-free portion of sGC. They also reported the presence of a small proportion of haem-oxidised enzyme in some sGC preparations by UV visible spectra. This observation corroborates the findings of Stasch *et al.* (2006), who demonstrated the presence of haem-oxidised sGC in living cells. Further evidence suggesting that under pathological conditions, sGC activators elicit greater cGMP elevating properties came from Zhou *et al.* (2008) who demonstrated that HMR 1066, another sGC activator, was able to elicit greater cGMP production in rat aortic smooth muscle cells exposed to oxidative stress than normoxic cells.

These observations led Stasch *et al.* (2006) to conclude that because oxidation of the haem group also creates a BAY 58-2667 sensitive state, a similar oxidative mechanism is likely in

diseased vessels. They also acknowledged that evidence for the existence of haem-free/oxidised sGC *in vivo* was circumstantial, but the profiles of the sGC activators are indistinguishable and so the physiological existence of oxidised and haem-free sGC could no longer be rejected.

Most recently, Roy *et al.* (2008), provided evidence that BAY 58-2667 and other sGC activators such as HMR 1766 target only the haem-free state of sGC. They suggested that oxidation of the haem group by pharmacological means or the proposed pathophysiological mechanisms creates a weakly bound haem state which has increased spontaneous haem loss, which allows BAY 58-2667 to bind to the haem-free pocket. The results reported are convincing but do not detract from the proposal of a so-called pathological state of sGC that can be targeted by sGC activators. The oxidation of sGC and subsequent haem loss, creating a larger proportion of NO-insensitive sGC, is plausible in the ischaemia-reperfusion setting, resulting from increased ROS. This makes sGC activators attractive pharmacological tools when targeting sGC in this environment. However, quantifying these states and characterising their dynamics is not yet possible.

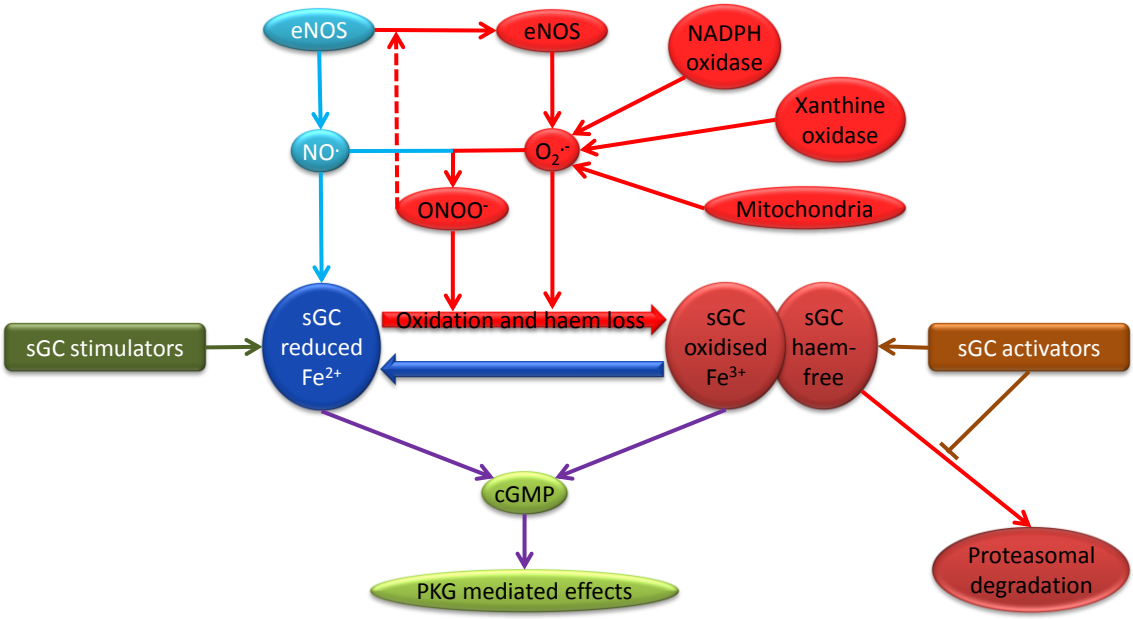

Figure 5.1 *Schematic demonstrating the redox balance between reduced and oxidised/haem-free sGC and the proposed mediators that could shift the redox towards the oxidised/haem-free state of sGC rendering it NO insensitive and a plausible target during early reperfusion. Blue colouring symbolises physiological activation of sGC, whilst red coloured components identify pathological stimuli oxidising sGC (Adapted from Stasch* et al. *2011).*

5.1.3 sGC degradation

The association of sGC with several proteins including heat shock protein (HSP) HSP90, HSP70 and eNOS has been described in aortic endothelial cells. This association has been suggested to facilitate the activation of sGC by NO produced by eNOS, preventing NO inactivation by superoxide and peroxynitrite (Venema *et al.*, 2003). As a result of these observations, Papapatropoulos *et al.* (2005), and Nedvetsky *et al.* (2008), reported that sGC protein levels are decreased in the presence of the HSP90 inhibitor geldanamycin. Studies by Mingone *et al.* (2008), and Nedvetsky *et al.* (2008), have demonstrated that haem-oxygenase 1 depletes sGC of haem and HSP90 stabilises long term expression of sGC. It has recently been suggested that the E_3 C terminus of heat shock cognate protein 70 (Hsc70)-interacting protein (CHIP), which interacts with the sGC/HSP90/HSP70 complex, plays a crucial role in mediating the ubiquitinylation of sGC (Xia *et al.*, 2007). In fact, Stasch *et al.* (2006), and Meurer *et al.* (2009), have reported that BAY 58-2667 is able to stabilise haem-free sGC and prevent CHIP-mediated ubiquitinylation, furthermore, increasing sGC protein levels above baseline. This effect is not seen by other cGMP generating or enhancing compounds such as BAY 41-2272, NO or PDE5 inhibitors (Hoffmann *et al.*, 2009).

5.1.4 Cinaciguat

Promising *in vivo* and *ex vivo* experimental studies led to a phase I clinical trial using BAY 58-2667 (Cinaciguat), with the ambition that it could be used for the treatment of acute decompensated heart failure. The authors of the trial reported that BAY 58-2667 had dose-dependent haemodynamic effects on diastolic blood pressure, mean arterial

pressure and HR and had desirable pre-load and after-load reducing cardiovascular effects (Frey *et al.*, 2008). Most recently phase IIb studies have been completed investigating the tolerability and efficacy of BAY 58-2667 given i.v. at high (150 and 100 μg/h) and low (50 and 25 μg/h) doses; however the results of these trials are yet to be published.

5.1.5 Hypotheses

The aim of this study was to investigate the effect of manipulating and targeting oxidised/haem-free sGC at reperfusion by means of a sGC activator. (4-(((4-carboxybutyl) [2- (5-fluoro-2-([4'-(trifluoromethyl) biphenyl-4-yl]methoxy)phenyl)ethyl] amino)methyl)benzoic acid) (BAY 60-2770) (see Figure 5.2), an aminodicarboxylic acid compound of the same class as BAY 58-2667 was selected for these experiments.

It was hypothesised that;

i. exogenous activation of sGC by BAY 60-2770 at reperfusion would limit infarct size;
ii. concomitant perfusion of BAY 60-2770 and the haem site oxidiser ODQ would afford greater protection than BAY 60-2770 perfusion alone;
iii. targeting both the reduced and oxidised/haem-free portions of sGC at reperfusion with the sGC stimulator BAY 41-2272 and the sGC activator BAY 60-2770 would afford greater protection than either compound alone;
iv. infarct limitation would be associated with elevated myocardial cGMP levels at reperfusion.

The specific experimental objectives were to:

1. Undertake a concentration response for BAY 60-2770 during early reperfusion in a rat isolated heart model
2. Concomitantly perfuse the haem site oxidiser ODQ, NO scavenger C-PTIO or sGC stimulator BAY 41-2272 with BAY 60-2770 during early reperfusion

3. Measure cGMP levels in LV and RV tissue samples from hearts perfused with BAY 60-2770

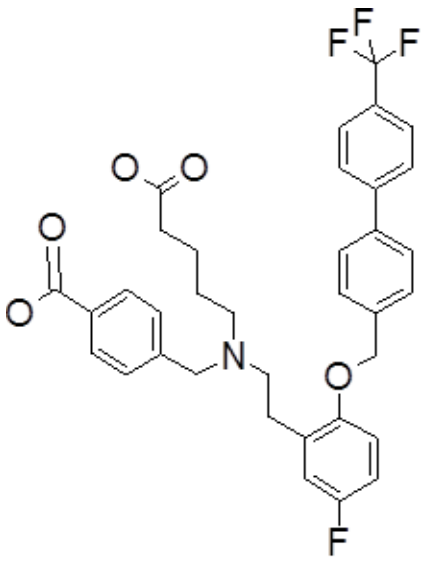

Figure 5.2 *Chemical structure of (4-(((4-carboxybutyl) [2- (5-fluoro-2-([4'-(trifluoromethyl) biphenyl-4-yl]methoxy)(phenyl)ethyl] amino)methyl)benzoic acid) (BAY 60-2770) (Schmidt, P.M., Schmidt, H.H.H.W., Hofmann, F., Stasch, J.-P. (2009) Handbook of Experimental Pharmacology: cGMP: Generators, Effectors and Therapeutic Implications Vol. 191, pp 315 Springer Berlin Heidelberg).*

5.2 Materials and Methods

5.2.1 Pharmacological compounds

All salts used to make modified KH solution were sourced from Fisher Scientific LTD (UK) and were of analytical or ultrapure quality. ODQ (selective sGC inhibitor) and C-PTIO (NO scavenger) were sourced from Tocris Bioscience (UK). BAY 41-2272 (NO-independent, haem-dependent sGC stimulator) and BAY 60-2770 (NO-independent, haem-independent sGC activator) were kind gifts from Bayer Pharma AG, (Wuppertal, Germany). BAY 41-2272 was dissolved in DMSO, the final concentration of DMSO in the KH was 0.05 % v/v.

5.2.2 Rat isolated heart perfusion

Rat isolated hearts were excised and retrograde perfused as described in Chapter 2. Once a period of 20 min haemodynamic stabilisation had been established, rat hearts were randomised and assigned to one of the groups mentioned in 5.2.3. All hearts were subjected to 35 min LDCA occlusion followed by 120 min of reperfusion unless otherwise stated.

5.2.3 Concentration response to BAY 60-2770: Study 1

A concentration response to BAY 60-2770 was carried out by perfusing hearts with BAY 60-2770 5 nM - 1 μM at reperfusion (see Figure 5.3). Although there are no published data using BAY 60-2770 in an isolated rat heart model, there are several reports of the structurally related sGC activator BAY 58-2667 being used. Krieg *et al.* (2009) reported infarct limitation when BAY 58-2667 was perfused at 50 nM and so a range

between 5 nM and 1 μM was chosen for BAY 60-2770. Rats used in this study were sourced from Harlan UK Ltd (Oxfordshire, UK).

Group 1, Control, (n=18). This group includes some hearts (n=4) that were perfused with the vehicle DMSO 0.05 % v/v from 30 min ischaemia until 10 min reperfusion. There was no statistical significant difference between control hearts perfused with or without DMSO vehicle and so all hearts were pooled in this group for statistical analysis.

Group 2, BAY 60-2770 1 μM, (n=7). BAY 60-2770 1 μM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 3, BAY 60-2770 500 nM, (n=8). BAY 60-2770 500 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 4, BAY 60-2770 50 nM, (n=8). BAY 60-2770 50 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 5, BAY 60-2770 5 nM, (n=6). BAY 60-2770 5 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 6, ODQ 2 μM, (n=6). ODQ 2 μM was perfused from 28 min ischaemia until 10 min reperfusion.

Group 7, ODQ 2 μM + BAY 60-2770 5 nM, (n=7). ODQ 2 μM was perfused from 28 min ischaemia and BAY 60-2770 5 nM from 30 min ischaemia, both until 10 min reperfusion.

Group 8, C-PTIO 30 μM (n=6). C-PTIO 30 μM was perfused from 28 min ischaemia until 10 min reperfusion.

Group 9, C-PTIO 30 µM + BAY 60-2770 5 nM, (n=6).C-PTIO 30 µM was perfused from 28 min ischaemia and BAY 60-2770 5 nM from 30 min ischaemia, both until 10 min reperfusion.

Group 10, BAY 41-2272 1 µM (n=6). BAY 41-2272 1 µM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 11, BAY 41-2272 1 µM + BAY 60-2770 5 nM (n=6). BAY 41-2272 1 µM and BAY 60-2770 5 nM were perfused from 30 min ischaemia until 10 min reperfusion.

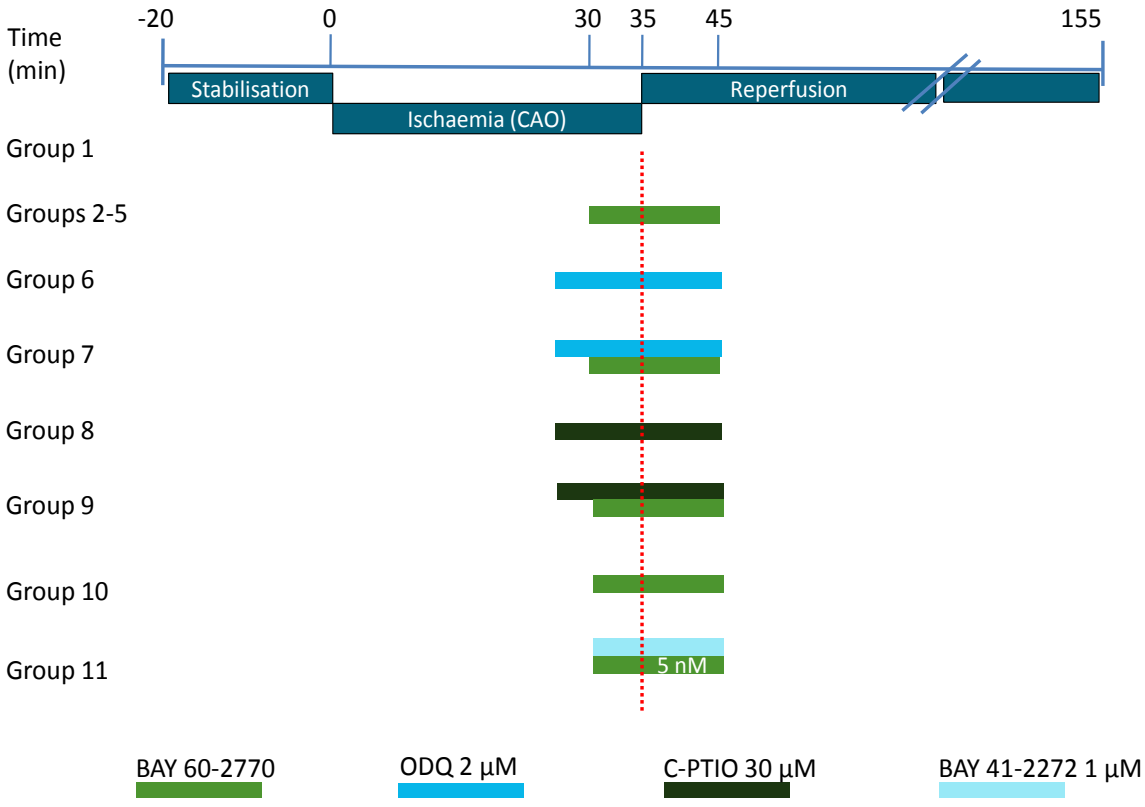

Figure 5.3 *Experimental protocol for groups 1-11 in Study 1. All hearts were stabilised for 20 min followed by 35 min regional ischaemia and then reperfused for 120 min. BAY 60-2770 was perfused at 5, 50, 500 nM and 1 µM.*

5.2.4 cGMP Radio immuno-sorbent assay: Study 2

To investigate further the pharmacological action of BAY 60-2770 and concomitant perfusion with BAY 41-2272, RIA was performed as described in Chapter 3.2.4. Tissue samples were prepared from rat hearts randomly assigned to one of 5 groups shown in Figure 5.4. Hearts that were subjected to regional ischaemia were treated as described for Study 1. Groups 1 and 2 have already been reported in Chapter 4. Animals used for RIA in this study were supplied by Charles River Laboratories Inc. (Maidenhead, UK).

Group 1, Naive, (n=6). Hearts were excised and washed in KH to remove any blood and then sectioned.

Group 2, 10 min reperfusion, (n=6). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min.

Group 3, 10 min reperfusion + BAY 60-2770 5 nM, (n=5). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BAY 60-2770 5 nM was perfused as described for Group 5 in 5.2.3.

Group 4, 10 min reperfusion + BAY 60-2770 5 nM + ODQ 2 µM, (n=5). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BAY 60-2770 5 nM and ODQ 2 µM were perfused as described for Group 7 in 5.2.3.

Group 5, 10 min reperfusion + BAY 41-2272 1 µM and BAY 60-2770 5 nM, (n=5). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BAY 41-2272 1 µM and BAY 60-2770 5 nM were perfused as described for Group 11 in 5.2.3.

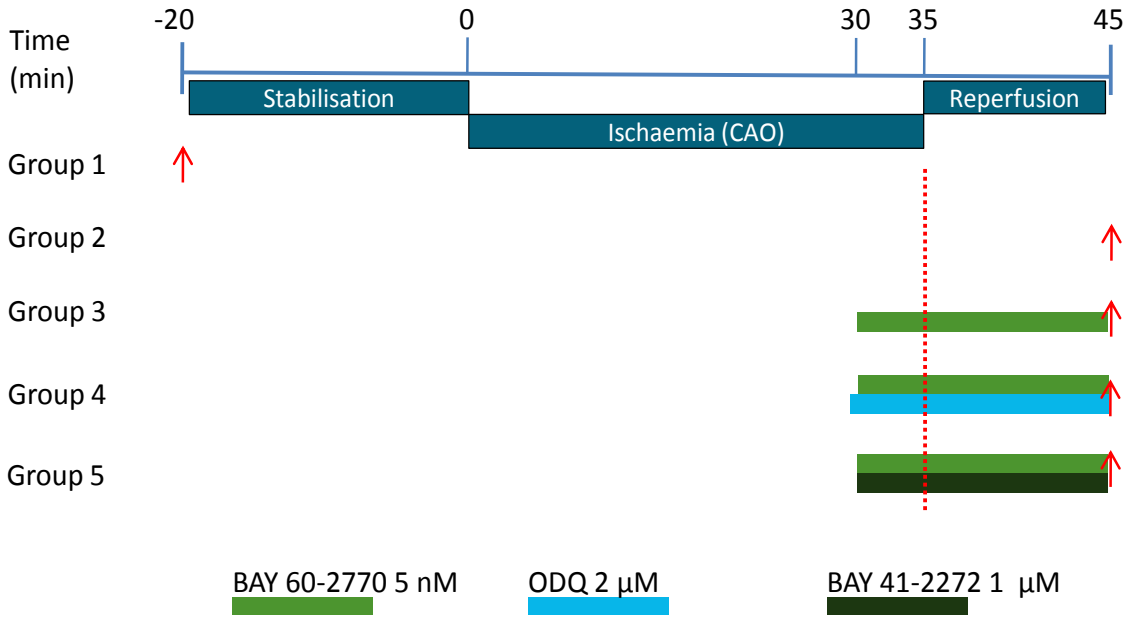

Figure 5.4 *Experimental protocol for hearts prepared for RIA in Study 2. Red arrows indicate time points at which hearts were sampled.*

5.3 Results

5.3.1 Summary of experiments

In Study 1 93 rats were used. Nine hearts were excluded from the study due to technical error; thus, 84 completed experiments are reported. In Study 2, 15 rats were used for RIA + 12 from Study 3 in Chapter 4. There were no exclusions and so data from 27 hearts are reported. The period of stabilisation before the onset of ischaemia was carried out to allow the hearts to stabilise and reach pre-determined criteria (see below). For a heart to be included and subjected to ischaemia, it had to achieve the following baseline cardiodynamic criteria;

CFR between 10 and 24 mL/min, LVEDP 5-10 mmHg, HR 200-350 BPM, LVDP greater than 50 mmHg and a steady sinus rhythm. Hearts were also excluded during analysis if there was inadequate delineation of the risk zone and infarcted tissue (poor staining).

5.3.2 Infarct size data: Study 1

Study 1 contains Langendorff perfusion experiments for the initial concentration response to BAY 60-2770; pharmacological oxidation of sGC with ODQ; inhibition of NO with the NO scavenger C-PTIO; and concomitant perfusion of both the sGC stimulator and activator. The area at risk for all hearts in all groups was between 38.6 and 55.8 % of the combined left and right ventricular tissue; there were no statistical differences between groups. Infarct size was expressed as a percentage of the risk zone, calculated as described in Chapter 2 and reported in Figures 5.5, 5.6, 5.7 and 5.8. Under control conditions (35 min ischaemia followed by 120 min reperfusion) hearts had infarct sizes of 33.0 $\pm$ 2.6 %

(n=18) compared to 22.0 ± 2.8 % (n=7) ($p<0.01$) for hearts treated with the highest concentration of BAY 60-2770. Treatment with 500 nM, 50 nM and 5 nM BAY 60-2770 also showed comparable infarct limitation (23.4 ± 2.2 % (n=8), 20.9 ± 2.3 % (n=8) and 19.7 ± 2.8 (n=6) % respectively). There were no statistical differences between BAY 60-2770 treated groups. All concentrations afforded between 29 and 41 % reduction in infarct size compared to controls, but there was no obvious concentration dependency in the range examined (Figure 5.5).

2 μM ODQ alone had no statistically significant effect on infarct size, (32.9 ± 2.2 % n=6). However, concomitant perfusion of ODQ with the lowest concentration of BAY 60-2770 reduced infarct size to 17.6 ± 2.0 % (n=7) (Figure 5.6). Perfusion with the NO scavenger C-PTIO produced infarct sizes similar to controls (32.0 ± 2.8 % n=6). Furthermore, C-PTIO did not abrogate the protection afforded by BAY 60-2770 (22.2 ± 2.2 % n=6) (Figure 5.7). Consistent with results reported in Chapter 4, perfusion of submaximal BAY 41-2272 (1 μM) did not afford statistically significant protection. Concomitant perfusion of BAY 60-2770 5 nM and BAY 41-2272 1 μM resulted in only a modest 21 % reduction in infarct size (24.8 ± 2.7 % n=6) (Figure 5.8).

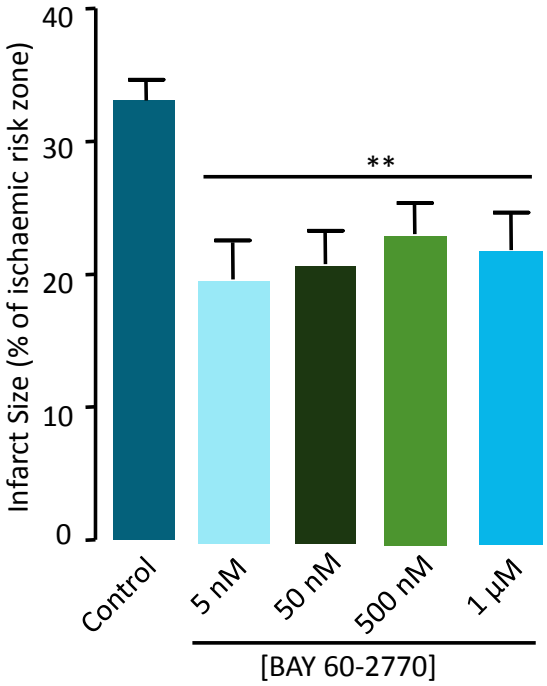

Figure 5.5 *Infarct size expressed as percentage of ischaemic risk zone for BAY 60-2770 concentration response (** p<0.01 vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-18).*

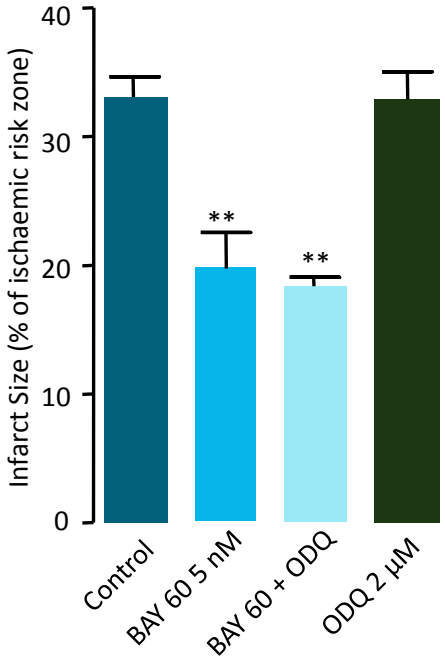

Figure 5.6 *Infarct size expressed as percentage of ischaemic risk zone for ODQ and ODQ + BAY 60-2770 treated hearts (** p<0.01 vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-18).*

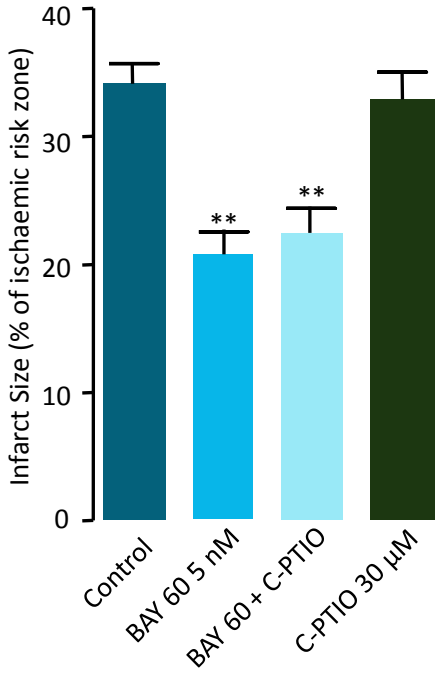

Figure 5.7 *Infarct size expressed as percentage of ischaemic risk zone for C-PTIO and C-PTIO + BAY 60-2770 treated hearts (** $p<0.01$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-18).*

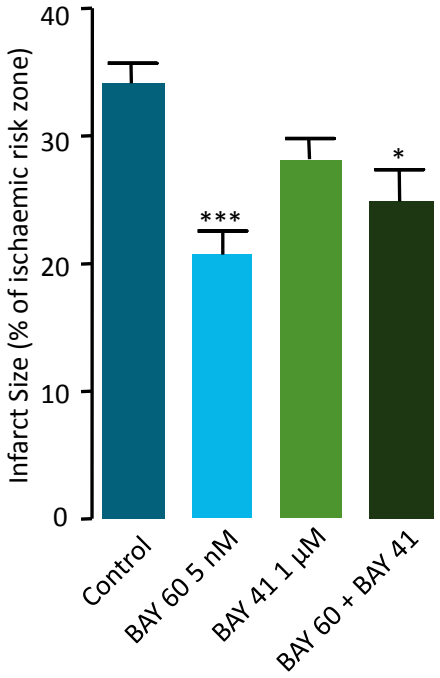

Figure 5.8 *Infarct size expressed as percentage of ischaemic risk zone for BAY 41-2272 and BAY 41-2272 + BAY 60-2770 treated hearts (* $p<0.05$, *** $p<0.001$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-18).*

5.3.3 Cardiodynamic data: Study 1

Baseline cardiodynamic data are presented in Table 5.1. There was no statistical difference between any of the parameters between groups. RPP dropped by as much as 85 % upon induction of ischaemia in all treatment groups, recovering partially throughout ischaemia and then dropping again upon reperfusion due to the tendency for the hearts to fibrillate. RPP recovered before gradually decreasing throughout reperfusion (Figures 5.9A, 5.10A, 5.11A & 5.12 A).

Upon induction of ischaemia, CFR dropped in all experiments by at least 30 % (Figures 5.9B, 5.10B, 5. 11B & 5.12B). CFR increased towards baseline once the ligature had been removed from the LDCA confirming successful reperfusion, and then decreased gradually during reperfusion.

Baseline cardiodynamic data. Study 1.

Treatment Group	n	CFR (mL/min)	HR (BPM)	LVDP (mmHg)	RPP (mmHg/min x 10^3)	Vol. LV and RV (cm^3)	Risk Zone Vol. (cm^3)	Risk Zone (% Vol. LV and RV)
Control	18	16.8 ± 0.6	315 ± 6	65.9 ± 3.1	20.7 ± 0.9	0.82 ± 0.02	0.39 ± 0.02	47.9 ± 2.2
BAY 60 1 µM	7	16.5 ± 1.1	336 ± 4	69.6 ± 4.8	23.4 ± 1.4	0.85 ± 0.02	0.46 ± 0.04	54.2 ± 4.1
BAY 60 500 nM	8	14.6 ± 0.6	322 ± 7	68.7 ± 3.7	22.1 ± 1.3	0.80 ± 0.05	0.34 ± 0.02	42.7 ± 1.8
BAY 60 50 nM	8	16.1 ± 1.0	331 ± 5	68.1 ± 4.0	22.5 ± 1.4	0.80 ± 0.02	0.40 ± 0.03	52.3 ± 3.3
BAY 60 5 nM	6	17.3 ± 1.0	319 ± 15	69.0 ± 5.0	22.1 ± 2.0	0.79 ± 0.03	0.36 ± 0.02	46.0 ± 2.1
ODQ	6	16.9 ± 0.9	316 ± 7	61.9 ± 1.7	19.6 ± 0.7	0.85 ± 0.04	0.37 ± 0.03	55.8 ± 3.5
BAY 60 + ODQ	7	15.5 ± 1.0	310 ± 7	69.5 ± 4.8	21.5 ± 1.7	0.86 ± 0.05	0.35 ± 0.05	43.1 ± 2.8
C-PTIO	6	14.7 ± 0.8	320 ± 6	67.4 ± 7.5	21.5 ± 2.4	0.85 ± 0.05	0.35 ± 0.01	41.7 ± 2.9
BAY 60 + C-PTIO	6	14.1 ± .07	333 ± 4	73.9 ± 6.6	24.7 ± 2.3	0.84 ± 0.01	0.38 ± 0.03	45.6 ± 3.4
BAY 41 1 µM	6	14.1 ± 0.6	313 ± 9	69.5 ± 4.4	21.6 ± 0.9	0.84 ± 0.02	0.32 ± 0.02	38.6 ± 1.6
BAY 60 + BAY 41	6	15.2 ± 1.6	323 ± 9	68.6 ± 5.3	22.1 ± 1.8	0.84 ± 0.02	0.38 ± 0.02	45.5 ± 2.9
Total	84							

Table 5.1 *Baseline cardiodynamic data. (BAY 60 = BAY 60-2770). No statistical differences between groups (ONE-way ANOVA + Newman-Keuls* post-hoc.

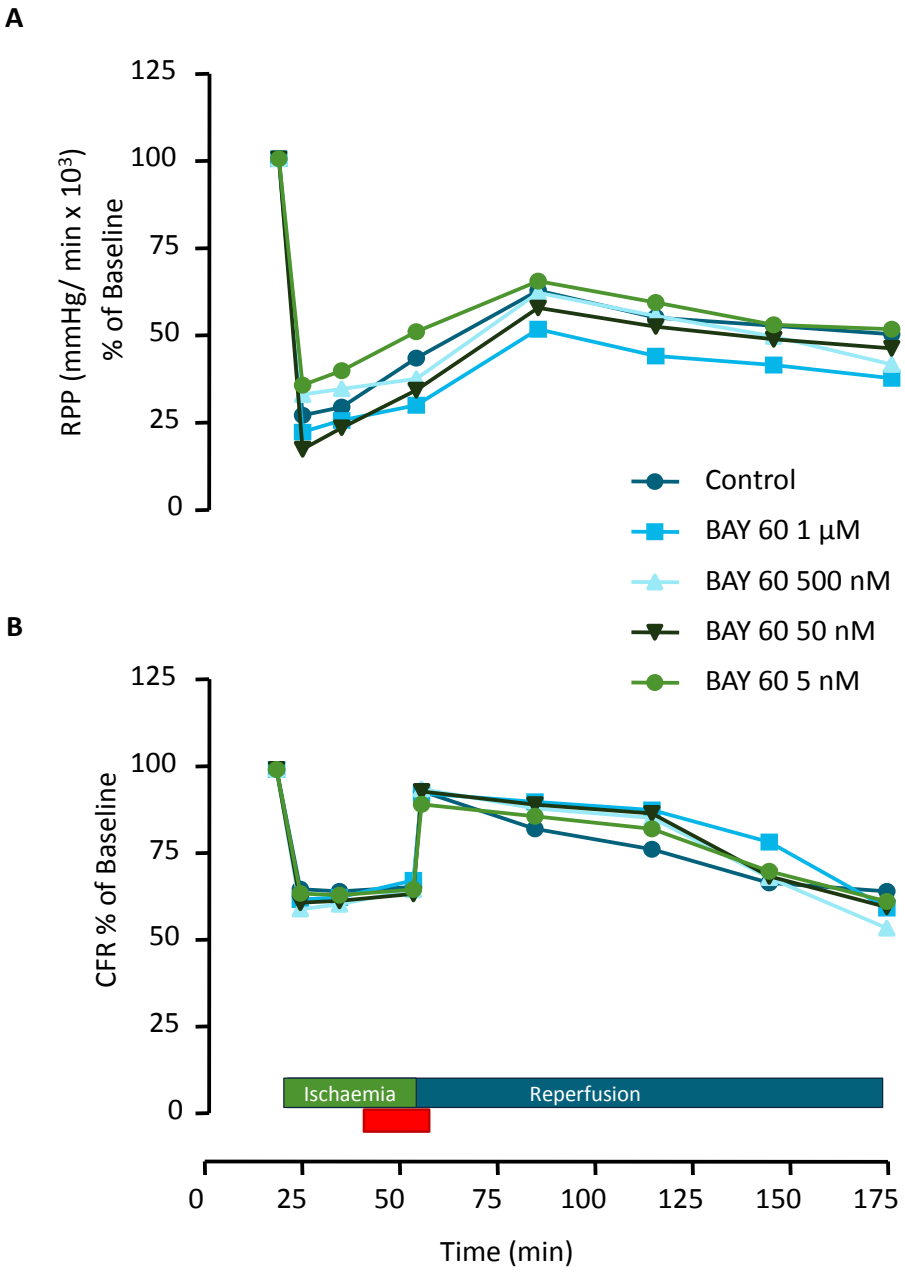

Figure 5.9 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 5.5. SEM bars have been removed for clarity. There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

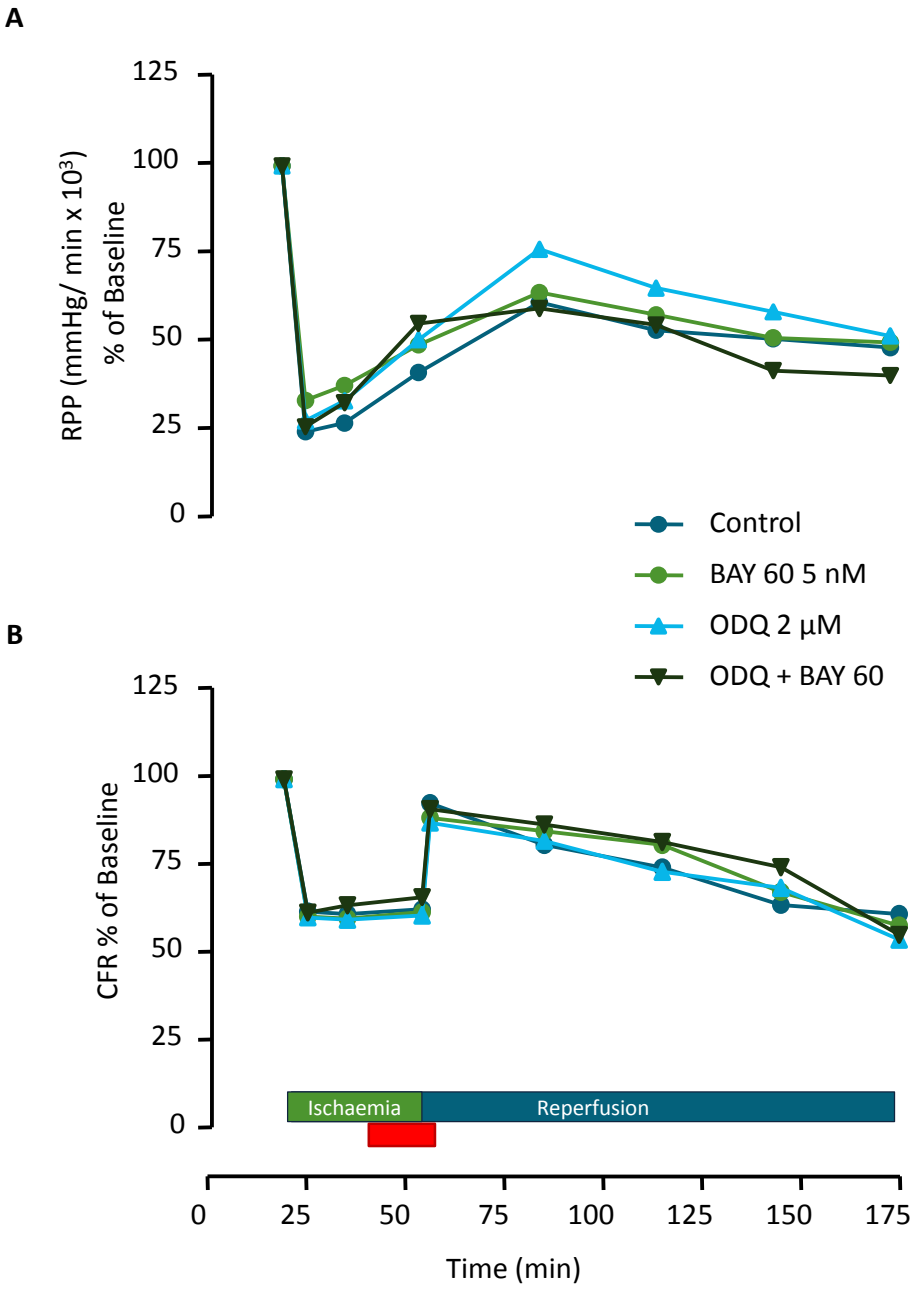

Figure 5.10 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 5.6. SEM bars have been removed for clarity There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

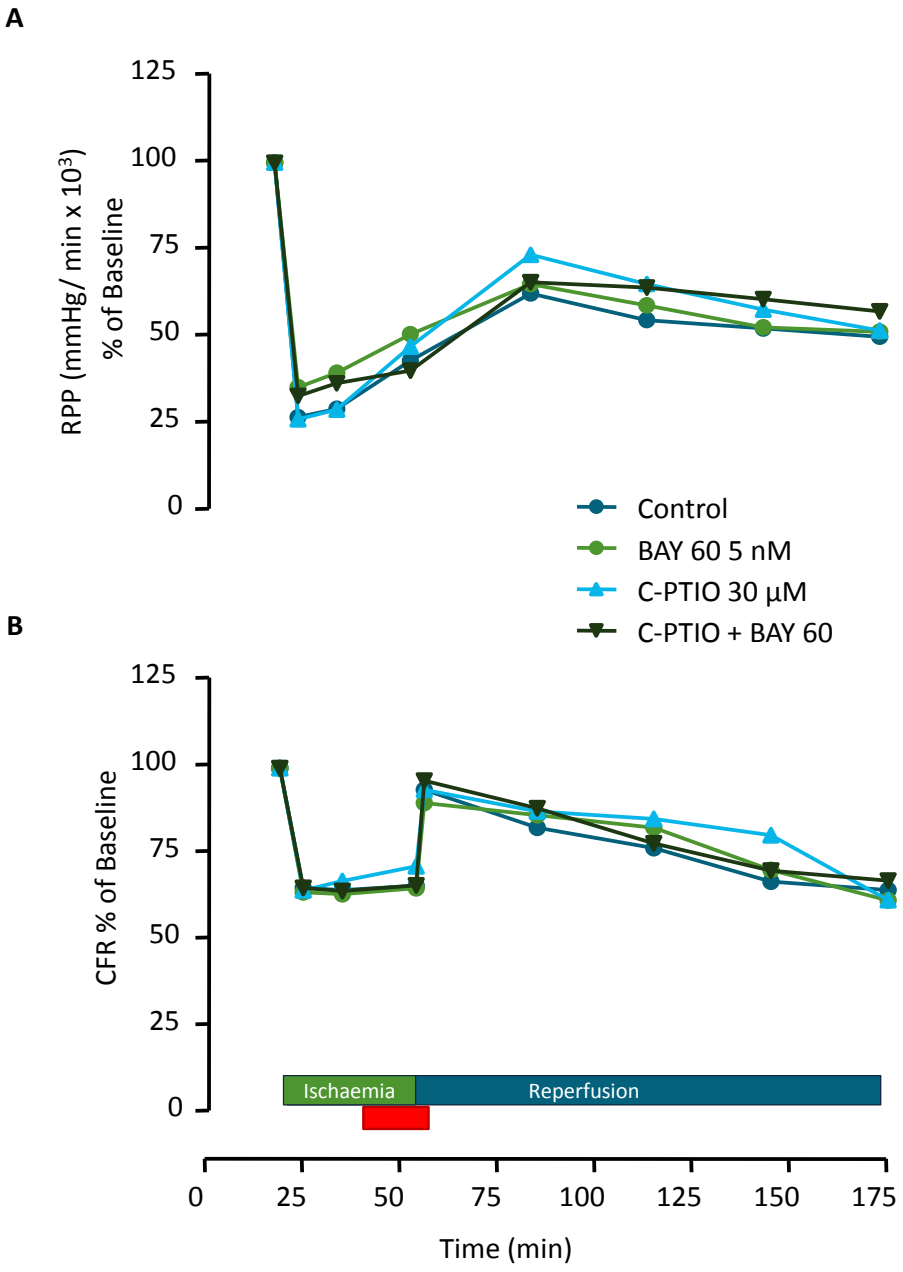

Figure 5.11 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 5.7. SEM bars have been removed for clarity. There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

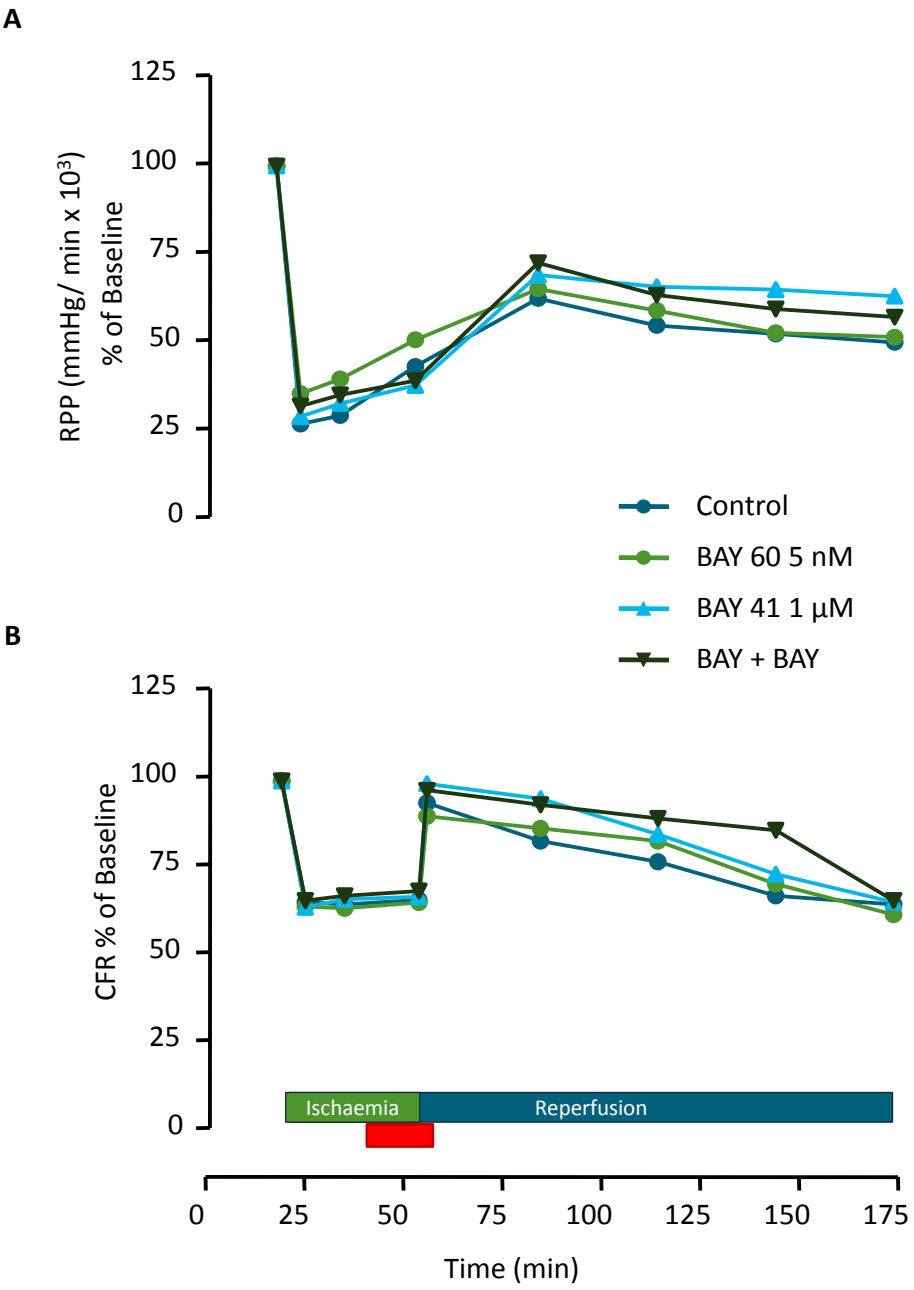

Figure 5.12 *Percentage change from baseline, rate pressure product [A] and coronary flow rate [B] from -1 min stabilisation [19 min] through 120 min reperfusion [175 min] for I-R experiments shown in figure 5.8. SEM bars have been removed for clarity. There were no statistically significant differences between treatment groups at each time point (repeated measures ANOVA). Red box indicates time at which drugs were perfused.*

5.3.4 RIA cGMP measurements: Study 2

Tissue levels of cGMP were measured in hearts that had been treated with or without BAY 60-2770 and subjected to 35 min regional ischaemia by occlusion of the LDCA. Measurements were also made in hearts perfused with BAY 60-2770 concomitantly with ODQ or BAY 41-2272. Tissue samples from concomitant BAY 60-2770 and ODQ perfused LV had cGMP levels 60 % higher than those perfused with BAY 60-2770 alone (20.16 ± 2.25 (n=5) vs. 12.60 ± 1.65 (n=5) fmol/mg tissue, $p<0.01$). An increase of 36 % from control was also seen in LV samples perfused with both the sGC stimulator and activator (17.11 ± 1.90 (n=5) vs. 12.60 ± 1.65 (n=5) fmol/mg tissue, $p<0.05$), an increase of 86 % compared to untreated hearts. RV samples which were not subjected to LDCA occlusion had cGMP levels greater than their adjacent LV in all groups (Figure 5.13 A and B).

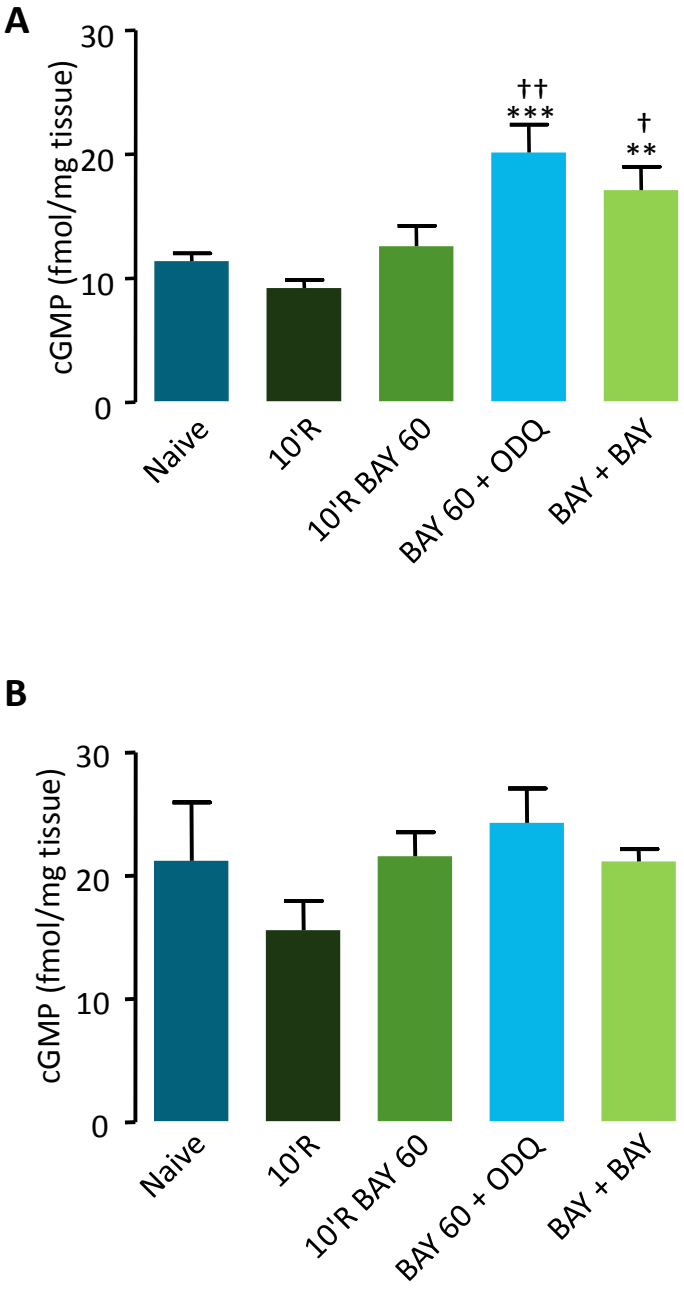

Figure 5.13 *cGMP levels in LV (A) and RV (B) heart samples perfused with or without BAY 60-2770 5 nM at reperfusion or concomitantly with ODQ 2 μM or BAY 41-2272 1 μM (**p<0.01, *** p<0.001 vs. 10'R, †p<0.05, †† p<0.01 vs. 10'R BAY 60) ONE-way ANOVA + Newman-Keuls post-hoc (n=5-6).*

5.4 Discussion

5.4.1 Summary of findings

The major findings of the studies in this chapter are:

i) BAY 60-2770 limited infarct size when given at reperfusion

ii) Concomitant perfusion with the haem oxidiser ODQ did not abrogate this protection

iii) Targeting both redox states of sGC could not afford additive/greater protection than either redox state alone

iv) Infarct size limitation was associated with elevated cGMP levels in the LV although there was not a direct correlation between elevated cGMP and reduced infarct size

The findings in these studies further support the notion that elevation of cGMP during early reperfusion contributes to infarct limitation. However, the data also suggest that there is not a linear relationship between cGMP production and infarct size.

5.4.2 Targeting oxidised/haem-free sGC limits infarct size

In order to explore the infarct limiting effects of targeting the oxidised/haem-free component of sGC, the sGC activator BAY 60-2770 was perfused during early reperfusion. All concentrations tested afforded significant infarct limitation suggesting that the EC50 must be in the picomolar range. However, to date there are no other published data using this compound in a model of ischaemia-reperfusion. The structurally similar compound BAY 58-2667 has been used in isolated rat and rabbit heart models, as well as *in situ* rabbit experiments (Cohen *et al.*, 2010; Krieg *et al.*, 2009). BAY 60-2770 differs only in the addition of a trifluoro group at the distal alkoxyaryl region and additional fluorine at the haem binding region. These modifications may increase the log P of

BAY 60-2770 and may increase its lipophilicity. Mechanistically it should act in the same way as BAY 58-2667, activating sGC by interaction with the α subunit at residue 371 and the β subunit between residues 231 and 310 (Stasch *et al.*, 2006). Using the structurally similar sGC activator, Krieg *et al.* (2009) demonstrated significant infarct reduction in the isolated rat heart when BAY 58-2667 was given at reperfusion. They reported almost twice the protection reported in this study; however in their experiments the sGC activator was perfused throughout reperfusion and they used a global ischaemia model. Whether the protective effects are attributed only during early reperfusion was not investigated. In an *ex vivo* rabbit heart model Cohen *et al.* (2010) reported reduction in infarct size of 54 %. However, BAY 58-2667 was perfused for 1 hour, starting 5 min prior to reperfusion. Although these results were obtained in a different species, it may suggest that infarct size reduction is greater when the sGC activator is perfused beyond the first few minutes of reperfusion.

Reductions in infarct size in hearts perfused with the sGC activator are supported by elevation in cGMP levels in hearts perfused with BAY 60-2770 at reperfusion. An increase of 27 % compared to untreated hearts was recorded suggesting that protection is mediated by increased cGMP levels at reperfusion. Krieg *et al.* (2009), also reported significant elevation in cGMP levels in isolated rat hearts perfused with BAY 58-2667. The increase they reported is much more pronounced than that observed in this study. They reported an elevation in cGMP levels of around 40 times compared to controls, although these elevated levels were 5 times lower than treated hearts in the current study. They did not

distinguish between left and right ventricle and infarct distribution in global ischaemia is less uniform, compared to the regional ischaemia model used here.

5.4.3 Oxidising sGC with ODQ potentiates BAY 60-2770 infarct limitation

In order to characterise the mechanistic action of BAY 60-2770 in the ischaemia-reperfusion setting further, the haem oxidiser ODQ was concomitantly perfused with BAY 60-2770 at reperfusion. Unsurprisingly, ODQ did not abrogate the protection afforded by BAY 60-2770, which is in support of the pharmacology of the sGC activators described by Stasch *et al.* (2006), which confirms the haem-independence of this class of compound. Furthermore, the infarct limitation in hearts perfused with both ODQ and BAY 60-2770 was similar to BAY 60-2770 alone. Again, this supports the work of Stasch *et al* (2006), who reported the ability of ODQ to potentiate BAY 58-2667 stimulated relaxation of aortic smooth muscle by oxidising reduced sGC rendering it sensitive to the sGC activator. Conversely, addition of BAY 58-2667 stabilised oxidised sGC from ubiquitin-mediated degradation (Hoffmann *et al.*, 2009). It is speculated by several research groups that this observation may be of clinical relevance if there is an increased pool of oxidised sGC, a conjecture being investigated in several cardiovascular pathologies (Schmidt *et al.*, 2009).

In support of these results and the published studies documenting the pharmacokinetics of sGC activators, cGMP levels were elevated by 120 % compared to untreated controls (Figure 5.13A). This is in agreement with Stasch *et al.* (2006), who reported that ODQ stabilises sGC and potentiates cGMP production when BAY 58-2667 was also added.

5.4.4 NO-independence of BAY 60-2770 mediated infarct limitation

Using the NO scavenger C-PTIO, it was demonstrated that BAY 60-2770 mediated protection was independent of NO. Concomitant perfusion of the NO scavenger and sGC activator afforded protection not dissimilar to that seen with BAY 60-2770 alone. There was a tendency for infarct size to be slightly greater in hearts perfused with both compounds, which may suggest that some of the infarct limitation is due to NO mediated sGC activation. Previous cell based studies have reported that the aminodicarboxylic acids produce cGMP additively to NO, not synergistically like the Bayer sGC stimulators (Schmidt *et al.*, 2003; Stasch *et al.*, 2002b; Stasch *et al.*, 2006). Therefore, it could be speculated the difference between the infarct size in the BAY 60-2770 group and that perfused concomitantly with C-PTIO is attributable to NO activated sGC.

In support of the NO-independence shown in this study, Krieg *et al.* (2009) reported that concomitant perfusion of BAY 58-2667 and the NOS inhibitor L-NAME did not significantly abrogate protection afforded by the sGC activator alone in an isolated rat heart model. However, a recent study by Downey's laboratory demonstrated that co-perfusion of L-NAME abrogated over 90 % of the infarct limitation afforded by BAY 58-2667 alone in an isolated rabbit heart model (Cohen *et al.*, 2010). Whether this divergence in observation is due to species differences remains to be elucidated; however, the concentration of L-NAME used in both studies was 200 μM, double the concentration our laboratory has used in the isolated rat heart (Burley & Baxter, 2007; D'Souza *et al.*, 2003).

5.4.5 Targeting both oxidised and reduced sGC

Based on the emerging evidence that there are both reduced and oxidised/haem-free sGC pools present in tissues (Stasch *et al.*, 2006), concomitant perfusion of both a sGC stimulator, BAY 41-2272 and a sGC activator, BAY 60-2770 was explored to determine whether targeting both states of sGC could limit infarct size beyond either drug perfused alone. Results suggest that targeting both states of sGC does not afford additive infarct limitation; in fact, infarct sizes for hearts perfused with both compounds were larger than hearts perfused with the sGC activator alone. Surprisingly cGMP levels in LV tissue samples perfused with both compounds at reperfusion are 36 % higher than those of tissue samples perfused only with the sGC activator. This suggests that targeting both states of sGC produces more cGMP; however, this does not translate into infarct limitation. I would speculate that cGMP produced by each of the sGC states is produced at spatially different locations within the cell. Whether in attempting to regulate the distal signals and activate PKG, phosphodiesterase prevent signalling remains to be elucidated. It is however well documented that only small increases in cGMP can elicit physiological responses (Evgenov *et al.*, 2006; Stasch *et al.*, 2002b). Quantification of cGMP produced by each state of sGC as well as the ratio of the forms of sGC would be desirable to explain these observations further.

5.5 Conclusions

Taken together, results from Studies 1 and 2 demonstrate that activation of sGC using the NO-independent, haem-independent activator, BAY 60-2770 limit infarct size when given during early reperfusion. Furthermore, this protection is mediated, in part by an increase in cGMP levels. The data in this study also demonstrate that protection can be afforded independently of endogenous NO and can be increased by oxidising the haem group of sGC. The hypothesis regarding targeting both states of sGC at reperfusion was refuted, yet the data suggest that total tissue cGMP production is not linearly linked to infarct limitation.

Chapter 6
sGC component of pGC mediated protection of reperfused myocardium

6.1 Introduction

6.1.1 Natriuretic peptides as an indicator of infarct size

As mentioned in Chapter 1, NPs play a fundamental role in cardiovascular homeostasis, their release being primarily regulated by pressure overload and myocardial stretch. In 1991, Mukoyama *et al.* reported for the first time that BNP levels were elevated during myocardial infarction. It was later reported that elevation of BNP correlates with infarct size, both in lethal (Arakawa *et al.*, 1994) and sublethal ischaemic insult (Kyriakides *et al.*, 2000). Since then several experimental studies have documented elevation of ANP (Arad *et al.*, 1994; Chen *et al.*, 1993; Zhang *et al.*, 2004) and BNP (D'Souza *et al.*, 2003; Hama *et al.*, 1995) in models of myocardial ischaemia. Most recently, numerous clinical studies confirmed the usefulness of measuring BNP levels in patients presenting with STEMI, and suggest that they are a good indicator of prognosis and infarct size (Fertin *et al.*, 2010; Neyou *et al.*, 2011; Seo *et al.*, 2011).

6.1.2 Natriuretic peptides during preconditioning

Knowledge that NP levels are closely linked to infarct size led to experimental studies investigating the infarct limiting properties of NPs given prior to, or post ischaemia. Previous work by our laboratory demonstrated that perfusion of BNP just before and throughout ischaemia in an isolated rat heart model of regional ischaemia reduced infarct size in a concentration dependent manner correlating with myocardial cGMP elevation (D'Souza *et al.*, 2003). Further exploration demonstrated that this protection was inhibited by K_{ATP} channel inhibitor, 5-HD (D'Souza *et al.*, 2003). Both ANP (Okawa *et al.*,

2003), and CNP (Hobbs *et al.*, 2004), have also been demonstrated to limit infarct size when administered prior to normothermic global ischaemia.

6.1.3 Natriuretic peptides at reperfusion

More recent studies have identified that NPs administered at reperfusion afford protection in both rat and rabbit models of ischaemia-reperfusion. Downey's group reported that administration of ANP just prior to reperfusion limited infarct size in the rabbit heart. They also demonstrated that the protection afforded required K_{ATP} activity as inhibition of the channel with 5-HD abrogated the protective effects of ANP (Yang *et al.*, 2006). BNP was also shown to limit infarct size in an *in situ* rat heart model, limiting infarct size in a concentration dependent manner (Ren *et al.*, 2007). Our laboratory reported similar results in an *ex vivo* rat heart model, demonstrating concentration dependent infarct limitation with BNP, which like previous studies was dependent on K_{ATP} channel activity (Burley & Baxter, 2007). Furthermore, we reported that NPs play a role in post-conditioning mediated protection as the non-specific NPR-A/NPR-B antagonist isatin abolished the infarct limitation afforded by a 6 x 10 sec post-conditioning protocol (Burley & Baxter, 2007). Most recently, George *et al.* (2010), reported that perfusion of BNP for 7 days post AMI significantly improved LV function and decreased LV remodelling in the rat heart.

6.1.4 PI3K/Akt and eNOS in natriuretic peptide mediated infarct limitation

Infarct limitation afforded by NPs has been demonstrated to require K_{ATP} activity as described above. It has also been demonstrated that protection was mediated by elevation of cGMP and distal PKG targets converging on the same effectors as NO/sGC signalling,

i.e. regulating calcium via PLB and L-type calcium channels and potassium efflux through the K_{ATP} channel (D'Souza *et al.*, 2003; Lincoln *et al.*, 2001; Lucas *et al.*, 2000).

Other signalling pathways have been suggested to play a part in NP mediated infarct limitation. D'Souza *et al.* (2004) reported that concomitant perfusion of L-NAME prior to LDCA occlusion abrogated the protection afforded by BNP alone. Similarly, perfusion of ODQ abolished BNP induced infarct limitation (D'Souza *et al.*, 2004). These results suggested that activation of NOS and sGC are required to afford BNP protection. Whether cGMP generated by pGC, sGC or both was needed to afford BNP protection remains unclear and little has been done to investigate this cardioprotective mechanism further. Similar observations were made in a reperfusion targeted treatment by Ren *et al.* (2007), who report that L-NAME reversed the protection afforded by BNP when given just prior to and throughout reperfusion in an *in situ* rat heart model. This was supported by Western blotting analysis that demonstrated that a cardioprotective dose of BNP significantly increased eNOS expression. The reversal of BNP induced infarct limitation by L-NAME at reperfusion has been demonstrated in an *ex vivo* rat heart model in our laboratory (Burley & Baxter, 2007). Concomitant perfusion of BNP and L-NAME just prior to and for the first 10 min of reperfusion abrogated the protection afforded by BNP and in fact increased infarct size beyond controls. In an isolated perfused rabbit heart model, Yang *et al.* (2006) demonstrated that the upstream activators of eNOS in the proposed RISK pathway are also essential for ANP mediated protection. Wortmannin, an inhibitor of PI3K, and PD98059, an inhibitor of ERK, independently blocked ANP mediated infarct limitation in the rabbit heart. Furthermore they reported that

concomitant perfusion of ODQ and ANP abrogated ANP mediated infarct limitation. Taken together these data strongly suggest that a pathway converging on sGC (PI3K/Akt/eNOS/NO/sGC) plays a critical role in NP mediated infarct limitation. However, biochemical confirmation of these observations is lacking. These studies also question whether there is a direct pGC/cGMP/PKG component to the RISK pathway or whether pGC mediated protection is mediated indirectly instead via PI3K/Akt/eNOS/NO/sGC.

6.1.5 Differential regulation and action of pGC and sGC mediated cGMP

Although it has long been documented that cGMP is produced by two forms of GC, a growing body of research suggests that the cGMP produced by different enzymes mediates different responses, regulated by specific PDEs (Fischmeister *et al.*, 2006). As mentioned previously in Chapter 1, pGC and sGC can mediate different calcium handling in cardiac myocytes. Castro *et al.* (2006), suggest that this differential action is a result of spatially separated pools of cGMP within the cell acting locally to elicit a response.

Using purified adult rat ventricular myocytes , Fischmeister's laboratory demonstrated the differential action of PKG on cGMP measured by recording the activity of the wild-type rat olfactory nucleotide-gated channel (Castro *et al.*, 2010). Their results suggest that PKG limits the production of sGC-mediated cGMP via PDE5 stimulation, in contrast to pGC mediated cGMP production, which is increased. They proposed that PKG initiates a positive feedback on pGC to produce more cGMP, compartmentalised by PDE2. They further suggested that PKG activated by sGC mediated cGMP production phosphorylates PDE5, limiting diffusion of cGMP locally (Castro *et al.*, 2010). These data support the

compartmentalisation theories of cGMP production. Furthermore, it demonstrates that pGC and sGC may elicit different responses through cGMP/PKG, highlighting the need to explore where and how pGC and sGC signalling converges, specifically during early reperfusion.

6.1.6 Hypotheses

The aim of this study was to investigate the signalling cascade between pGC and sGC and whether elevation of cGMP by pGC during ischaemia-reperfusion is mediated by recruiting PI3K/Akt/eNOS/NO/sGC.

It was hypothesised that;

i. Perfusion of BNP during early reperfusion would elevate phosphorylated Akt and this elevation would be inhibited by the PI3K inhibitor Wortmannin;
ii. BNP would elevate NO levels in the myocardium;
iii. BNP mediated elevation of cGMP during early reperfusion would be blocked by ODQ

The specific experimental objectives were to:

1. Semi-quantify the change in pAkt (by Western blot) in normoxic and ischaemia-reperfused rat isolated perfused heart
2. Measure cGMP levels in hearts perfused with BNP
3. Measure NOx by OBC in coronary effluent collected from hearts perfused with BNP

6.2 Materials and Methods

6.2.1 Pharmacological compounds

All salts used to make modified KH solution were sourced from Fisher Scientific LTD (UK) and were of analytical or ultrapure quality. ODQ (selective sGC inhibitor) was sourced from Tocris Bioscience (UK). Wortmannin (PI3K inhibitor) and rat BNP-32 were purchased from Sigma-Aldrich (UK).

6.2.2 Rat isolated heart perfusion

Rat isolated hearts were excised and retrograde perfused as described in Chapter 2. Once a period of 20 min haemodynamic stabilisation had been established, rat hearts were randomised and assigned to one of the groups mentioned in 6.2.3. All hearts were subjected to 35 min LDCA occlusion followed by 10 min of reperfusion unless otherwise stated.

6.2.3 Western blotting: Study 1

Animals used in this study were sourced from Harlan UK Ltd (Oxfordshire, UK). Two sets of samples were prepared for Western blotting. Groups 1-4 underwent stabilisation and were treated with 15 min of normoxic drug perfusion (Figure 6.1), whilst groups 5-8 underwent a standard ischaemia-reperfusion protocol and were treated from 5 min prior to until 10 min after reperfusion (Figure 6.2).

To determine the effects of BNP on PI3K/Akt activity, Western blotting was performed to analyse the phosphorylation of Akt, using polyclonal immunoglobulins. As described previously, Akt is a kinase proposed to be involved in the RISK pathway of cell survival against reperfusion injury.

Hearts that had been perfused for Western blotting analysis were sectioned as described in Chapter 3.2.4 and each portion was further divided into 4-5 pieces, blotted dry, snap frozen in liquid nitrogen, and stored at -80 ° C until required for analysis. Samples were powdered by hand with a mortar and pestle and kept cool on a bed of dry ice. The powdered tissue was then homogenised in lysis buffer with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, UK). The homogenates were sonicated for 3 sec and then centrifuged for 15 min at 13,000 g. The supernatant was aliquoted and kept on ice. Total protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, UK). Protein quantification was achieved by the reaction of protein with cupric (Cu^{2+}) reduced to cuprous (Cu^{+}) cations, in an alkaline biuret solution. A purple chromophore is formed when the cuprous ions react with BCA. 10 µL of aliquoted sample was mixed with 200 µL of working reagent in a 96-well plate and incubated for 30 min at 37 °C. Absorbance at 540 nm was measured (Dynex Technology MRX TC Revolution 4.22, UK). Protein concentration was determined and calculated using a standard curve produced from different concentrations (0, 20, 250, 500, 10^3, $10^{3.3}$, $10^{3.6}$, $10^{3.9}$ and 10^4 µg/mL) of BSA in lysis buffer.

For each sample ((tissue homogenate in Laemmli sample buffer (4 % w/v sodium dodecyl sulphate (SDS), 20 % v/v glycerol, 10 % 2-mecaptoethanol, 0.0004 % bromophenol blue, 0.125 m Tris HCl), 1:1 ratio), 30 µg of protein was loaded. Proteins were then separated using a mini-PROTEAN 3 cell and tank system (Bio-Rad Laboratories Inc., UK) and wet transferred to polyvinylidene difluoride membrane (PVDF), (Hybond P, Amersham Biosciences Ltd., UK). The PVDF membrane was then immersed in 5 % w/v

skimmed milk powder solution for 3 h, to reduce the amount of non-specific binding of proteins during subsequent steps of the assay. PVDF membranes were probed with pAkt or tAkt primary antibodies (1:1000) at 4 °C overnight on a roller plate. PVDF membranes were washed in TBS-Tween (3 x 15 mini washes), then probed with a goat-anti rabbit horseradish peroxidase (HRP)-conjugate secondary antibody (1:10,000) for 1 hour, followed by further washing steps as described above. Bands were detected using Western blotting Luminol reagent (GE Healthcare, UK) which was evenly spread over the PVDF membranes and placed in a developing cassette for 5 min. Using double coated film sensitive to both blue and green light chemiluminescence systems, images were developed. Films were left to dry for two hours before being scanned using a Canon MP610 all in one printer, scanner, copier. Densitometry was performed using computer imagery software ImageJ 1.45q. Mean grey values were then converted into optical densities using a standard curve produced from a Kodak calibrated step tablet with known optical density values for each grade of colour.

Normoxic treatment protocols

Group 1, Control, (n=5). 20 min stabilisation followed by 15 min perfusion.

Group 2, BNP 10 nM, (n=5). 20 min stabilisation followed from 15 min perfusion with BNP 10 nM (concentration determined by previous studies in our laboratory).

Group 3, Wortmannin 100 nM, (n=5). 20 min stabilisation followed by 15 min perfusion with Wortmannin 10 nM (concentration determined from previous studies in our laboratory).

Group 4, BNP 10 nM + Wortmannin 100 nM, (n=5). 20 min stabilisation followed by 15 min concomitant perfusion of BNP 10 nM and Wortmannin 100 nM.

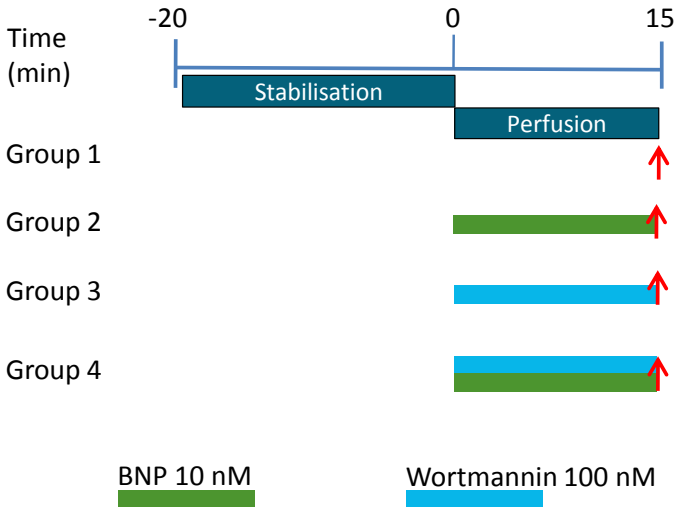

Figure 6.1 *Experimental protocol for groups 1-4 in Study 1. All hearts were stabilised for 20 min followed by 15 min normoxic perfusion. Red arrows indicate time points at which hearts were sampled for Western blotting.*

Ischaemia-reperfusion treatment protocols

Group 5, Control, (n=5). 20 min stabilisation followed by 35 min LDCA occlusion and then 10 min reperfusion.

Group 6, BNP 10 nM, (n=5). BNP 10 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 7, Wortmannin 100 nM, (n=5). Wortmannin 100 nM was perfused from 30 min ischaemia until 10 min reperfusion.

Group 8, BNP 10 nM + Wortmannin 100 nM (n=5). BNP 10 nM + Wortmannin 100 nM were perfused from 30 min ischaemia until 10 min reperfusion.

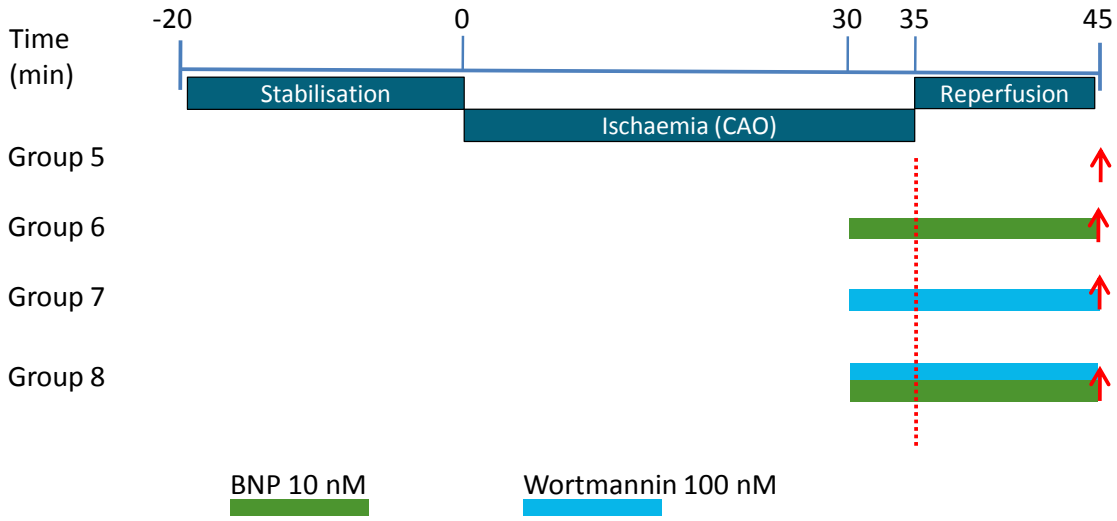

Figure 6.2 *Experimental protocol for groups 5-8 in Study 1. All hearts were stabilised for 20 min followed by 35 min regional ischaemia and then reperfused for 10 min. Red arrows indicate time points at which hearts were sampled for Western blotting.*

6.2.4 cGMP Radio immuno-sorbent assay: Study 2

To investigate the cGMP elevating action of BNP given during early reperfusion RIA was carried out as described in Chapter 3.2.4. Tissue samples were prepared from rats randomly assigned to one of four groups described below (see Figure 6.3). Animals used for RIA in this study were supplied by Charles River Laboratories Inc. (Maidenhead, UK).

Group 1, Naive, (n=6). Hearts were excised and washed in KH to remove any blood and then sectioned.

Group 2, 10 min reperfusion, (n=6). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min.

Group 3, 10 min reperfusion + BNP 10 nM, (n=5). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BNP 10 nM was perfused as described for Group 6 in 6.2.3.

Group 4, 10 min reperfusion + BNP 10 nM + ODQ 2 µM, (n=5). Hearts were stabilised, subjected to LDCA occlusion for 35 min and reperfused for 10 min. BNP 10 nM and ODQ 2 µM were perfused concomitantly from 30 min ischaemia to 10 min reperfusion.

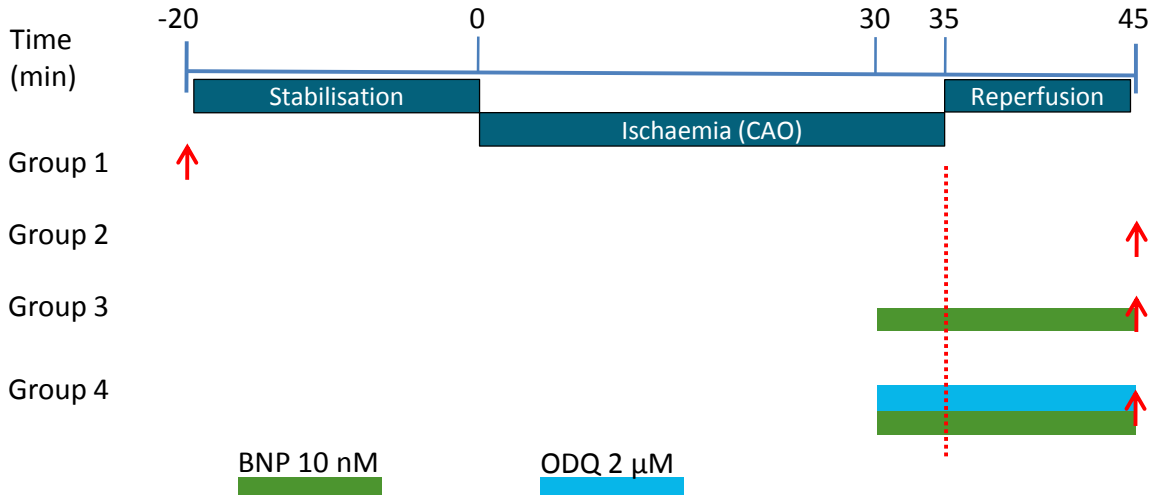

Figure 6.3 *Experimental protocol for hearts prepared for RIA in Study 2. Red arrows indicate time points at which hearts were sampled for cGMP measurement.*

6.2.5 NO_2^- measurement by ozone based chemiluminescence: Study 3

To explore downstream targets of BNP signalling, OBC was employed to investigate its action on NO levels in the myocardium as described in Chapter 4.2.6. Hearts were cannulated and stabilised as described in Chapter 2 and then perfused for a further 15 min either with KH buffer alone or for 15 min with BNP 10 nM. Coronary effluent was collected for 2 sec on completion of treatment and snap frozen in liquid nitrogen (see Figure 6.4).

Group 1, Control, (n=4). Hearts were stabilised, followed by a further 15 min perfusion. Coronary effluent was then collected.

Group 2, 15 min BNP 10 nM, (n=4). Hearts were stabilised, followed by 15 min BNP 10 nM perfusion. Coronary effluent was then collected.

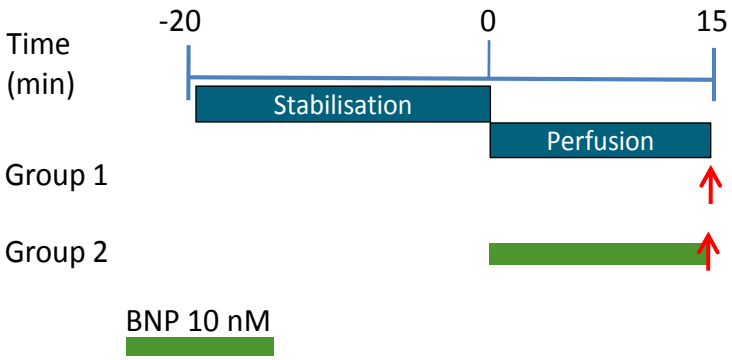

Figure 6.4 *Experimental protocol for OBC Study 3. Red arrows indicate time points at which coronary effluent was collected for OBC.*

6.3 Results

6.3.1 Summary of experiments

In Study 1 44 rats were used. Four hearts were excluded from the study due to technical error; thus, 40 completed experiments are reported. In Study 2, 10 rats were used for RIA + 12 from Study 3 in Chapter 4. There were no exclusions and so data from 22 hearts are reported. In Study 3, 8 rats were used for OBC measurements, with no exclusions so data for 8 experiments are reported. The period of stabilisation before the onset of ischaemia was carried out to allow the hearts to stabilise and reach pre-determined criteria (see below). For a heart to be included and subjected to ischaemia, it had to reach the following baseline cardiodynamic parameters;

CFR of between 10 and 24 mL/min, LVEDP of 5-10 mmHg, HR of 200-350 BPM, LVDP greater than 50 mmHg and a steady sinus rhythm.

6.3.2 Western blotting analysis: Study 1

Total and phosphorylated (Ser 473) Akt immunoreactivity measurements were made to evaluate PI3K/Akt activity upon treatment with BNP (Figure 6.5). Following 20 min stabilisation and 15 min perfusion of BNP, pAkt/tAkt ratio increased compared to controls, but did not reach statistical significance ($p=0.163$ for ONE-way ANOVA, $n=5$). Measurements made in tissue samples taken from hearts that had undergone 35 min LDCA occlusion and 10 min reperfusion showed a similar pattern of results. BNP treatment resulted in an increased pAkt/tAkt ratio compared to control, ($p<0.05$ vs. Control, $n=5$). Furthermore perfusion of Wortmannin alone and concomitantly with BNP limited phosphorylation of Akt ($p<0.05$ vs. BNP, $n=5$) (Figure 6.5B)

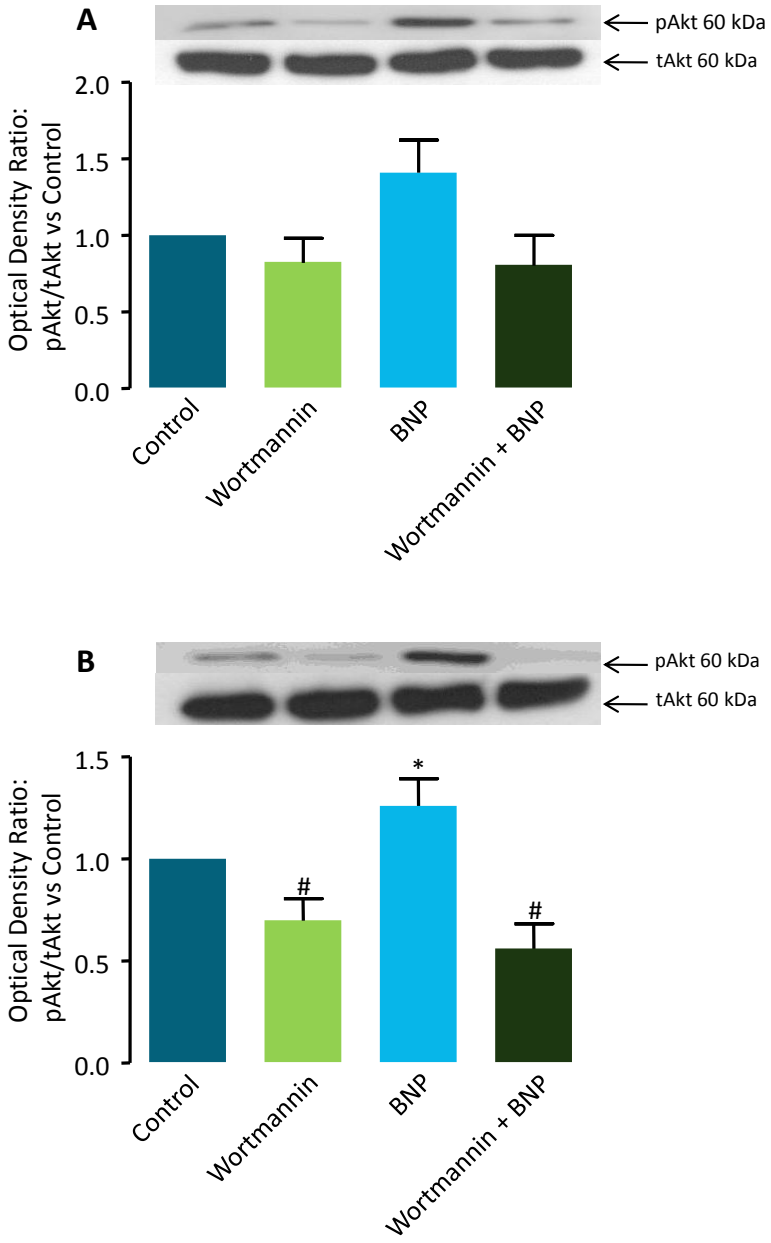

Figure 6.5 *Representative Western blots illustrating changes in pAkt in myocardial tissue samples from stabilised [A] and ischaemia-reperfused [B] hearts treated with BNP 10 nM, Wortmannin 100 nM or BNP and Wortmannin concomitantly. Perfusion of BNP during reperfusion increased the pAkt/tAkt ratio (*$p<0.05$ vs. Control). Wortmannin perfused alone or concomitantly with BNP at reperfusion abrogated this increase (# $p<0.05$ vs. BNP). ONE-way ANOVA + Newman-Keuls post-hoc (n=5)*

6.3.3 RIA cGMP measurements: Study 2

Tissue levels of cGMP were measured in hearts that had been treated with or without BNP 10 nM and subjected to 35 min regional ischaemia by occlusion of the LDCA. Measurements were also made in hearts perfused with BNP concomitantly with ODQ 2 μM. In LV samples, cGMP levels were reduced in tissue that had been subjected to ischaemia-reperfusion compared to naïve tissue. In LV, perfusion of BNP showed a 25 % increase in the mean from 9.20 ± 0.70 (n=6) to 11.49 ± 1.40 (n=5) fmol/mg tissue, compared to untreated controls. Similar differences between means (23 %) were seen in tissue samples perfused with both BNP and ODQ; however statistical significance was not reached (p=0.329 for ONE-way ANOVA, n=5). RV samples, which were not subjected to LDCA occlusion, had cGMP levels greater than their adjacent LV in all groups. There was a 23 % decrease from 15.60 ± 2.36 (n=5) to 11.97 ± 1.62 (n=5) fmol/mg tissue of cGMP in RV tissue samples perfused with BNP compared to control (p=0.218 for ONE-way ANOVA) (Figure 6.6 A and B).

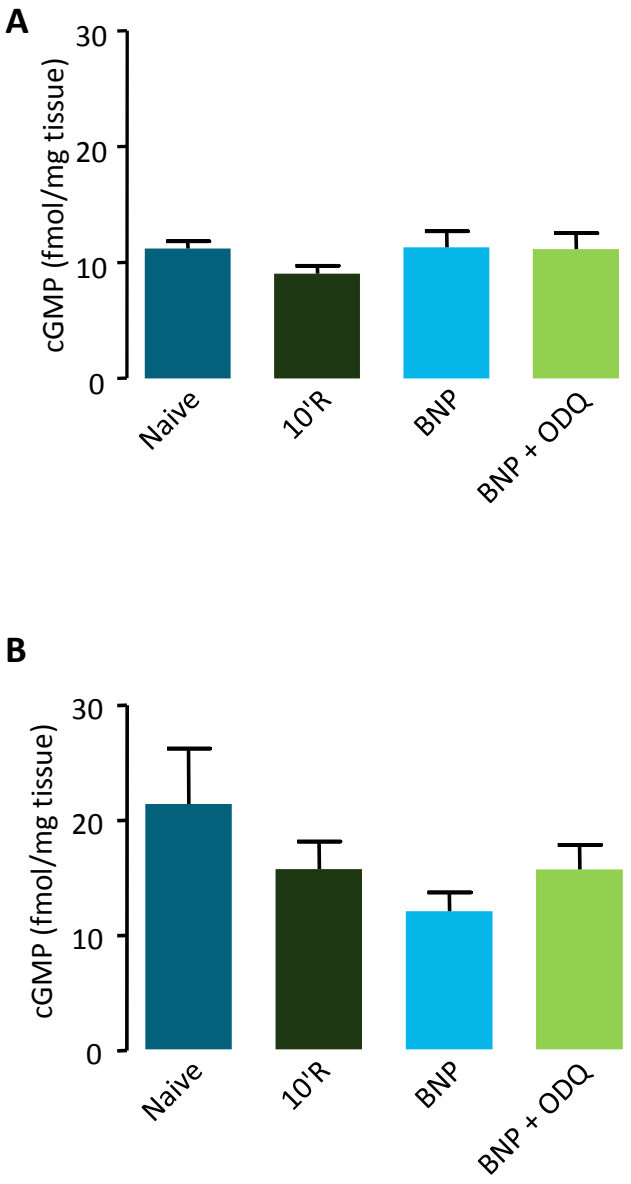

Figure 6.6 *cGMP levels in LV (A) and RV (B) heart samples perfused with or without BNP 10 nM or concomitantly with ODQ 2 μM ($p>0.05$) ONE-way ANOVA ($n=5$-6).*

6.3.4 NO_2^- levels measured by ozone based chemiluminescence: Study 3

Nitrite levels measured in coronary effluent from Langendorff perfused hearts after 20 min stabilisation and 15 min further perfusion were 3189 ± 234 pmoles/min (n=4). NO_2^- levels in coronary effluent collected from rat hearts stabilised for 20 min followed by 15 min perfusion with BNP 10 nM were elevated by 146 % to 7865 ± 489 pmoles/min ($p<0.001$, n=4) (see Figure 6.7).

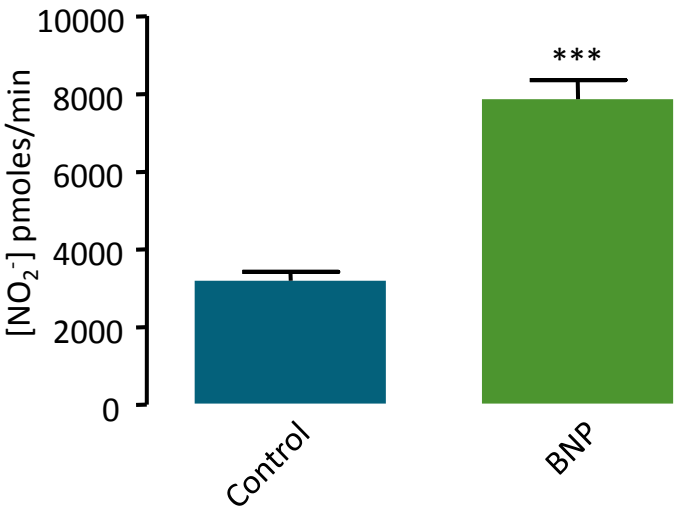

Figure 6.7 *NO_2^- levels in coronary effluent samples of hearts stabilised for 20 min followed by 15 min further perfusion or 15 min perfusion with BNP 10 nM measured by OBC (***p<0.001 vs. control) unpaired two-tail t-test (n=4).*

6.4 Discussion

6.4.1 Summary of findings

The major findings of the studies in this chapter are:

i) PI3K/Akt activity was modestly increased during reperfusion in the presence of BNP
ii) ODQ did not block BNP mediated cGMP production in the myocardium
iii) Nitrite levels were elevated in coronary effluent of hearts perfused with BNP

The findings of these studies form a body of preliminary work and so extensive conclusions cannot be drawn. However, they do suggest that NP mediated protection is associated with the activation of much of the PI3K/Akt/eNOS/NO/sGC/cGMP pathway and compliment earlier studies.

The modest elevation of pAkt seen in hearts treated with BNP during early reperfusion supports the notion that pGC signals through the PI3K/Akt pathway during early reperfusion. This biochemical analysis is in agreement with infarct studies mentioned earlier that reported loss of protection in rabbit hearts perfused with ANP and the PI3K inhibitor Wortmannin (Yang *et al.*, 2006). Taken together with the work of Ren *et al.* (2007), who reported increased levels of eNOS expression following BNP perfusion, the data suggest that pGC signals through the PI3K/Akt/eNOS part of the RISK pathway to afford infarct limitation. Further support for this paradigm comes from the preliminary NO_2^- measurements made in coronary effluent from hearts perfused with BNP under normoxic conditions. The results demonstrate that BNP perfusion can elevate NO convincingly by 146 %, supporting the previous infarct study that demonstrated that

inhibiting NO by L-NAME during BNP perfusion abrogates infarct limitation (Burley & Baxter, 2007).

The cGMP measurements recorded in this study are somewhat surprising. BNP perfusion only produced modest (25 %) increase in cGMP compared to untreated controls. More surprising are the cGMP levels, which were reduced in BNP perfused RV tissue compared to untreated controls. Concomitant perfusion of BNP and ODQ resulted in very similar (11.34 vs. 11.49 fmol/mg tissue) cGMP levels as hearts perfused only with BNP. Considering that ODQ abrogated infarct limitation afforded by ANP, suggesting the need for sGC in NP mediated protection (Yang *et al.*, 2006), the cGMP measurements in this study suggest that it may not be total cGMP level mediated but maybe a localised production of cGMP.

6.5 Conclusions

The results of these studies provide preliminary biochemical evidence that NP/pGC mediated infarct limitation requires activation of PI3K/Akt/eNOS. Furthermore, they suggest that in terms of infarct limitation pGC and sGC may be closely associated. Taken together these results suggest a need to investigate further the relationship between pGC and sGC mediated cGMP production and ultimate infarct limitation.

Chapter 7
General Discussion

Discussion

7.1 Fundamental findings

The major findings from the studies included in this thesis are summarised as follows;

Production of cGMP during early reperfusion via exogenous targeting of sGC is cardioprotective by means of infarct limitation. This was demonstrated in Chapter 3 with the use of the sGC stimulator BAY 41-2272, which significantly limited infarct size in the isolated rat perfused heart when administered during early reperfusion. This observation was also demonstrated in Chapter 5 when administration of BAY 60-2770, a sGC activator during the same period significantly limited infarct size compared to control experiments. In Chapter 4, it was shown that perfusion with an NO donor during early reperfusion could afford infarct limitation; further corroborating the notion, that sGC is a key mediator in limiting reperfusion injury.

Pharmacological observations of the mechanisms by which the sGC stimulators and activators elevate cGMP levels are translated in the ischaemia-reperfusion infarct limitation model. Experiments in Chapter 3 showed that concomitant perfusion of the sGC stimulator BAY 41-2272 with the haem site oxidiser ODQ abrogated the protection afforded by the sGC stimulator alone, suggesting haem dependence in its action. Conversely, in Chapter 5 it was demonstrated that concomitant perfusion of the sGC activator BAY 60-2770 with ODQ showed a trend towards increased protection above BAY 60-2770 only perfusion. This supports previous cell based models that suggest that BAY 60-2770 acts in a haem-independent manner. The NO component of both BAY compounds was also explored, confirming that endogenous NO is not required for either

BAY compound to afford protection. This was reported in Chapters 4 with concomitant perfusion of BAY 41-2272 with the NOS inhibitor L-NAME and in Chapters 3 and 5 with concomitant perfusion of C-PTIO with either BAY 60-2770 or BAY 41-2272.

In Chapter 5, it was demonstrated that targeting both the reduced and oxidised portions of sGC by concomitantly perfusing BAY 41-2272 and BAY 60-2770 could not afford greater infarct limitation than either treatment alone. In fact, concomitant perfusion resulted in reduced infarct limitation. It was also demonstrated in Chapter 4 that exogenous NO and BAY 41-2272 could act synergistically to afford greater infarct limitation than the sum of the two independent treatments.

Finally, total tissue cGMP measurements reported in Chapters 3, 4 and 5 suggest that there is linear relationship between total cell cGMP production and infarct limitation.

7.2 Context within the broad ischaemia-reperfusion setting

The data presented in this thesis focus only on one lyase enzyme, GC and the immediate product of its activity, cGMP. As eluded to throughout this thesis, the intracellular signalling that culminates in limitation of infarct size is both large and diverse. Like many other studies this work demonstrates that targeting a specific component of the so-called RISK pathway can afford infarct limitation. The fact that inhibition of some components of the pathway does not exacerbate infarct size beyond control levels demonstrates the dynamic non-linear structure of the signalling. An example of this can be taken from infarct studies documented in Chapters 3 and 4. Perfusion of the haem site oxidiser ODQ did not produce infarct sizes greater than control experiments. Similarly, perfusion of the

NOS inhibitor L-NAME or the NO scavenger C-PTIO produced infarct sizes comparable to controls. This suggests that other components of the pathway can be activated, or that components can be bypassed by non-linear signalling.

Adjunct data in the form of RPP and CFR recorded during the infarct experiments highlight no haemodynamic or functional differences between groups. These data in the form of CFR suggest that any infarct limitation documented for a given treatment group is independent of vasodilatation. However, specific investigation of any vasoactive properties of the interventions used would require an adapted protocol. Hearts would be perfused at constant flow and not constant pressure and any changes in vascular tone would be recorded as a change in pressure. This model is better suited to investigating vasoactive parameters, but is not optimal for infarct studies as constant pressure represents physiological conditions more robustly.

RPP is a reliable indicator of heart function, represented as a function of both HR and LVDP. Heart rate fluctuates very little throughout the described Langendorff experiments and so any large changes in RPP are because of changes in LVDP. No differences were reported for RPP between different treatment groups irrespective of their ability to limit infarct size. Although this may appear surprising, the percentage of the total heart damaged by CAO in our model varies only between approximately 8-17 %, insufficient to observe differences in LVDP. These observations are consistent with previous studies that report comparable limitation in infarct size following pharmacological interventions, yet document no difference in either LVDP or RPP (Burley & Baxter, 2007; D'Souza *et al.*, 2003; Hausenloy *et al.*, 2002)

The data presented in this thesis are limited to infarct size and biochemical studies. Experiments in Chapters 3 and 5 demonstrate that during early reperfusion both the reduced and oxidised forms of sGC are present in the myocardium. This conclusion can be made based on the cell based pharmacological studies conducted by Schmidt *et al.* (2003), and Stasch *et al.*(2006), who demonstrated that the sGC activators act only on oxidised or haem-free sGC. Both BAY 41-2272 and BAY 60-2770 were able to significantly elevate cGMP levels in LV myocardial tissue samples. In agreement with comments made by Stasch *et al.* (2006) supported by the above-mentioned experiments, a portion of the sGC present in the myocardium during early reperfusion is in the so-called pathological oxidised state. There is accumulating evidence to suggest that cGMP is produced in co-localised pools, depending on whether the particulate or soluble cyclase catalysed its conversion from GTP. How this transfers to RISK pathway signalling remains unclear, yet evidence suggests that total cGMP levels are not the limiting factor in affording protection. It also remains unclear whether sGC in different redox states is localised to spatially distinct areas of the cell, possibly further contributing to cGMP localisation. Investigating this further may highlight so-called cardioprotective cGMP, or more specifically infarct limiting cGMP that originates from specific localised pools that signals through the RISK pathway. It may also be of benefit to explore the production of cGMP in the myocardium over a wider time course, specifically during late ischaemia and early reperfusion. Continuous recordings of myocardial cGMP during this period would allow exploration of the specific production/hydrolysis of the cyclic nucleotide and ascertain the optimum period for elevating cGMP. In our studies, we were limited to only one reperfusion sampling time point. Further investigation is required to document whether

an earlier sampling time point, possibly 2 min reperfusion would have highlighted greater elevations of myocardial cGMP, before PDEs shift the equilibrium towards hydrolysis.

Specifically targeting sGC has been shown to be a promising therapeutic target. As discussed in Chapter 5, successful clinical trials have been carried out using both the sGC stimulator Riociguat and the sGC activator Cinaciguat in the treatment of primary pulmonary hypertension and acute decompensated heart failure respectively. The obvious benefits of using these compounds over other drugs are their targeting more distally in the signal transduction pathway, potentially limiting undesirable effects. A desirable property of both cGMP-elevating compounds reported in this thesis is their independence of NO. There could be real potential in their use as adjunct therapies to NO donors, which have well characterised tolerance issues when used chronically, although it is not clear if vascular tolerance contributes to a reduction in cardioprotection.

Development and introduction of new therapies specifically for the treatment of reperfusion injury post AMI has been slow considering the extensive body of research reporting the infarct limiting properties of many compounds (Downey & Cohen, 2009). It is conceivable that part of the problem is the number of protective agents being reported. Clinical trials have been conducted investigating the adjunct therapy of NPR targeting alongside PPCI. Carperitide, an NPR1 agonist afforded infarct limitation, reduced malignant arrhythmias and lowered the incidence of ST-segment elevation (Kitakaze *et al.*, 2007). Considering the data presented in Chapter 6 that suggests NPR mediated infarct limitation signals through the same upstream kinases as sGC to elevate cGMP, it may therefore prove beneficial to target downstream of the NPR nearer the proposed effector

kinases. This concept was investigated in a preliminary proof of concept clinical study that reported that a single dose of CSA could limit infarct size in human patients. CSA has been demonstrated to act directly on the mPTP, proposed as one of the end effectors in the RISK pathway (Piot *et al.*, 2008). Furthermore there was no detrimental effect on LV remodelling following assessment at 6 months post AMI (Mewton *et al.*, 2010). More comprehensively designed clinical trials need to be conducted in association with more specifically designed *in vivo* and *ex vivo* studies.

7.3 Limitations and obstacles

In Chapter 3, it was concluded that BAY 41-2272 afforded infarct limitation when given at reperfusion and was associated with elevation of total tissue cGMP levels. It was further concluded that the protection afforded was sGC mediated and required the haem to be in the reduced state. cGMP measurements recorded in comparable experiments perfused with ODQ would have potentially supported this observation further by demonstrating a reduction in cGMP levels compared to BAY 41-2272 treated hearts. The practicalities of preparing large numbers of whole tissue samples for RIA and the time it takes to carry out the initial heart perfusion were the reasons these experiments were not carried out.

As mentioned previously, measuring NO levels in any biological sample is difficult. Total NO_2^- levels presented in Chapters 4 and 6 provide superficial evidence of NO changes in the myocardium. The somewhat inconsistent data investigating the NO donor NOC-9 in the infarct size and OBC models suggests that the concentration of the NO donor chosen for OBC was too low. Although at this concentration (1 nM) infarct limitation was observed, it was not a sufficiently high concentration to produce quantities of NO that

were measureable in the coronary effluent. As described in Chapter 4, the biology of this particular NONOate results in rapid production of NO over a short period. It is conceivable that the NO produced following perfusion of NOC-9 was metabolised or even reduced to NO_2^- before it could be measured. Increasing the concentration of NOC-9 perfused for the OBC experiments may have elicited a measureable elevation in NO in the coronary effluent, however the concentration chosen was for comparison with infarct limitation documented in earlier experiments.

EPR may provide more precise tissue levels of NO however employing these methods in an isolated perfused heart model is difficult. The spin traps required to trap NO cause technical issues when trying to perfuse them though the Langendorff apparatus alongside NO manipulating tools such as those used in this thesis. The need for iron in the spin trap mixture produces a viscous liquid that blocks the flow of buffer through the apparatus which is problematic when attempting to perfuse pharmacological tools. If apparatus were being dedicated solely to EPR measurements, it would be possible to record more consistent tissue NO levels in this model.

In Chapter 6, Western blotting analysis suggested that pAkt levels were elevated during early reperfusion in the presence of BNP. Sampling time points were chosen so consistency was kept with infarct studies previously reported where BNP was perfused until 10 min reperfusion. In future studies it may be of benefit to explore an earlier reperfusion time point to investigate the possibility that there may be a more marked elevation in pAkt expression immediately after reperfusion. I believe that the concentration of BNP (10 nM) used in the current studies should still be employed in

future experiments as there is robust evidence to suggest that it is cardioprotective in terms of infarct limitation when administered during early reperfusion (Burley & Baxter, 2007).

A general limitation of this thesis is that the cGMP measurements made are of total tissue cGMP. Although they provide convincing evidence that cGMP levels are elevated after certain pharmacological interventions, they do not provide subcellular detail of cGMP production. This would indeed be desirable to further explore the compartmentalisation hypothesis (described in Chapter 1.26, 1.28 and 6.1.5) of cGMP and whether this plays a role in tissue salvage signalling in the myocardium. It is conceivable that it is not total cGMP elevation that is important in terms of protection but elevation of specific pools associated with local downstream targets in the RISK pathway. Using a cell based model, it would be useful to employ fluorescence resonance energy transfer (FRET) to investigate specifically where cGMP is being produced following treatments and how this may change following a sustained period of hypoxia. Leroy *et al.* (2008), have employed this method in the exploration of cAMP distribution in adult rat ventricular myocytes, reporting that there are temporary cAMP compartments formed during β-adrenergic receptor stimulation with isoprenaline.

Stasch's laboratory have recently published data that demonstrates the possibility of monitoring changes in the cellular sGC haem status (Hoffmann *et al.*, 2011). They conclude that this method is limited to recombinant expression systems, utilising an engineered sGC variant but propose that in time may be used *in vivo.* It would be of benefit to utilise this technique in the ischaemia-reperfusion setting, helping us to

understand the changes in the redox state of sGC and specifically target cGMP pools that could maximise infarct limitation.

Recent studies by Brouckaert's laboratory have demonstrated the utilisation of siRNA technologies to investigate the necessity for specific isoforms of sGC in inhaled NO mediated reduction in pH and in infarct size following index ischaemia (Nagasaka *et al.*). It may be of benefit to utilise this technique as an alternative to the pharmacological methods used to explore the mechanisms that afford infarct limitation. Using siRNA to silence the genes required to produce components of the RISK pathway would provide an alternative approach to using pharmacological tools to manipulate this pathway, specifically exploring the downstream targets of cGMP such as PKG. It may also be possible to manipulate the production of different states of sGC to control artificially the redox equilibrium in the ischaemia-reperfusion setting.

Other methods employed to simulate AMI and investigate infarct limiting interventions include *in situ* models whereby animals are allowed to recover following LDCA occlusion for a period of time and then sacrificed for analysis of their myocardium. This model is by definition suited to exploring recovery over time and is not suitable for exploring the immediate effects of pharmacological intervention. Early reperfusion is the period in which the RISK pathway has been reported to be activated and so the *ex vivo* model reported in this thesis is deemed most suitable. A similar argument can be made for the reperfusion duration used in these experiments. Two hours is chosen as it is a sufficient length of time for the washout of metabolites and dehydrogenase enzymes that may be present in the infarcted tissue immediately following reperfusion. Failure to wash these

enzymes out of the heart sufficiently would give false positive staining results. Similarly, over longer periods, the viability of the heart is reduced (described in Chapter 2). Pacing is a possibility and would allow longer reperfusion, however, eliminating spontaneous electrical activity and associated arrhythmias would not be physiologically representative, particularly during early reperfusion when there is a high occurrence of these events. It has been documented that anti arrhythmic interventions can contribute to infarct limitation (Wit & Duffy, 2008).

7.4 Correlation between cGMP elevation and infarct limitation

In an attempt to explore the relationship between infarct size and total LV cGMP levels, infarct size data against corresponding treatment groups that measured cGMP levels have been plotted (Figure 7.1). Analysis of this data shows that there is correlation between the two variables, specifically that when infarct size is reduced, total LV cGMP levels increase. Spearman's rank correlation coefficient reports an r-value of -0.7 and a p value of 0.04. This observation demonstrates that pharmacological targeting of cGMP elevation during early reperfusion is a worthwhile therapeutic target to investigate further. It would be desirable to collect further cGMP measurements from heart samples treated with other pharmacological interventions matched to infarct studies to build a more extensive data set and profile the correlation further. It may also be of interest to investigate the corresponding infarct size/cGMP data for hearts perfused with NPR agonists to ascertain whether there are comparable observations. If further studies support the observation shown in Figure 7.1, cGMP measurements immediately post MI maybe of clinical benefit in determining the extent of injury. As described in Chapter 6.1.1, BNP measurements in

patients presenting with STEMI provide a good indication of the extent of injury and prognosis.

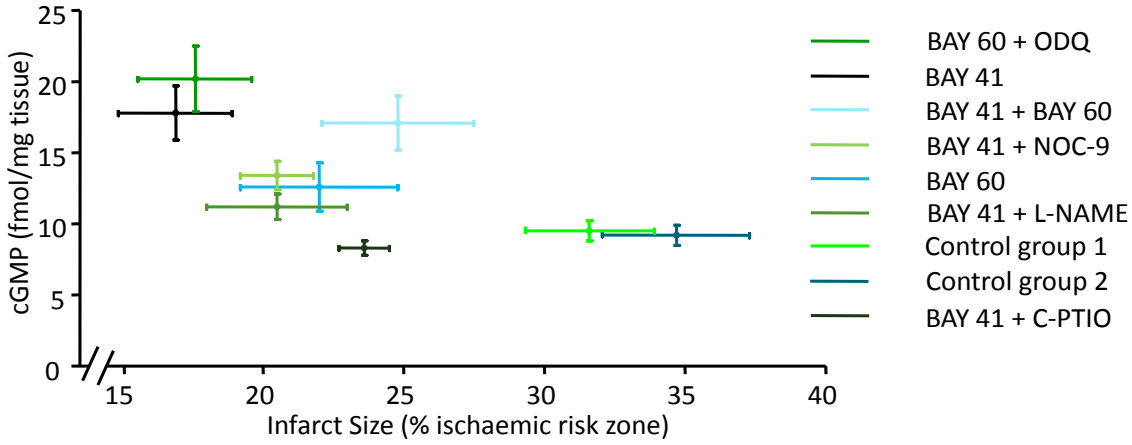

Figure 7.1 *Representative schematic of the relationship between infarct size and LV total cGMP levels in corresponding experimental groups. Spearman's test shows negative correlation with an r value of -0.7 ($p<0.05$, $n=6$-18). The major outlier being the concomitant perfusion of both the sGC stimulator and activator (BAY 41 + BAY 60), which resulted in large cGMP elevation compared to controls but corresponding infarct experiments did not show the expected reduction in infarct size. SEM shown for both infarct size and LV total cGMP levels.*

7.5 Concluding thoughts

The experiments reported in this thesis have been conducted to explore the role of targeting sGC during early reperfusion to afford cardioprotection by means of infarct limitation. Using novel sGC stimulators and activators as pharmacological tools it has been possible to address the questions posed and come to some key conclusions:

Can cardioprotection be afforded by exogenous stimulation/activation of sGC?

The experiments in Chapters 3 and 5 clearly demonstrate that perfusion of the sGC stimulator BAY 41-2272 and the sGC activator BAY 60-2770 limit infarct size when

administered during early reperfusion. Furthermore, the protection afforded is associated with total LV cGMP elevation.

Can sGC stimulation afford protection independently of NO?

Both BAY 41-2272 and BAY 60-2770 afforded protection independently of NO as shown by reduced infarct sizes in experiments in which concomitant perfusion of the NOS inhibitor L-NAME or NO scavenger C-PTIO with the BAY 41-2272 or C-PTIO and BAY 60-2770.

Can greater protection be afforded by targeting different redox states of sGC?

Experiments in Chapter 5 suggest that targeting both the reduced and oxidised states of sGC do not afford increased protection by means of reduced infarct size. Furthermore, targeting the oxidised form of sGC did not afford greater protection than targeting the reduced form of sGC.

The results reported in this thesis demonstrate that the sGC/cGMP pathway plays an important role in the ischaemia reperfusion injury setting. Further exploration to investigate this signalling pathway is warranted, specifically investigating the localised production of cGMP and how these changes affect infarct size. There is clearly a need to continue investigating the ischaemia-reperfusion field and develop pharmacological adjunct therapies to PPCI, possibly cGMP elevating compounds.

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