

INVESTIGATING THE MECHANISMS OF NITRITE-MEDIATED CARDIOPROTECTION

by

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ABSTRACT

Nitrite is able to elicit cardioprotection against myocardial IRI when administered as a preconditioning agent. Activation of the classical NO-sGC-cGMP-PKG pathway under hypoxic and/or acidic conditions has been implicated in this protection, but the exact mechanism remains unclear. Herein, we investigated whether nitrite mediates cardioprotection by (1) PKG1 α oxidation and/or the (2) ALDH2 pathway. Using an isolated Langendorff mouse model of IRI, nitrite (100 μ M) was administered as a preconditioning agent in (1) PKG1 α WT and Cys42Ser KI mice or (2) ALDH2 WT and KO mice.

We demonstrate that nitrite improves cardiac function (LVDP; $p < 0.01$; $n = 8-12$) and coronary flow rate (CFR; $p < 0.001$; $n = 8-12$) post-IRI *via* the PKG1 α oxidation pathway. However, we observed no significant difference following nitrite treatment in the PKG1 α WT and KI mice ($p > 0.05$; $n = 8-12$), thus suggesting a dual mechanism involving the NO-cGMP-PKG pathway in the cardiomyocytes. In contrast, we offer evidence to support ALDH2 involvement in nitrite-mediated improvements in CFR ($p < 0.001$; $n = 7-11$) and suggest it may also be involved in cardioprotection.

The study provides novel evidence supporting the involvement of both the PKG1 α oxidation and ALDH2 pathways in nitrite-mediated effects at the level of the cardiac microvasculature and cardiomyocytes in a murine model of myocardial IRI.

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ABBREVIATIONS

4-HNE	4-hydroxy-2-nonenal
ADP	Adenosine Monophosphate
ALDH	Aldehyde Dehydrogenase
ALDH2	Aldehyde Dehydrogenase 2
AMI	Acute Myocardial Infarction
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
ANP	Atrial Natriuretic Peptide
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BAD	Bcl-2-associated death promoter protein
BAX	Bcl-2-associated X protein
BH₄	Tetrahydrobiopterin
bp	Base Pairs
Ca²⁺	Calcium ion
CABG	Coronary Artery Bypass Graft
CaCl₂	Calcium Chloride
cGMP	Cyclic Guanosine Monophosphate
CFR	Coronary Flow Rate
CHD	Coronary Heart Disease
CK	Creatine Kinase

CPTIO	2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; NO scavenger
CSI	Chemical Shift Imaging
CVD	Cardiovascular Disease
Cys	Cysteine
DNA	Deoxyribonucleic Acid
EDRF	Endothelium-derived Relaxing Factor
EET	Epoxyeicosatrienoic Acid
eNOS	Endothelial Nitric Oxide Synthase
ES	Embryonic Stem (in reference to cells)
ETC	Electron Transport Chain
FMD	Flow-mediated Dilatation
GDN	Glyceryl Dinitrate
GI	Gastrointestinal
GIK	Glucose-Insulin-Potassium
GPCR	G-Protein Coupled Receptor
GSK-3β	Glycogen synthase kinase – 3beta
GTN	Glyceryl trinitrate
H⁺	Hydrogen ion
H₂O₂	Hydrogen Peroxide
HET	Heterozygous
IHD	Ischaemic Heart Disease
iNOS	Inducible Nitric Oxide Synthase
IP	Intraperitoneal

IPC	Ischaemic Preconditioning
IPost	Ischaemic Post-conditioning
IRI	Ischaemia Reperfusion Injury
K⁺	Potassium ion
K_{ATP}	ATP-Sensitive Potassium Channel
KCl	Potassium Chloride
KHB	Krebs-Henseleit Buffer
KH₂PO₄	Potassium Phosphate
KI	Knock In
KO	Knock Out
LAD	Left Anterior Descending Artery
LDH	Lactate Dehydrogenase
LDL	Low Density Lipoproteins
L-NIO	(N ⁵ -(1-Iminoethyl)-L-ornithine, dihydrochloride); NOS inhibitor
LPS	E.Coli Lipopolysaccharide
LVDP	Left Ventricular Developed Pressure
LVdP/dt Max	Maximal change in Left Ventricular Pressure/time; indicative of cardiac contractility
LVdP/dt Min	Minimal change in Left Ventricular Pressure/time; indicative of cardiac relaxation
LVEDP	Left Ventricular End Diastolic Pressure
LVP	Left Ventricular Pressure
MACE	Major Adverse Cardiac Event
MDA	Malondialdehyde

MgSO₄	Magnesium Sulfate
MI	Myocardial Infarction
MMP	Mitochondrial Membrane Potential
mPTP	Mitochondrial Permeability Transition Pore
MVO	Microvascular Obstruction
Na⁺	Sodium ion
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaHCO₃	Sodium Hydrogen Carbonate
NaNO₂	Sodium Nitrite
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NO₃⁻	Inorganic Nitrate ion
NOS	Nitric Oxide Synthase
O₂	Oxygen atom
O₂⁻	Superoxide anion
ODQ	[1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one]; sGC inhibitor
Oligo	Oligonucleotide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDE5	Phosphodiesterase 5
Pi	High energy phosphate
PKC	Protein Kinase C
PKG	Protein Kinase G

PKG1α	Protein Kinase G 1alpha
PPCI	Primary Percutaneous Coronary Intervention
RACK	Receptors for Activated C Kinase
RCF	Relative Centrifugal Force
RIC	Remote Ischaemic Conditioning
rIPost	Remote Ischaemic Post-conditioning
RISK	Reperfusion Injury Salvage Kinase
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
Ser	Serine
sGC	Soluble Guanylate Cyclase
SNAP	S-nitroso-N-acetylpenicillamine; NO donor
STEMI	ST Segment Elevation Myocardial Infarction
TAC	Transaortic Constriction
TCA	Tricarboxylic Acid
TTC	Triphenyltetrazolium Chloride
UV	Ultraviolet
WT	Wild Type
XOR	Xanthine Oxidoreductase

1. INTRODUCTION

1.1. Coronary Heart Disease

Cardiovascular disease (CVD) is the leading cause of deaths worldwide. The largest proportion of these deaths is attributed to Coronary Heart Disease (CHD), which is estimated to be 7.4 million in 2012 [World Health Organisation, 2015]. Despite being largely preventable through the adoption of lifestyle changes to limit risk factors, CHD also remains the single principal cause of mortality and morbidity in the United Kingdom. The British Heart Foundation (BHF) reported 69,000 deaths due to CHD in 2014, 41,000 of which were considered 'premature' (occurring under the age of 75) [British Heart Foundation, 2015]. Furthermore, BHF estimated the financial burden on the National Health System to be £15.2 billion per annum [British Heart Foundation, 2015].

CHD, which is referred to in some literature as Ischaemic Heart Disease (IHD), is characterised by the occlusion of the coronary circulation due to plaque build-up within the artery wall (known as atherosclerosis). High levels of cholesterol in the blood (hypercholesterolemia; largely influenced by diet and a sedentary lifestyle) promotes the accumulation of low-density lipoprotein (LDL) particles within blood vessels. Subsequent oxidative modification of these particles results in the formation of atheroma, which narrows the artery and reduces the flow of oxygenated blood to cardiomyocytes [Longo et al., 2012]. This period of reduced oxygen provision is defined as acute myocardial ischaemia (AMI) and if prolonged to a duration in excess of 20 minutes, can trigger myocardial infarction (MI) [Hausenloy and Yellon, 2013] [Petersen, 2007] [Burley et al., 2007].

It is estimated that approximately one third of patients sustaining MI will die shortly after ischaemia onset [Burley et al., 2007]. Surviving patients however, will be at risk of additional complications following standard interventional treatments, such as thrombolytic therapy (drug administration to dissolve blood clots) or primary percutaneous coronary intervention (PPCI; stent insertion into affected arteries). Although these interventions can restore blood flow to the ischaemic cardiac tissue and limit infarct size, they do carry their own risk of further cardiomyocyte death. The abrupt biochemical and metabolic changes that occur in response to blood flow restoration results in a phenomenon known as myocardial ischaemia-reperfusion injury (IRI) [Hausenloy et al., 2005b] [Hausenloy and Yellon, 2013].

2.2. The phenomenon of Myocardial Ischaemia-Reperfusion Injury

In 1960, myocardial IRI was initially proposed by Jennings and colleagues [Jennings et al., 1960]. The authors demonstrated in a canine model of ischaemia, that myocardial injury and increased infarct size was associated primarily with reperfusion rather than the ischaemic event itself [Jennings et al., 1969] [Yellon and Hausenloy, 2007] [Hausenloy and Yellon, 2007]. The pathophysiology of myocardial IRI can be divided into four recognised categories: (1) reperfusion-induced arrhythmias, (2) myocardial stunning, (3) microvascular obstruction (MVO) and (4) lethal reperfusion injury.

(1). **Reperfusion-induced arrhythmias:** There is evidence of ventricular arrhythmias following reperfusion in patients undergoing PPCI. The irregular rhythms usually self-terminate or are otherwise easily treated pharmacologically [Hearse and Tosaki, 1987].

(2). **Myocardial stunning:** Contractile dysfunction occurs following the re-establishment of blood flow to ischaemic myocardium due to the effects of intracellular ion concentrations on the myofibril contractile apparatus. However, these effects are reversible [Kloner et al., 1998].

(3) **MVO:** The major contributing factors influencing MVO are capillary damage affecting vasodilatation and/or compression by nearby cell swellings, platelet micro-thrombi and neutrophil plugging [Ito, 2006]. MVO presents as sluggish coronary blood flow with a characteristic velocity profile associated with increased infarction, left ventricular remodelling and a reduced ejection fraction. These changes are not reversible [Hausenloy and Yellon, 2013].

(4) **Lethal reperfusion injury:** Defined as the death of cardiomyocytes as a result of reperfusion which were previously viable at the end of an ischaemic period and considered non-reversible. Interventions carried out in both experimental models of MI and in patients suggest that lethal reperfusion injury may contribute up to 50% of the final infarct size [Yellon and Hausenloy, 2007]. This pathophysiology is central to the present study and the key contributory factors are discussed in depth below (see sections 1.2.1., 1.2.2. and 1.2.3.).

It has been proposed that myocardial ischaemia in the absence of reperfusion results in an infarct size of approximately 70% [McAlindon et al., 2014]. Following thrombolytic or PPCI therapy and subsequent reperfusion, infarct size is reduced by 40% with the remaining 30% attributed to myocardial IRI [McAlindon et al., 2014]. Therefore, identifying novel cardioprotective strategies to reduce infarct size further is paramount.

1.2.1. Mitochondrial function in the healthy heart

In a healthy heart, the cardiomyocytes are aerobic and the energy requirements of myofibril contraction are provided by the mitochondria through mitochondrial oxidative phosphorylation [Mitchell and Moyle, 1967]. As such, there is a high abundance of mitochondria present in the cardiomyocytes (approximately 30% of cell volume) [Givvimani et al., 2015]. At the level of the mitochondria, energy substrate adenosine triphosphate (ATP)

is formed under aerobic conditions through the well-established respiration pathways of glycolysis, the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC). Oxidative phosphorylation elicited *via* the ETC permits sensitivity to oxygen deficiency, thus allowing the supply of ATP to match ATP demand if the work load of the heart was to increase [Skarka and Ostadal, 2002] [Halestrap and Pasdois, 2009]. In addition to its role in energy production, the mitochondria also maintain a chemiosmotic gradient known as the 'mitochondrial membrane potential' (MMP). The MMP plays an essential role in regulating the calcium ion homeostasis and subsequent calcium signalling required for normal functioning of the cardiomyocytes and contraction of the cardiac myofibrils [Mitchell, 1979]. Previous studies have shown that disruption or collapse of the chemiosmotic gradient is a major contributing factor of myocardial IRI [Mathur et al., 2000] [Skarka and Ostadal, 2002].

Ion movement in and out of the cardiomyocytes is determined by several channels and exchangers at the sarcolemmal membrane: sodium-hydrogen (Na^+/H^+) exchanger, sodium-calcium ($2\text{Na}^+/\text{Ca}^{2+}$) exchanger, sodium-potassium (Na^+/K^+) ATPase and sodium channels. This ion movement, and thus cardiomyocyte pH, is influenced by changes at the level of the mitochondria, particularly during incidences of ischaemia and reperfusion [Hausenloy and Yellon, 2013] [Kwong and Molkentin, 2015]. Calcium efflux is additionally influenced by a non-selective channel at the mitochondrial membrane, which is known as the mitochondrial permeability transition pore (mPTP). The mPTP was first characterised by Haworth and Hunter (1979) and has since been found by numerous studies to have an important role in cell metabolism [Elrod et al., 2010] [Giorgio et al., 2013] [Kwong et al., 2014], calcium ion movement [Korge et al., 2011] [Bernardi and von Stockum, 2012] and cardiac development [Hom et al., 2011]. Furthermore, studies have demonstrated a pathological role, which is discussed in detail in section 1.2.3 [Kwong and Molkentin, 2015].

1.2.2. Alterations in mitochondrial function during myocardial ischaemia

During a prolonged period of ischaemia (>20 minutes), insufficient levels of oxygen (hypoxia) are sensed by the oxygen-sensitive ETC, therefore causing oxidative phosphorylation to cease. This subsequently leads to a decrease in the ATP bioavailability for the cardiomyocytes and a concomitant rise in the ATP breakdown products adenosine diphosphate (ADP), adenosine monophosphate (AMP) and high-energy phosphate (Pi) [Zweier and Talukder, 2006]. Compensatory adoption of an alternative, anaerobic form of respiration produces lactic acid as a by-product, leading to acidification. The presence of H⁺ ions lowers intracellular pH and prevents contracture of the cardiac myofibrils through a potent inhibitory effect on a trigger site intended for calcium binding [Hausenloy and Yellon, 2013]. Furthermore, H⁺ ions are able to independently induce their own extrusion through Na⁺-H⁺ exchangers on the cardiomyocyte sarcolemmal membrane thus triggering an influx of sodium (Na⁺) ions into the cell in response. Increased cellular influx of Na⁺ ions through the Na⁺ ion channels and Na⁺/H⁺ exchanger, and decreased efflux *via* the Na⁺/K⁺ ATPase leads to a sodium overload during ischaemia [Lansbery et al., 2006] [Palovic et al., 2007], which is supported by sodium chemical shift imaging (CSI) of isolated rat hearts subjected to ischaemia [Weidensteiner et al., 2002]. This high concentration of Na⁺ ions threatens collapse of the MMP gradient and disruption of ionic and metabolic homeostasis, which in turn leads to necrotic cell death of the cardiomyocytes [Halestrap et al., 2004] [Halestrap, 2006]. This cascade of changes in the cardiomyocytes during ischaemia and subsequent anaerobic respiration is shown in figure.

1.1.

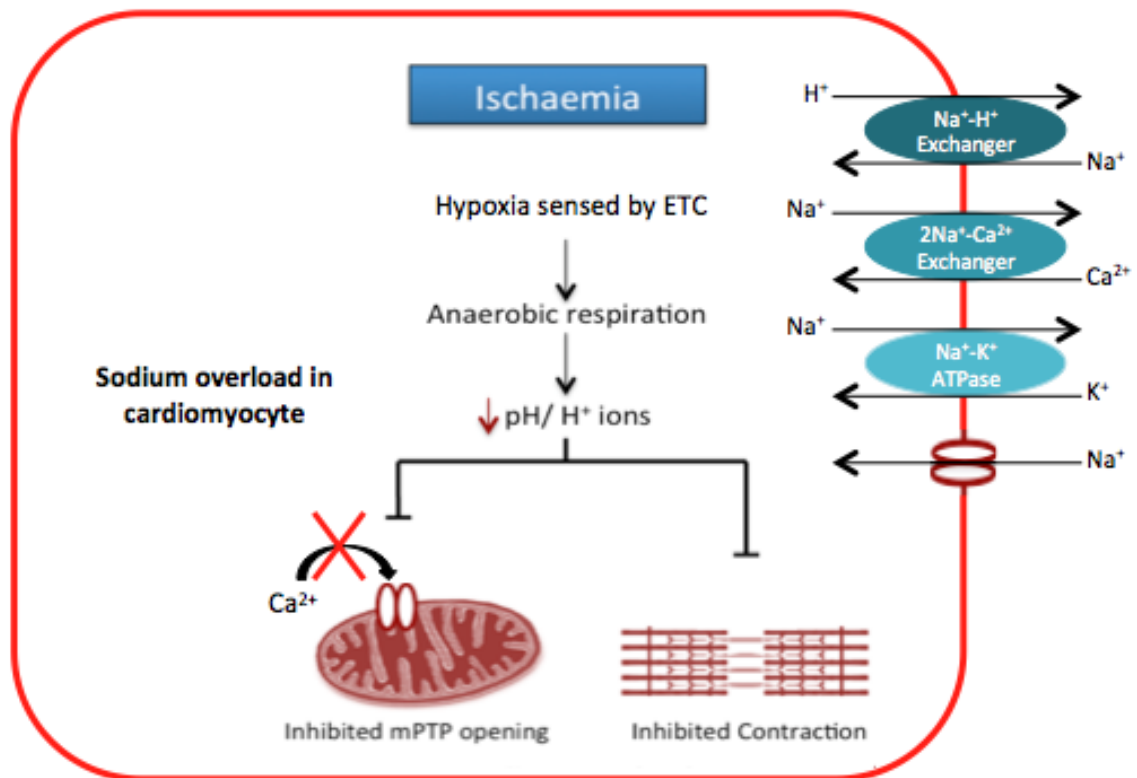


Figure.1.1: Cascade of changes that occur within the cardiomyocyte in response to prolonged ischaemia.

Hypoxia is sensed by the ETC as insufficient oxygen levels fail to support aerobic respiration. Compensatory anaerobic respiration takes place within the cardiomyocyte in response, producing lactic acid as a by-product, leading to acidification and triggering a movement of ions through channels and exchangers at the sarcolemmal membrane, which results in a sodium ion overload within the cell; placing the myocytes at risk of necrotic cell death. The presence of H^+ ions and a low pH additionally inhibit myofibril contracture and opening of the mPTP (preventing the binding of calcium ions to a specific trigger site).

Figure. 1.1 adapted from Hausenloy and Yellon, 2013.

1.2.3. Alterations in mitochondrial function during myocardial IRI

Recent studies have reported that an alteration in mitochondrial dynamics during myocardial IRI is the principal mediator in the onset of tissue injury [Kwong and Molkenin, 2015]. For example, this includes membrane potential collapse [Halestrap, 2009], cessation of energy production [Kwong and Molkenin, 2015] and cell death in response to intrinsic killing mechanisms [Lesnefsky et al., 2001]. As a result, the fate of the cardiomyocyte is critically

dependent upon preserving mitochondrial function [Crompton et al., 2002] [Hausenloy et al., 2009].

The identification of mitochondrial permeability transition pore (mPTP) as a critical determinant of cardiomyocyte death was initially described by the pioneering work of Crompton and Colleagues using isolated adult rat mitochondria in cardiomyocytes [Crompton et al., 1987] [Crompton and Costi, 1988]. The authors reported the involvement of the mPTP as the proponent of mitochondrial dysfunction in the heart following reperfusion after a period of ischaemia [Halestrap, 2009]. Subsequent studies in isolated cardiomyocytes [Leyssens et al., 1996] and Langendorff perfused hearts [Griffiths and Halestrap, 1993] [Griffiths and Halestrap, 1995] demonstrated the key involvement of the mPTP in the phenomenon IRI. The mPTP is known to be sensitive to calcium, oxidative stress, phosphate and ADP [Halestrap, 2009], which are conditions notably present in incidences of anoxia/re-oxygenation-induced injury in the heart and other tissues [Halestrap et al., 2004] [Hausenloy et al., 2009]. *In vitro* studies have demonstrated pore opening in response to high concentrations of Ca^{2+} ions and rapid pore closure after calcium chelation [Halestrap, 2009]. These results suggest that calcium ion presence is the primary trigger of mPTP opening. It is however notable that a pathological rise in Ca^{2+} concentration alone is likely to be ineffective without the presence of additional influences, such as oxidative stress [Kim et al., 2006] and pH changes [Halestrap, 2009], altering the sensitivity of the pore complex [Halestrap and Pasdois, 2009].

During ischaemia, despite an influx of calcium ions into the cardiomyocyte, the mPTP remains closed due to the aforementioned potent inhibitory effect of H^+ ions preventing Ca^{2+} binding to a specific trigger site [Gunter and Pfeiffer, 1990]. However, at reperfusion, there is a rapid removal of lactate and a re-establishment of physiological pH, which eliminates the inhibitory

block and permits subsequent opening of the mPTP in response to the pathological rises in Ca^{2+} concentration [Halestrap, 2009]. Opening of the non-selective channel allows uncontrolled movement of molecules, most-detrimentally protons, through the inner mitochondrial membrane and subsequent disruption of the MMP. This in turn leads to respiratory chain uncoupling and prevention of ATP synthesis at the level of the reenergised mitochondria, culminating in ATP depletion and necrotic cell death [Halestrap and Pasdois, 2009].

MMP disruption also exacerbates calcium overload and myofibril hypercontracture as accumulated calcium is released into the cardiomyocyte cytoplasm [Halestrap et al., 2004]. In addition, re-activation of the ETC triggers a release of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) [Kalogeris et al., 2012], which contributes to oxidative stress and increases the sensitivity of the mPTP complex to react to basal concentrations of calcium [Skarka and Ostadal, 2002]. Levels of circulating ROS are exacerbated by further release from xanthine oxidase, which is an enzyme present in endothelial cells and able to metabolise superoxide anions and NADPH oxidase present in migrating neutrophils. An increase in the ROS level also induces the rupture of the sarcoplasmic reticulum and this in turn leads to an instantaneous calcium ion 'overload' within the cytoplasm of cardiomyocytes, triggering hypercontracture of cardiac myofibrils [Hausenloy and Yellon, 2013].

A further consequence of mPTP opening at reperfusion is mitochondrial swelling. This occurs due to an increase in permeability of the inner mitochondrial membrane, which lends itself to the movement of small osmolytes. In contrast, the mitochondrial matrix protein concentration remains high, thus exerting osmotic pressure. As the matrix expands, pressure is placed upon the outer membrane, which has the potential to eventually rupture, releasing pro-apoptotic

proteins able to activate a caspase pathway leading to apoptotic cell death [Halestrap and Pasdois, 2009] [Skarka and Ostadal, 2002]. These changes at the level of the mitochondria in response to reperfusion are schematically illustrated in figure. 1.2.

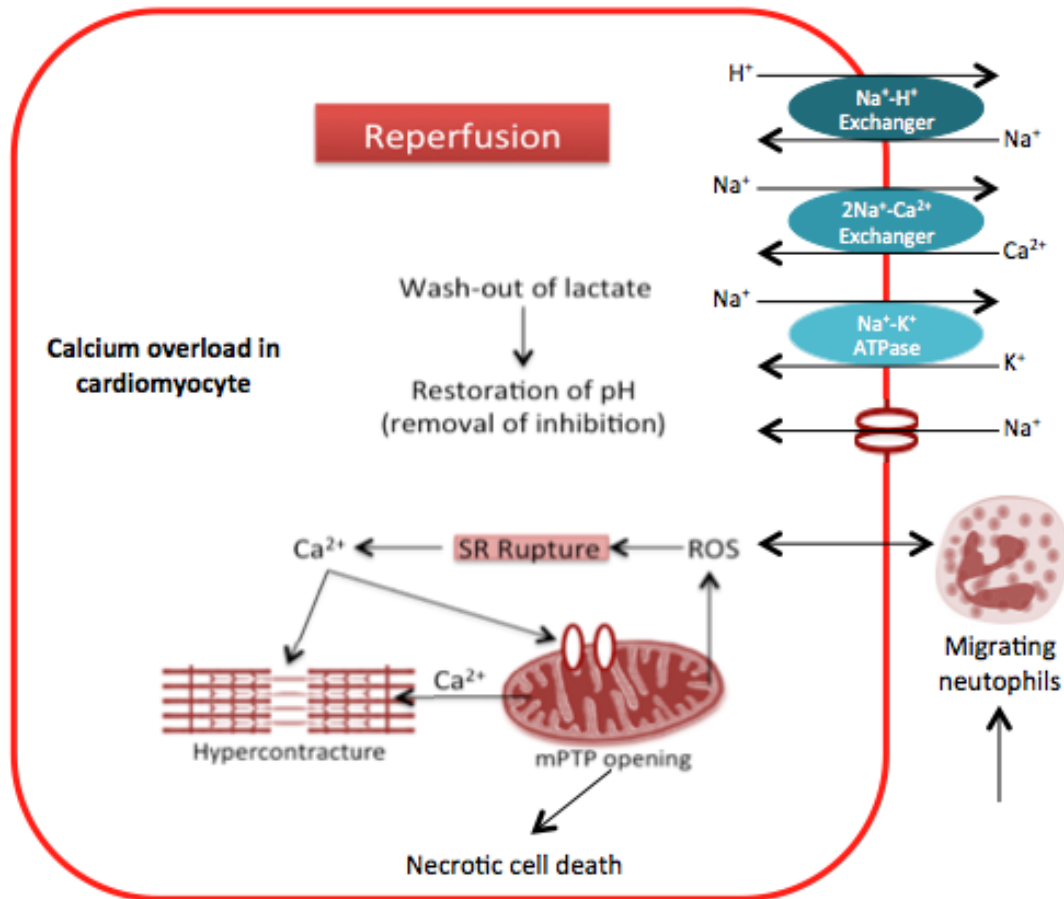


Figure.1.2.Cascade of changes that occur within the cardiomyocyte in response to reperfusion. Restoration of physiological pH following a wash-out of lactate at reperfusion removes the inhibitory effect at the level of the myofibrils and the mPTP; permitting both to respond to the calcium overload in the cell cytoplasm, thus triggering hypercontracture of the cardiac myofibrils and mPTP opening. The rise in Ca^{2+} concentration is due to reactive oxygen species (ROS) – released from the mitochondria and the endothelial cells following the conversion of metabolites released from migrating neutrophils – which trigger the rupture of the sarcoplasmic reticulum. Additional Ca^{2+} enters the cytoplasm through exchangers at the sarcolemmal membrane and from the reenergised mitochondria. Uncoupling of oxidative phosphorylation and depletion of ATP at the level of the mitochondria can trigger necrotic cell death.

Figure. 1.2. adapted from Hausenloy and Yellon, 2013.

1.3. Established methods of cardioprotection against myocardial IRI

Although re-establishment of blood flow to ischaemic heart muscle may be considered detrimental, reperfusion is vital if the changes elicited by a prolonged ischaemic episode are to be reversed and tissue integrity salvaged [Turer and Hill, 2010]. Methods designed to induce adaptive responses to incidences of ischaemia and minimise the damage elicited by reperfusion are termed as 'conditioning'. Conditioning may be applied before or after an ischaemic event. There are three mechanical strategies by which the heart can be protected against myocardial IRI through the application of non-lethal episodes of ischaemia: (1) ischaemic preconditioning, (2) ischaemic post-conditioning and (3) remote ischaemic conditioning. These methods are described below (see sections 1.3.1 – 1.3.3). Alternatively, several pharmacological strategies have been found to be therapeutic in incidences of IRI (see section 1.5.).

1.3.1. Ischaemic preconditioning

Ischaemic preconditioning (IPC) involves applications of ischaemia and reperfusion before an ischaemic event. It was first described in 1986 by Murry and colleagues using a canine model. The authors reported that four 5 min cycles of non-lethal periods of ischaemia and reperfusion (through cyclic left anterior descending (LAD) artery occlusion), followed by a prolonged 40 min period of ischaemia reduced infarct size by 75% when compared to control animals [Murry et al., 1986]. These results highlighted a potential to induce adaptive conditioned responses with cardioprotective potential, but only when an ischemic event can be predicted.

1.3.2. Ischaemic post-conditioning

The premise of ischaemic post-conditioning (IPost) is almost identical to that of IPC, but brief cycles of ischaemia and reperfusion are applied following a prolonged period of ischaemia at the onset of the reperfusion phase [Zhao et al., 2003]. This phenomenon was first described by Zhao and colleagues where they observed approximately 40%-50% reduction in infarct size in a canine model following 60 min LAD occlusion and three 30 sec cyclic phases of occlusion and reperfusion before a final 3 h reperfusion phase [Zhao et al., 2003]. Similar levels of cardioprotection have also been demonstrated in humans. Previous studies have reported that four 60-sec cycles of low-pressure inflation and deflation of an angioplasty balloon in patients undergoing PPCI reduces infarction size by 36% [Staat et al., 2005] and 39% [Thibault et al., 2008], respectively. In addition, there is an on-going phase 3 DANAMI-3 trial of 2,000 patients to investigate the effects of an IPost intervention of four 30-sec cycles of low-pressure inflation and deflation of an angioplasty balloon versus conventional treatments in STEMI patients [Hofsten et al., 2015]. Post-conditioning has also been shown to be effective *ex vivo* through the application of periods of hypoxia and re-oxygenation to harvested atrial appendages [Sivaraman et al., 2007].

1.3.3. Remote ischaemic conditioning

Both IPC and IPost require invasive intervention to be applied directly to the heart and the coronary vasculature, which carries an element of risk. Remote ischaemic conditioning (RIC) offers an alternative strategy by which the heart is protected through the application of non-lethal episodes of ischaemia and reperfusion to areas at a distance from the heart (remote tissues). RIC was first described by McClanahan and colleagues in 1993, who demonstrated that brief applications of renal artery occlusion and reperfusion conferred protection at the level of the heart in a rabbit model [McClanahan et al., 1993]. A number of studies have also

demonstrated that RIC can also be applied to the upper/lower limbs, intestines, kidneys or lungs [Hausenloy and Yellon, 2008]. Several studies on a small patient population have reported RIC to be cardioprotective [Cheung et al., 2006] [Hausenloy et al., 2007] [Venugopal et al., 2009]. For example, Thielmann and colleagues have previously reported that the application of three 5-min cycles of upper limb ischaemia (through the inflation and deflation of a blood pressure cuff at 200mmHg) in a study of 329 patients undergoing coronary artery bypass graft (CABG) surgery, reduced troponin I (a biomarker indicative of myocardial injury) by approximately 17% and lowered mortality rates and incidences of cardiac events [Thielmann et al., 2013]. In contrast, one of the first major trials to produce negative results came from Bonser's group. Rahman and colleagues utilised three time 5-min cycles of forearm ischaemia-reperfusion in 162 CABG patients in a double-blinded, placebo-controlled trial. No reduction in myocardial injury was observed, as indicated by 48-hr post-operative measurements of troponin. [Rahman et al., 2010] [Sivaraman et al., 2015]. Furthermore, a very recent study by Hausenloy and colleagues reported that in the large patient population ERICCA trial (1612 patients), CABG patients subjected to four 5-min inflations and deflations of a blood pressure cuff (to 200mmHg) showed no statistical difference in clinical end-points such as death from cardiovascular causes and non-fatal infarction levels between conditioned and unconditioned groups. [Hausenloy et al., 2015]. Reasons for this discrepancy in results include patient population size, patient co-morbidities and the use of other pharmacological agents that could be deemed cardioprotective. For example, many patients undergoing CABG surgery have been taking medication such as beta blockers or glyceryl trinitrate (GTN) and have been exposed volatile anaesthetics pre-surgery, all of which have the ability to mimic IPC and elicit a degree of cardioprotection. This can in turn mean that RIC is unable to confer any further benefit [Heusch et al., 2015][Sivaraman et al., 2015].

1.4. The RISK pathway and its importance in cardioprotective intervention

Numerous research articles have been published which have attempted to elucidate the mechanism(s) by which mechanical conditioning methods elicit their cardioprotective effects [Hausenloy et al., 2005b] [Yellon and Downey, 2003] [Sivaraman and Yellon, 2014]. Previous studies have implicated the involvement of a signalling cascade of various pro-survival kinases, which has been termed the 'reperfusion injury salvage kinase pathway' or RISK pathway [Hausenloy and Yellon, 2004]. A simplified representative illustration of the key components of the RISK pathway is shown in figure. 1.3.

To mimic mechanical conditioning (pre- and/or post-conditioning), administration of various pharmacological agonists and antagonists (e.g. volatile anaesthetics and adenosine acting upon adenosine receptors, atrial natriuretic peptide affecting soluble guanylate cyclase (sGC), phosphodiesterase 5 (PDE5) inhibitors modulating cyclic guanylate monophosphate (cGMP) levels, naxolone δ receptors antagonist and protein kinase C (PKC) antagonist Delcasertib) of the RISK pathway have also been investigated [Liu et al., 1991] [Davis et al., 1983] [Yang et al., 2006a] [Kukreja et al., 2007] [Schultz et al., 1995] [Churchill and Mochly-Rosen, 2007]. A number of animal studies have reported that these pharmacological inhibitors can abolish the cardioprotection afforded by IPC and IPost, whilst agonists mimic the effects, thus highlighting activation of the RISK pathway as an essential factor in mechanical methods of protection [Hausenloy and Yellon, 2007] [Sivaraman and Yellon, 2014] [Chang et al., 2012].

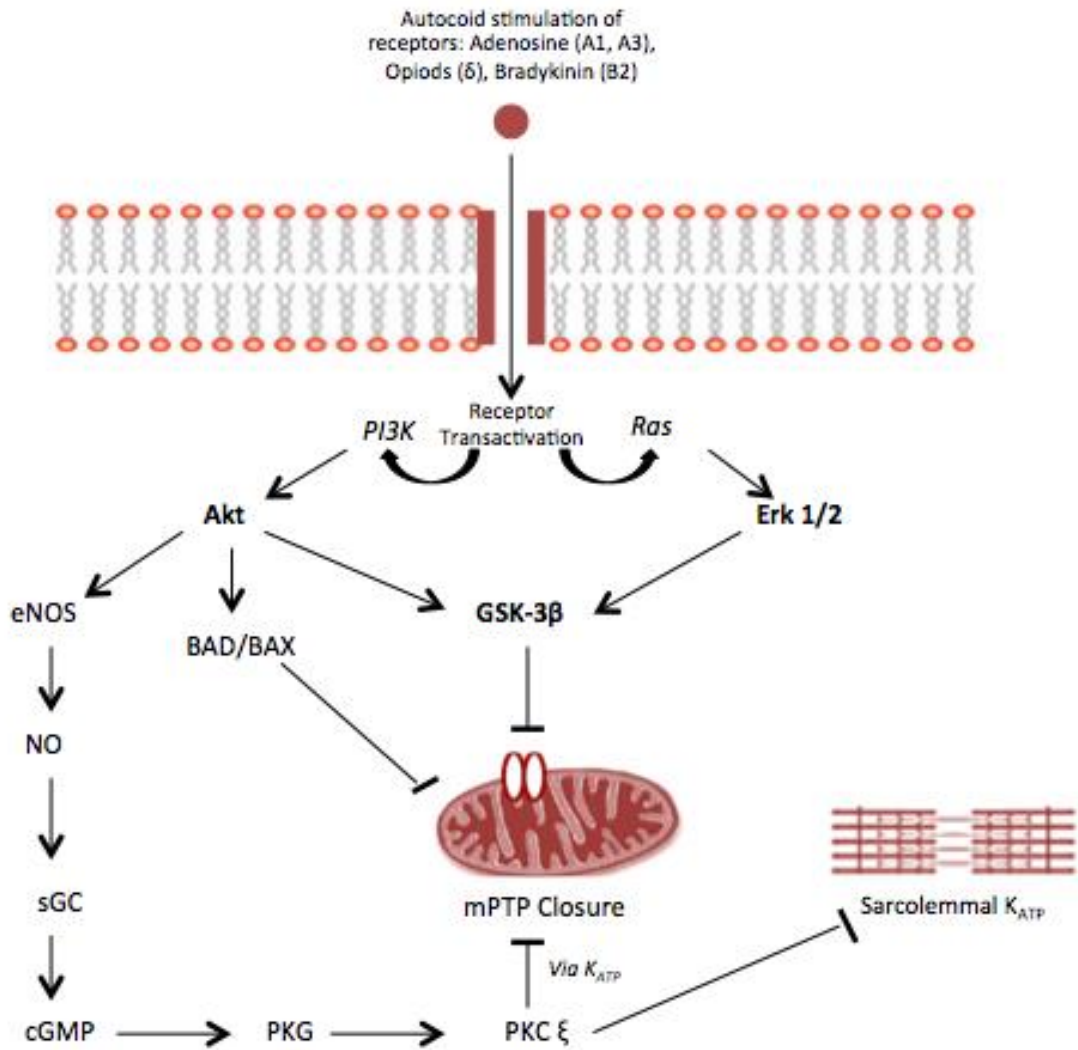


Figure.1.3. Schematic illustration providing an overview of the RISK pathway components involved in cardioprotective signalling.

Signalling cascade known as the RISK pathway has been shown to be recruited during mechanical ischaemic conditioning methods of eliciting cardioprotection. Pathway begins at the cardiomyocyte plasma membrane with the activation of G-protein-coupled receptors by autocooid substances. Activation of two key pathways, PI3K-Akt and Ras-Erk 1/2 (with downstream effectors including eNOS, BAD/BAX and GSK-3 β), which terminate at the mitochondria and sarcolemma, mediate cardioprotection through mPTP closure and an influence on calcium loading.

Figure. 1.3 adapted from Hausenloy et al., 2009.

1.4.1. The involvement of the RISK pathway in ischaemic preconditioning (IPC)

The application of IPC stimuli to isolated perfused rat hearts demonstrated phosphorylation of both Akt and downstream target GSK3 β (able to mediate inhibition of the mPTP to elicit

cardioprotection) prior to the prolonged ischaemic period [Tong et al., 2000]. Furthermore, inhibition of Akt and GSK3 β proved their imperative involvement in the preconditioning process [Tong et al., 2002]. Activation at the point of reperfusion may occur due to the initial activation that took place in response to sufficient IPC stimulus prior to ischaemia [Hausenloy et al., 2005b]. In addition, mediators such as ROS have been implicated in the activation of Erk 1/2 [Samavati et al., 2002], although its role in IPC is debated [Behrends et al., 2000]. At reperfusion, IPC has been demonstrated to phosphorylate p70S6K thus inducing the inactivation of pro-apoptotic factors BAD and BAX and promoting cell survival [Hausenloy et al., 2005a].

1.4.2. The involvement of the RISK pathway in ischaemic post-conditioning (IPost)

Administration of adenosine or adenosine receptor agonists at reperfusion has been shown to elicit cardioprotection and mimic IPost effects. Furthermore, endogenously produced adenosine has a delayed washout time in incidences of ischaemic post-conditioning. These results suggest that activation of the adenosine receptor, and therefore recruitment of the RISK pathway plays an essential role at reperfusion [Philipp et al., 2006]. Furthermore, the application of a standard IPost stimulus of six 10-sec cycles of ischaemia and reperfusion following a prolonged ischaemic event in a rat heart model was shown to increase Akt phosphorylation. Inhibition of Akt abrogated the reduction in infarct size and subsequently the IPost effect [Tsang et al., 2004]. This protection was additionally abolished by inhibition of the Erk 1/2 component of the RISK pathway suggesting that both Akt and Erk 1/2 are required to mediate the protection induced by IPost [Yang et al., 2004]. Further downstream, post-conditioning triggers the phosphorylation of p70S6K and endothelial nitric oxide synthase (eNOS). P70S6K induces the inactivation of pro-apoptotic factors BAD and BAX whilst eNOS prevents mPTP opening through its release of nitric oxide (NO) into the cGMP-

PKG-PKC pathway. Together these downstream effectors elicit cardioprotection [Tsang et al., 2004] [Hausenloy et al., 2005b].

1.4.3. Direct mPTP inhibition in IPC and IPost: The eNOS-cGMP-PKG signalling pathway

The signal cascades of the RISK pathway activated during either IPC or IPost have been reported to converge at the mPTP [Hausenloy and Yellon, 2007], suggesting that dysfunction at the level of the mitochondria is pertinent to the pathophysiology of myocardial IRI.

A number of signal transduction pathways are also recruited by the binding of autocooids (including adenosine, bradykinin and endogenous opioids and other growth factors) to specific G-protein coupled receptors (GPCRs) present at the plasma membrane of cardiomyocytes [Hausenloy et al., 2009] [Sivaraman and Yellon, 2014]. These pathways express a cardioprotective signal to specific components of the mitochondria such as the ATP-sensitive mitochondrial potassium K_{ATP} channel and/or the mPTP. One such pathway is the PI3K-Akt-eNOS-cGMP-PKG cascade; linking GPCRs to the mitochondria in IPC and IPOST and appearing central to many of the mechanical interventions currently available [Cohen et al., 2006]. Protein kinase G (PKG) in particular (along with its downstream effectors), have been highlighted as a critical mediator of both IPC and IPost in myocardial IRI [Hausenloy et al., 2009] [Burley et al., 2007]. Cytosolic PKG is thought to phosphorylate a pool of the PKC- ϵ isoform, which in turn opens the mitochondrial K_{ATP} channel, and elicits protection *via* mPTP inhibition in both IPC and IPost [Costa et al., 2005]. It is therefore the eNOS-cGMP-PKG pathway that forms a focal point of the present study. This particular cascade is highlighted in figure.1.4.

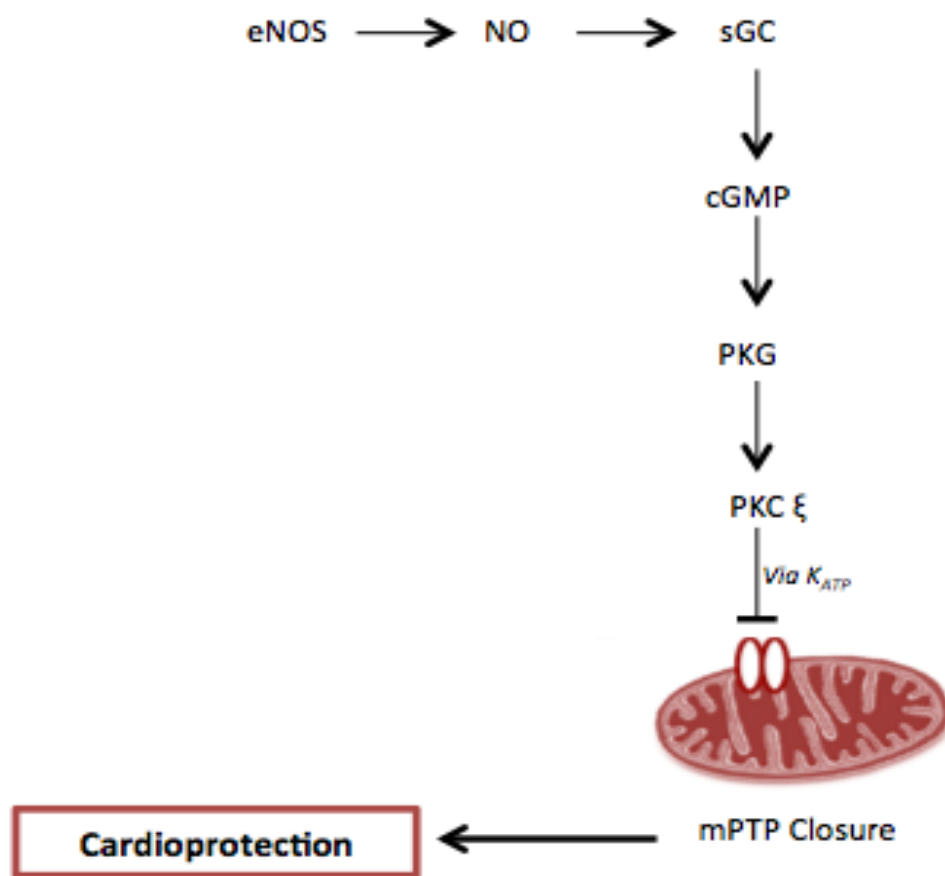


Figure.1.4. The eNOS-cGMP-PKG signalling pathway.

The eNOS-cGMP-PKG pathway was experimentally demonstrated to be central to many of the pharmacological and mechanical interventions used to combat damage elicited by myocardial IRI. The pathway terminates at PKCε, which acts upon potassium channels at the level of the mitochondria to reduce mPTP opening at reperfusion; an event central to the pathology of reperfusion injury. Cardioprotection elicited *via* this pathway is the focus of the present study.

1.5. Potential Pharmacological agents to protect against myocardial IRI

The RISK pathway provides a series of novel targets by which cardioprotection may be elicited through pharmacological activation/upregulation or inhibition/downregulation of cascade components [Hausenloy and Yellon, 2004] [Hausenloy and Yellon, 2007]. A selection

of pharmacological agents that have been considered and/or trialled for therapeutic use against myocardial IRI are discussed below:

1.5.1. Pharmacological agents reported to have beneficial effects against myocardial IRI

Adenosine: Previous animal studies have demonstrated that adenosine is protective against myocardial IRI in both IPC and IPost [Kis et al., 2003] [Yang et al., 2004] [Park et al., 2006]. In addition, Liu and colleagues have reported that intracoronary administration of an adenosine receptor agonist (N6-1-(phenyl-2R-isopropyl)adenosine) produced a reduction in infarct size similar to that observed in preconditioned hearts, thus supporting a role for adenosine in IPC [Liu et al., 1991]. In 1999, to test whether adenosine reduces myocardial infarct size in man, the Acute Myocardial Infarction Study of Adenosine (AMISTAD) trial was conducted and has shown promising results. In this 236 patients study, infusion of adenosine showed a relative reduction in infarct size of 33% compared to the control group [Mahaffey et al., 1999]. The AMISTAD-II randomised trial examined the effects of three-hour adenosine infusions at two different concentrations prior to reperfusion in 2118 ST-segment elevation MI (STEMI) patients. Myocardial infarct size was reduced with the higher adenosine dose of 70µg/kg/min [Ross et al., 2005]. Post-hoc analysis also showed that patients treated with adenosine had improved survival outcome [Kloner et al., 2006].

Bradykinin: Intra-arterial infusions of bradykinin were found to reduce infarct size to a similar extent as IPC in an *in vivo* rabbit model of IRI. These beneficial changes were blocked by the administration of a bradykinin receptor antagonist [Wall et al., 1994]. Further animal studies have demonstrated a crucial role for bradykinin and its action at B2 receptors in IPC [Manolis et al., 2010]. The involvement of bradykinin in cardioprotection in humans is a debated topic.

Patients administered with bradykinin prior to isolated coronary artery bypass graft (CABG) surgery recorded reduced creatine kinase levels (CK; a biomarker measurement indicative of cardiac injury) [Wang et al., 2009]. Whilst a trial in healthy males using a forearm endothelium-mediated vasomotor dysfunction model suggested no role for bradykinin in humans [Sharma et al., 2015].

Atrial Natriuretic Peptide (ANP): Administration of the ANP has been shown experimentally to reduce myocardial infarct size in a rabbit model through the activation of pro-survival kinases within the RISK pathway [Yang et al., 2006b]. In addition, Kitakaze and colleagues have demonstrated in the J-WIND-ANP trial of 569 STEMI patients that an intravenous administration (0.025µg/kg/min) of ANP analogue carperitide over 72 hours reduced infarct size by approximately 15% (determined by CK area under the curve calculations) and improved left ventricular ejection fraction [Kitakaze et al., 2007].

Glucose-insulin-potassium (GIK) therapy: Various clinical trials have considered the therapeutic use of GIK therapy with mixed results. The CREATE-ECLA trial in which 20,201 STEMI patients were given an intravenous infusion of GIK or placebo for 24 hrs after reperfusion showed no statistical difference in mortality 30 days post-procedure [Mehta et al., 2005]. However, more encouraging results were produced by the IMMEDIATE trial of 357 STEMI patients. Patients received an intravenous GIK or placebo infusion for 12 hours (started by paramedics in the ambulance on the way to the PPCI centre) prior to reperfusion. The authors reported a reduction in infarct size and in-hospital mortality in the individuals given GIK therapy, suggesting that the effectiveness of treatment is dependent on the time point at which it is administered [Selker et al., 2012].

Cyclosporine: The immunosuppressant drug cyclosporine is able to elicit cardioprotection through its interaction with cyclophilin D, which is a key component of the mPTP. Cyclosporine prevents pore formation and subsequently inhibits the mPTP opening, thus protecting mitochondrial integrity [Sivaraman and Yellon, 2014]. In a small pilot trial by Piot and colleagues (2008), 58 patients with acute STEMI were given a 2.5mg/kg dose of cyclosporine or saline solution prior to undergoing PPCI treatment. The cyclosporine group showed a significant reduction in infarct size (MRI scan 5 days post-procedure) and CK levels [Piot et al, 2008]. However, a very recent study (CIRCUS trial) in 790 patients investigated whether cyclosporine treatment could reduce death and hospitalisation for patients with heart failure. This trial showed no statistical difference between patients treated with cyclosporine or placebo [Frolich et al., 2013] [Cung et al., 2015].

1.6. Nitric Oxide and its involvement in cardioprotection

Nitric oxide (NO) is a signalling molecule and plays an essential role in the cardiovascular system, such as in vascular smooth muscle cell proliferation, angiogenesis, vasodilatation, regulation of blood pressure, and inhibition of platelet aggregation [Moncada and Higgs, 1993] [Bailey et al., 2014] [Bohlen, 2015]. Reduced NO bioavailability has been implicated in several cardiovascular pathologies, such as hypertension, atherosclerosis, heart failure and IRI [Rochette et al., 2013] [Lundberg et al., 2015]. Therefore restoration and/or improvement of NO bioavailability has a potentially therapeutic role [Palmer et al., 1988] [Moncada and Higgs, 1993].

1.6.1. The mechanism of NO production

In 1980 Furchgott and Zawadzki used sandwich preparations of blood vessels with either intact or damaged/rubbed endothelium to discover the ability of endothelium to generate

endothelium-derived relaxing factor (EDRF). EDRF was later identified as endogenously produced NO. NO is synthesised by nitric oxide synthase (NOS) from the substrate L-arginine in the presence of various cofactors, such as nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH₄) and [Moncada et al., 1989]. To date, three NOS isoforms have been identified: neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed physiologically and generate only moderate amounts of NO in order to maintain basal levels. Inducible NOS (iNOS) however, is expressed following an increase in inflammatory cytokines, and therefore produces higher levels of NO as part of the innate immune system [Nathan, 1997] [Rosselli et al., 1998] [Omar et al., 2016].

1.6.2. NO classical signalling pathway and bioavailability in cardioprotection

Once NO is generated, it is well established that NO mediates its downstream functions through the 'classical' NO-sGC-cGMP-PKG pathway, which is vital for the physiological effects in the heart and the vasculature [Francis et al., 2010]. Specifically targeting components of the eNOS-cGMP-PKG pathway has highlighted its importance in cardioprotection. Gross and colleagues demonstrated that the protective effects of epoxyeicosatrienoic acids (EETs) against myocardial IRI in a rat model of infarction were completely attenuated by the specific eNOS inhibitor L-NIO [Gross et al., 2013]. In addition, eNOS knock-out mice subjected to myocardial IRI showed that infarct size was significantly augmented when compared to their wild-type littermates [Huang et al., 1995].

The cardioprotective role of cyclic guanosine monophosphate (cGMP) has been studied extensively in various myocardial IRI models [Cohen et al., 2006] [Kass, 2012]. Sildenafil is a phosphodiesterase-5 (PDE5) selective inhibitor and a pharmacological agent able to elicit cardioprotection by raising the abundance of cGMP intracellularly through the prevention of its

hydrolysis by PDE5 enzymes [Blanton et al., 2012] [Kass, 2012]. Madhani and colleagues have previously shown that administration of sildenafil at the onset of reperfusion in a murine model of IRI attenuated the infarct size. Furthermore, co-administration of KT5823 (PKG inhibitor), significantly increased the infarct size, thus supporting the previously established importance of the cGMP-PKG pathway during myocardial IRI [Costa et al., 2005] [Das et al., 2008] [Burley et al., 2007]. However, there has been a lack of clinical trials in humans considering the role of PDE5 inhibitors in this context [Sivaraman and Yellon, 2014].

Previous studies have also shown that L-arginine analogues, NO donors, NOS selective inhibitors and NOS gene deletion improved NO bioavailability following myocardial IRI [Jones and Bolli, 2006] [Omar et al., 2016]. Nakano and colleagues assessed the role of NO in classical IPC using an *ex vivo* rabbit model of myocardial ischaemia-reperfusion. Hearts were perfused with NO-donor, S-nitroso-N-acetylpenicillamine (SNAP), as a preconditioning agent prior to the application of 30 min global ischaemia followed by 120 min reperfusion. SNAP treatment elicited a cardioprotective effect similar to that observed following IPC, with a statistically significant reduction in infarct size when compared to the control [Nakano et al., 2000].

There is however disparity between the effectiveness of NO donors experienced in pre-clinical animal studies and human trials [Bice et al., 2016]. Glyceryl trinitrate (GTN) is a NO donor, able to mediate coronary vasodilatation and improve oxygen supply-demand balance in the myocardium [Sivaraman and Yellon, 2014]. It is one of the most commonly used treatments in acute MI, left-sided heart failure and angina pectoris in humans [Sogo et al., 2000]. Unfortunately, it is well-documented in the clinical setting that GTN induces tolerance. Prolonged or high-dose administration of GTN results in a diminished haemodynamic

response (primarily affecting vasodilatation) [Csont and Ferdinandy, 2005]. Furthermore, an alternative NO donor tested clinically in man, isosorbide dinitrate, was found in two separate trials to be ineffective in reducing infarct size when reperfusion was confirmed [Hildebrandt et al., 1992] [Morris et al., 1995]. It is proposed that the discrepancy between the results afforded by animal studies and clinical trials is due to numerous factors which include, comorbidities present in patients with AMI and/or undergoing cardiac surgery included in the trials, which are not replicated in the healthy juvenile animals used in animal models [Bice et al., 2016].

Overall, the importance of the eNOS-cGMP-PKG pathway in cardioprotection against myocardial IRI, paired with the ineffectiveness of NO donors in the clinical setting, highlights a requirement for an alternative therapy able to substitute NO and activate this pathway.

1.7. Nitrite as a potential substitute for NO

Nitrite was previously viewed to be biologically inactive product of NO [Omar and Webb, 2014] [Bailey et al., 2014], but studies by Benjamin et al. (1994) and Lundberg et al. (1994) have demonstrated opposing evidence that nitrite could be reduced under hypoxic and/or acidic conditions to produce NO. Therefore, confirming an alternative source of NO bioavailability when comparing to the classical NO pathway described in section 1.5.2. [Omar and Webb, 2014].

1.7.1. Dietary nitrate conversion in the digestive system

Inorganic nitrate (NO_3^-) is present in beetroot and in leafy vegetables such as spinach and cabbage [Kapil et al., 2010] as well as cured meats [Kim and Conca, 1990]. Once ingested, within 24 hrs 75% of nitrate is excreted via the kidneys, with the remaining 25% taken up

into the salivary glands [Omar and Webb, 2014]. Nitrate-rich saliva is released into the oral cavity and nitrate is converted to nitrite by commensal bacteria (*Actinomyces*, *Veillonella* and *Rothia* species) found on the surface of the tongue [Doel et al., 2005]. After swallowing, the highly acidic conditions of the stomach results in the protonation of nitrite to nitrous acid in addition to the spontaneous generation of NO. Some nitrite will be absorbed by the upper GI tract and re-enter the circulation via the 'enterosalivary recirculation pathway' of nitrate [Bailey et al., 2014].

1.7.2. Generation of NO by reductase enzymes

Zweier and colleagues demonstrated that NO could be generated under hypoxic conditions independently of NOS enzymes and were the first group to suggest its involvement in NO provision to the ischaemic heart [Zweier et al., 1995]. It has since been identified that nitrite is reduced to NO under hypoxic and/or acidic conditions by a plethora of nitrite reductases [Kapil et al., 2010] [Pellegrino et al., 2009]. These include, xanthine oxidoreductase (XOR) [Li et al., 2001], mitochondrial cytochrome p450 enzymes [Castello et al., 2006] [Omar and Webb, 2014], haem-globins (deoxygenated forms of haemoglobin and myoglobin) [Rassaf et al., 2007], aldehyde dehydrogenase 2 (ALDH2) [Arif et al., 2015] and endothelial nitric oxide synthase (eNOS) [Vanin et al., 2007]. Such reductases are expressed constitutively in the blood and in tissues (e.g. vessels and the myocardium) [Farah and Reboul, 2015].

1.7.3. Nitrite in cardioprotection

The benefits of dietary nitrate and nitrite have been demonstrated in various animal models of myocardial IRI [Ashmore et al., 2014] [Raaf et al., 2009] [Bryan et al., 2007]. For instance, Kapil and colleagues have reported a similar level of infarct reduction to that exhibited in nitrite-perfused isolated hearts through dietary nitrate supplementation in a murine model

[Kapil et al., 2010]. In addition, Bryan et al. (2007) provided mice with an oral supplementation of nitrite in drinking water prior to their subjection to IRI. The authors reported that nitrite supplementation substantially decreased infarct size (approximately 48%). Overall, these results suggest that therapeutic uses for dietary nitrate and nitrite supplementation offer protection when given prior to an ischaemic event (pre-conditioning) [Kapil et al., 2010]. In addition, Webb and colleagues have previously investigated the role of nitrite following myocardial IRI in a rat Langendorff model [Webb et al., 2004]. Sodium nitrite (NaNO_2 ; 10 and $100\mu\text{M}$) was administered as a preconditioning agent before and during a period of global normothermic ischaemia. The authors showed that nitrite (10 and $100\mu\text{M}$) significantly reduced infarct size via XOR-catalysed reduction to NO with additional improvements to left ventricular function when compared to controls [Webb et al., 2004]. The results of this study were further supported by Duranski et al. (2005), in which they demonstrated a dose-dependent effect of intraventricular bolus administration of NaNO_2 in an *in vivo* murine model of ischaemia-reperfusion. The authors reported a reduction in infarct size (~67%) and suggested the effect was NO-dependent as the protection was abrogated in the presence of NO scavenger CPTIO [Duranski et al., 2005]. The same research group also demonstrated cytoprotective effects of nitrite against IRI in the liver [Duranski et al., 2005]. Other studies have highlighted the beneficial effects of nitrite in other organs such as the brain [Jung et al., 2006] [Phillips et al., 2009], kidney [Tripatara et al., 2007] and hindlimb [Kumar et al., 2008].

To date, various nitrite reductases have been investigated. For example, Ghosh and colleagues demonstrated that dose-dependent vasodilatation in response to nitrite was almost completely abolished by the presence of XOR inhibitor allopurinol [Ghosh et al., 2013]. Additional studies utilising eNOS knock-out [Wood et al., 2013] and myoglobin deficient

[Totzeck et al., 2012] mice demonstrated the importance of these reductase enzymes in nitrite-mediated vasodilatation and blood pressure control. Furthermore, in a study measuring nitrite-induced vasorelaxation in rat aortae, relaxation was also attenuated by the presence of the ALDH2 enzyme inhibitor cyanamide [Arif et al., 2015]. It has been proposed that nitrite mediates its cardiovascular effects through reduction to NO by the aforementioned nitrite reductases under conditions of hypoxia and acidosis [Lundberg et al., 2008] [Kapil et al., 2010]. Nitrite-NO subsequently elicits powerful protective effects in the heart and the vasculature through signal transduction *via* the classical sGC-cGMP-PKG pathway [Pellegrino et al., 2009] [Totzeck et al., 2012].

There is encouraging evidence in the clinical arena that nitrite is also able to confer cardioprotection against ischaemia-reperfusion in human studies/trials. Webb and colleagues demonstrated that dietary supplementation of beetroot juice (a rich source of nitrate) restored flow-mediated dilatation (FMD) responses, reduced by ~60% in response to mild ischaemic insult, to baseline levels in the forearms of healthy males [Webb et al., 2008]. In addition, Ingram and colleagues have shown in 10 patients with inducible myocardial ischaemia and in 19 healthy subjects induced with whole-arm ischaemia-reperfusion that a low dose of sodium nitrite improved functional responses in the ischaemic myocardium and provided protection against vascular IRI, respectively [Ingram et al., 2013]. Recently, phase II trials have also been conducted to examine the potential role of nitrite therapy in AMI. The large NIAMI trial (280 patients) conducted by Siddiqi and colleagues involved the intravenous administration of sodium nitrite (70 μ mol; 5 min infusion) immediately before the reperfusion phase as a form of peri-conditioning in STEMI patients. The multi-centre study showed that there was no significant changes in the infarct size or CK and troponin I levels at both 6-8 days and 6 months post-AMI [Siddiqi et al., 2014]. In addition, Jones and colleagues assessed the

effects of an intracoronary infusion of sodium nitrite (NaNO_2 ; 10mL of $1.8\mu\text{mol}$) or a sodium chloride (NaCl) placebo prior to balloon dilatation in 80 STEMI patients [Jones et al., 2015]. The authors reported no change in infarct size (as determined by MRI) or biomarkers CK and troponin T levels. However, there was an improvement in myocardial salvage index and a lower number of major adverse cardiac events (MACEs) recorded 1 year post-procedure in patients given nitrite treatment [Jones et al., 2015]. The clinical application of therapeutic nitrite treatment in humans does however require further investigation.

Overall, these studies highlight the effectiveness of nitrite when administered as a preconditioning agent prior to a prolonged ischaemic event [Ingram et al., 2013]. In addition, these results suggest that nitrite confers cardioprotection from a transient presence of the molecule hours before ischaemia onset [Pride et al., 2014]. Although there is compelling evidence that nitrite is protective, the precise mechanism(s) remains to be fully elucidated. A recent study in healthy subjects reported that administration of sodium nitrite caused a marked radial vasodilatation under normoxic conditions (physiological levels of oxygen) and was abolished during hypoxia and hyperoxia [Omar et al., 2015]. These results suggest an alternative mechanism(s) by which nitrite may elicit cardioprotection.

Therefore, the main objective of the present study is to decipher the mechanism(s) by which nitrite mediates cardioprotection, and we consider two potential mediators: PKG 1 α oxidation and the aldehyde dehydrogenase 2 (ALDH2) enzyme.

1.8. The PKG 1 α oxidation pathway: a novel mediator of cardioprotection?

It has been generally accepted that nitrite is reduced to NO by several nitrite reductase enzymes under hypoxic and/or acidic conditions and mediates cardioprotection [Omar and

Webb, 2014]. Furthermore, previous studies have postulated that NO can trigger mPTP closure and elicit cardioprotection against myocardial IRI through the recruitment of the sGC-cGMP-PKG pathway; a component of the RISK pathway of pro-survival protein kinases [Cohen et al., 2006]. However, compelling data from recent studies poses the notion of an alternative, novel PKG1 α oxidation pathway in the vasculature [Prysyazhna and Eaton, 2015], and we hypothesise that nitrite may be able to elicit cardioprotection via PKG1 α oxidation, independently of the sGC-cGMP-PKG pathway as described in section 1.8.1.

1.8.1. Direct PKG1 α oxidation: dimer formation independent of sGC-cGMP-PKG pathway

Post-translational modifications of proteins are a means by which function and activity can be regulated. Such modifications include phosphorylation, glycosylation, acetylation, hydroxylation and oxidation. Such mechanisms permit alterations in cell oxidant levels to induce changes in enzymatic function [Cremers and Jakob, 2013], thus influencing signal transduction and enabling a form of 'redox signalling' [Prysyazhna and Eaton, 2015]. This process is particularly relevant to incidences of increased oxidative stress, such as during myocardial IRI. There is experimental evidence to support oxidative modification of cGMP-dependent PKG as an alternative, direct approach of activating the kinase with the ability to have an impact on the cardiovascular system [Prysyazhna and Eaton, 2015]. Burgoyne and colleagues have previously demonstrated that exposing isolated rat hearts to oxidative stress via hydrogen peroxide (H₂O₂) reduced coronary perfusion pressure (e.g. vasorelaxation of the coronary vasculature) [Burgoyne et al., 2007]. Furthermore, cumulative concentration of H₂O₂ to isolated rat thoracic aortic vessels induced vasorelaxation in the presence of sGC inhibitor (ODQ) and also increased PKG disulphide dimer formation [Burgoyne et al., 2007] [Burgoyne et al., 2013].

PKG exists as two isoforms: Type 1 (PKG1) and Type 2 (PKG2). Mammals possess two PKG encoding genes, *prkg1* and *prkg2*, able to produce PKG1 and PKG2 isoforms respectively, which are homodimers of two identical subunits (75 and 85 kDa) with a similar three-domain architecture [Francis et al., 2010]. PKG1 is predominantly expressed in the cardiovascular system and exists as splice variant isozymes PKG1 α (present in the heart, lungs and cerebellum) and PKG1 β (highly expressed in platelets and hippocampal neurons) [Geiselhoringer et al., 2004]. PKG1 α has been found to be sensitive to redox oxidation due to the presence of 11 cysteine residues in its structure (five of which are susceptible to oxidation)[Takio et al., 1984]. Disulphide bond formation is a cysteine modification able to form in signalling kinases and influence enzymatic activity [Cremers and Jakob, 2013], protein interactions [Banky et al., 2003] and cellular localisations [Brennan et al., 2006]. Burgoyne and colleagues have demonstrated the formation of an intermolecular disulphide bond between cysteine 42 (Cys42) residues on adjacent PKG1 α monomeric chains to form a PKG dimer in response to H₂O₂ [Burgoyne et al., 2013]. In Western blots, the PKG monomer band shifts from a 75kDa to 150kDa as a dimeric form. This disulphide formation is representative of PKG1 α oxidation-induced activation. In addition, the authors have shown that the vasodilatory response to H₂O₂ was abolished in the presence of PKG inhibition (Rp-8-Br-cGMP), but not with sGC inhibition (ODQ). These results highlight a novel alternative PKG activation pathway *via* PKG1 α oxidation to form a disulphide dimer bond in response to oxidative stress independently of the classical (NO-cGMP-PKG) pathway [Prysyazhna and Eaton, 2015].

1.8.2. Evidence supporting nitrite-mediated vasorelaxation via PKG1 alpha oxidation in the vasculature

It has become apparent that redox activation of PKG1 α oxidation is a principle mechanism by which vasorelaxation occurs both *ex vivo* and *in vivo*. Pryszyzhna and colleagues utilised transgenic PKG1 α Cys42Ser knock-in (KI) mice (“redox dead”; unable to sense or undergo redox signalling) and their WT littermates to assess the involvement of this pathway in the vasodilatory effects of H₂O₂. The authors demonstrated that dose-dependent vasodilatation of the resistance vessels was blocked in the PKG α Cys42Ser KI mice when compared to the WT mice, resulting in hypertension and confirming the importance of PKG1 α oxidation in blood pressure mediation [Rudyk et al., 2012]. In addition, Rudyk and colleagues have shown that glyceryl trinitrate (GTN) induces vasorelaxation *via* the PKG1 α oxidation pathway. In this study, they reported that GTN caused PKG1 α disulphide dimerisation in isolated resistance (mesenteric) and conduit (aortic) vessels. Furthermore, PKG1 α Cys42Ser KI mice displayed a blunted response to GTN and a markedly impaired reduction in blood pressure, therefore supporting a role for the PKG1 α oxidation pathway *in vivo*. KI littermates also exhibited a lack of GTN tolerance [Rudyk et al., 2012] [Pryszyzhna and Eaton, 2015].

Furthermore, oxidative activation of PKG1 α decreases calcium levels and therefore contraction within vascular smooth muscle cells; a mechanism that likely contributes to the observed vasodilatation [Muller et al., 2012]. In addition, detrimental effects of over-activation *via* PKG1 α oxidation have also been reported in mice subjected to septicaemia. Rudyk and colleagues demonstrated that PKG1 α Cys42Ser WT and KI mice induced with bacterial endotoxin LPS (E.Coli Lipopolysaccharde) caused a three-fold increase in hypotension in the WT mice when compared the KI littermates, accompanied by an increase

in the disulphide dimer. PKG1 α Cys42Ser KI mice were largely resistant to hypertension, lowered cardiac output and organ injury usually associated with sepsis [Rudyk et al., 2012].

1.8.3. Potential role for nitrite-mediated cardioprotection via PKG1 alpha oxidation

Pellegrino et al. (2009) have previously demonstrated that nitrite can elicit negative inotropic and lusitropic effects in normoxic isolated rodent hearts via eNOS-independent NO production and cGMP-PKG pathway activation. The protocol did not however subject the hearts to a period of ischaemia or subsequent reperfusion.

Echocardiograms from PKG1 α Cys42Ser KI mice showed a reduced diastolic volume, which is a potential indicator of diastolic dysfunction and impaired relaxation of cardiac myofibrils [Prysyazhna et al., 2012b]. Additionally, isolated perfused PKG1 α Cys42Ser KI mouse hearts subjected to a prolonged period of ischaemia, demonstrated increased ischaemia-induced end-diastolic pressures when compared to their WT littermates. [Prysyazhna et al., 2012b] Pulse wave Doppler analysis of mitral inflow velocity in PKG1 α Cys42Ser KI mice was suggestive of a stiffer myocardium, again with impaired relaxation compared to WT mice as determined by a decreased ratio of early to late ventricular filling velocities [Prysyazhna et al., 2012]. This study suggested that PKG1 α disulphide dimerization and thus, redox signalling, plays a vital role in the maintenance of diastolic relaxation, particularly during periods of myocardial ischaemia. It is therefore a pathway that could be utilised therapeutically.

There is however opposing evidence to suggest that PKG1 α in fact prevents beneficial responses to cardiac stressors (e.g. during ischaemic event) and its oxidation is a hallmark of heart disease. Recently, Nakamura et al. (2015) used a TAC (transaortic constriction) model to induce pressure overload in PKG1 α WT mice and PKG1 α Cys42Ser KI mice. Intact heart

function was subsequently assessed using echocardiography and tissues were collected post-mortem for histological analysis of molecular signalling. TAC-induced chamber dilatation, contractile depression, ventricular hypertrophy and fibrosis were blunted in redox-dead PKG1 α Cys42Ser KI mice. Furthermore, Nakamura and colleagues demonstrated that PKG1 α disulphide dimer was increased in patients with heart failure when compared to non-failing hearts, thus suggesting that PKG1 α is oxidised in both rodent models and patients with heart disease [Nakamura et al., 2015]. Although this study demonstrates that responses to PKG1 α oxidation may be variable in terms of therapeutic potential, to our knowledge there is no study to date that has examined the role of PKG1 α oxidation in nitrite-mediated cardioprotection. As such, in the present study we aim to investigate whether nitrite is able to circumvent the well-established NO-sGC-cGMP-PKG pathway and elicit cardioprotection through PKG1 against an *in vitro* model of myocardial IRI *via* PKG1 α oxidation.

1.9. ALDH2 enzyme: a potential mediator of cardioprotection?

The generation of ROS during myocardial IRI places the heart in a state of oxidative stress. This has well-established detrimental effects at the level of the mitochondria, such as rupture of the sarcoplasmic reticulum and induction of calcium overload as previously described in section 1.2.3. However, ROS have further deleterious effects through the accumulation of ROS-derived reactive aldehydes [Budas et al., 2009]. Aldehyde dehydrogenases (ALDHs) are vital enzymes, which catalyse the oxidation of reactive aldehydes to non-reactive, non-cytotoxic aldehydes and therefore offer a further target for mediating cardioprotection in incidences of oxidative stress such as ischaemia and reperfusion [Chen et al., 2010].

1.9.1. Aldehydes and aldehyde toxicity in Myocardial IRI

Aldehydes are generated endogenously through numerous physiological processes such as the catabolic breakdown of amino acids and transmitter substances [Chen et al., 2010] or lipid metabolism/peroxidation and carbohydrate metabolism [Esterbauer et al., 1991] [Marchitti et al., 2008]. Aldehydes can enter the cells and form adducts with DNA, lipids and proteins and therefore cause damage and inactivation (e.g. inhibition of metabolic enzyme glyceraldehyde 3-phosphate and the proteasome in addition to impairment of energy generation at the level of the mitochondria) [Esterbauer et al., 1991] [Petersen et al., 2004].

During myocardial IRI, reactive aldehydes generate 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) [Lee et al., 2001]. Conditions of intense oxidative stress such as ischaemia and reperfusion are accompanied by an increase in the presence of ROS. ROS are able to induce the lipid peroxidation of polyunsaturated fatty acids (such as arachidonic acid and linoleic acid) present at the plasma membrane to form 4-HNE, which readily reacts with amino acid residues of various proteins. If accumulated in high concentrations, 4-HNE is able to directly inhibit ALDH2 activity and subsequently induce further exacerbating toxic aldehyde levels in the mitochondria [Doorn et al., 2006]. In the myocardium, 4-HNE inhibits a plethora of metabolic enzymes, and as a cytotoxin it has been shown to have a direct inhibitory effect on contractility of myofibrils at the level of the sarcolemma, which is able to induce arrhythmias in isolated myocytes [Aberle et al., 2004] [Bhatnagar, 2006].

The aldehyde dehydrogenase (ALDH) family of genes are a group of enzymes able to remove aldehydes from the body. Aldehyde dehydrogenase 2 (ALDH2) is emerging as a crucial enzyme in the protection of the heart against ischaemic damage [Chen et al., 2008] [Churchill et al., 2009]. ALDH2 is one of 19 members of the wider gene family to play a role in the

detoxification of reactive aldehydes in a variety of organs [Vasiliou and Nebert, 2005] and is particularly abundant in those with a high capacity for oxidative ATP generation such as the heart and the brain [Stewart et al., 1996]. ALDH2 is acknowledged primarily for its role in ethanol (or alcohol) metabolism by detoxifying acetaldehyde [Budás et al., 2009]. In addition, approximately 40% of the East Asian population, estimated to be 540 million individuals worldwide, carry a mutant ALDH2*2 allele, therefore resulting in a severe reduction in the ALDH2 enzymatic activity when compared to the wild-type ALDH2*1 allele [Yoshida et al., 1984]. Decrease in the ALDH2 activity is associated with insensitivity to GTN treatment and has potential implications with regard to relative risk for cardiovascular diseases [Chen et al., 2010]. Therefore, increasing our understanding of ALDH2 activity and its involvement in processes such as cardioprotection is pertinent.

1.9.2. Mitochondrial ALDH2 and cardioprotection

Chen and colleagues have previously demonstrated that activation of PKC ϵ in the ischaemic heart with ethanol or agonist $\psi\epsilon$ RACK (receptors for activated C-kinase) increased ALDH2 activity and decreased infarct size when compared to controls [Chen et al., 2008]. Furthermore, Budás and colleagues corroborated the importance of PKC ϵ activating ALDH2 in a Langendorff model of IRI and showed that acute administration of ethanol in mutant mice lacking PKC ϵ conferred cardioprotection. ALDH2 activity was correlated to a statistically significant reduction in infarct size and creatine kinase [Budás et al., 2010]. In addition, ALDH2 inhibition through high levels of GTN (tolerance phenomenon, discussed in section 2.7.3.) and cyanamide (ALDH2 inhibitor) abolished the attenuation of infarction [Nossaman et al., 2012]. Previous studies have also suggested that not only is ALDH2 activity of major importance but also ALDH2 expression [Yu et al., 2014]. For instance, in an experimental model of myocardial IRI in anaesthetised rats, expression levels of ALDH2 were reduced when

compared to a sham group. Furthermore, in the same study, rats subjected to three 5 min cycles of lower limb ischaemia post-reperfusion as a form of remote ischaemic post-conditioning (rIPost) demonstrated elevated levels of ALDH2 expression in the left anterior myocardium. ALDH2 expression was also attenuated when these rats were exposed to rIPost alongside PI3K inhibitor wortmannin. Overall, these results highlight the importance of ALDH2 in cardioprotection and that rIPost *via* the PI3K/Akt pathway plays an essential role in the mechanism [Yu et al., 2014].

To demonstrate the direct cytoprotective role of ALDH2, experimental studies utilising pharmacological selective agonist N-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide (ALDH activator-1 or Alda-1) have also been investigated [Eaton et al., 1999] [Chen et al., 2001] [Chen et al., 2008] [Gomes et al., 2014]. Treatment with Alda-1 in an *in vivo* rodent model of myocardial infarction increased ALDH2 activity by approximately two-fold and subsequently lowered infarct size by 60% [Chen et al., 2010]. In addition, Alda-1 treatment has shown positive results in heart failure models [Gomes et al., 2014]. Gomes and colleagues reported that following 6 week treatment with Alda-1 in a myocardial infarction-induced heart failure rat model improved cardiac function, left-ventricular compliance, diastolic function and mitochondrial function when compared to the control group. Furthermore, the benefits of Alda-1 treatment in both cases is linked to a reduction in cytotoxic aldehyde 4-HNE [Chen et al., 2010] [Gomes et al., 2014].

1.9.3. ALDH2 and nitrite: potential to influence its cardioprotective effects

The role of ALDH2 as a glyceryl trinitrate (GTN) reductase enzyme and its subsequent involvement in GTN-mediated vasodilatation has been demonstrated in numerous studies [Munzel et al., 2005] [Chen et al., 2002] [Li et al., 2006]. Predominantly, mitochondrial ALDH2

catalyses the step-wise reduction of GTN to 1,2-glyceryl dinitrate (1,2-GDN) and NO, which is then able to elicit downstream effects *via* the classical NO pathway [Ferriera and Mochly-Rosen, 2012]. ALDH2 therefore plays an important role in the bioactivation of GTN in the treatment of patients with cardiovascular issues such as AMI, angina and heart failure [Chen and Stamler, 2006]. Furthermore, ALDH2 inhibition prevents GTN conversion and attenuates its potent vasodilatory effects [Chen et al., 2005]. Although its therapeutic potential in the clinical arena is well-established, continuous GTN treatment can induce tolerance, and therefore lead to free radical production and increased morbidity [Gori and Parker, 2008]. Chen and colleagues reported the involvement of the ALDH2 pathway as a novel mediator of GTN tolerance [Chen et al., 2005]. The authors showed that there was a significant decrease in GTN bioactivity in the aortas from ALDH2 KO mice. Furthermore, in rabbit aortas given high doses of GTN to elicit tolerance, ALDH2 activity was reduced by ~50% with similar results produced in rat hearts *ex vivo* [Chen et al., 2002]. The study also demonstrated that GTN tolerance and subsequent ALDH2 inactivation was associated with an increase in infarct size [Chen et al., 2008]. ALDH2 involvement in GTN bioactivation has also been demonstrated in human subjects. Treatment of healthy volunteers with disulfiram, an ALDH2 inhibitor, blocked GTN responses observed in volunteers not given the drug. Furthermore, individuals carrying the ALDH2*2 gene mutation demonstrated attenuated responses [Mackenzie et al., 2005].

The production of nitrite as a by-product in the conversion of GTN to 1,2-GDN and the involvement of ALDH2 in this process highlights a potential influence of ALDH2 in nitrite-mediated cardioprotection. Martin Feelisch's group used mass spectrometry-based proteomic studies to demonstrate changes that occur in response to nitrite in rat hearts [Perlman et al., 2009]. Perlman and colleagues showed protein modifications, which included changes to the

ALDH2 enzyme, in response to acute nitrite exposure and for the first time linked such changes to nitrite-mediated cardioprotection [Perlman et al., 2009]. Nossaman et al. (2012) compared the cardiovascular responses to GTN and sodium nitrite administration through the measurement of systemic arterial pressures in the intact chest rat and utilised inhibitors cyanamide (ALDH2 inhibitor), allopurinol (XOR inhibitor) and ODQ (sGC inhibitor) to elucidate the underlying mechanisms. The results showed a significant vasodilatory effect of both GTN and sodium nitrite in the systemic vascular beds. ODQ attenuated vasodilatory responses, thus suggesting a principle involvement of sGC (e.g. *via* the NO-sGC-cGMP-PKG pathway). Furthermore, responses to GTN were inhibited by cyanamide, thus suggesting a role for ALDH2 in GTN-mediated vasorelaxation [Nossaman et al., 2012]. In addition, responses to NaNO₂ were attenuated by both cyanamide and allopurinol therefore proposing that both ALDH2 and XOR enzymes are involved in nitrite bioactivation.

Based on these findings, we propose that ALDH2 may potentially play an essential role in nitrite-mediated cardioprotection. As such, to assess whether ALDH2 is involved, we aim to investigate the effects of nitrite in a mouse model of myocardial IRI by utilising ALDH2 WT and KO mice.

1.10. Summary

Following myocardial infarction, IRI occurs as a result of blood flow restoration to an ischaemic area. Necrotic cell death of cardiomyocytes is largely attributable to dysfunction at the level of the mitochondria due to the opening of the mPTP, release of ROS, calcium overload, and cardiac myofibril hypercontracture. Protection against IRI can be elicited mechanically or pharmacologically through various processes beneath the umbrella term 'conditioning'. These methods largely rely upon the recruitment of an extensive signalling pathway of survival protein kinases (termed as the RISK pathway), which often terminates the mPTP opening and subsequently prevents MMP disruption.

Nitrite, a naturally occurring anion, is reduced under conditions of hypoxia and/or low pH (acidic conditions) by a plethora of nitrite reductases to endogenous NO. Several animal studies have established the ability of nitrite to elicit cardioprotection when administered prior to an ischaemic event or present transiently, thus categorising nitrite as a preconditioning agent. It has been widely accepted that nitrite-mediated cardioprotection utilises signal transduction through a specific part of the RISK pathway, such as the activation of the NO-sGC-cGMP-PKG cascade (classical activation of the NO pathway).

In parallel studies, Burgoyne et al. (2007) and Pryszyzna et al. (2012) have offered evidence to support the involvement of an alternative PKG activation mechanism, known as the PKG1 α oxidation pathway. Previous studies have shown that PKG1 α oxidation elicits vasodilatation (independent of the classical NO pathway) and may also contribute to the maintenance of diastolic relaxation in the heart following oxidative stress. PKG1 α oxidation involves disulphide bond formation between Cysteine 42 residues on adjacent monomeric chains to shift PKG from its monomeric form at 75kDa to a PKG dimer at 150kDa. This redox

mechanism elicits downstream effects and mimics those of the standard NO-sGC-cGMP-PKG pathway, by directly activating PKG1 α oxidation and circumventing sGC-cGMP. In parallel to these findings, nitrite has recently been demonstrated to trigger vasodilatation under normoxic conditions in human studies by Omar and colleagues, therefore suggesting that nitrite may potentially have an alternative mechanism to mediate vasodilation [Omar et al., 2015]. As such, in the present study we aim to determine whether nitrite is able to circumvent the classical activation of the NO-sGC-cGMP-PKG pathway and to elicit cardioprotection against myocardial IRI *via* the PKG1 α oxidation pathway.

The generation of ROS during myocardial IRI has deleterious effects through the accumulation of ROS-derived reactive aldehydes, which are cytotoxic at the level of the mitochondria. Aldehyde dehydrogenases (ALDHs) are vital enzymes, which catalyse their oxidation to non-cytotoxic aldehydes and therefore offer a further target for mediating cardioprotection. ALDH2 is one of many ALDH genes with a vital role in the detoxification of reactive aldehydes such as 4-HNE, which is able to impair ATP production from the mitochondrial ETC, directly inhibiting cardiac contractility and inducing opening of the mPTP.

Increased ALDH2 activation decreases infarct size following MI and therefore pharmacological agents, such as Alda-1 (ALDH2 activator), may offer therapeutic potential. ALDH2 activation has also been linked to improvements in ventricular function in heart failure and ischaemic post-conditioning. Furthermore, Nossaman et al. (2012) have demonstrated that the potent vasodilator activity of GTN treatment in rodents was inhibited by ALDH2 inhibitor cyanamide, thus suggesting that ALDH2 has a vital role. Additionally, we and others have shown that the vasodilatation exhibited by nitrite administration was attenuated by cyanamide, thus suggesting ALDH2 involvement in the bioactivation of nitrite

to NO. We therefore speculate that ALDH2 is involved as an alternative reductase enzyme in the reduction of nitrite to NO for subsequent signal transduction and cardioprotection via the NO-cGMP-PKG pathway. As such, the second objective of the present study is to elucidate ALDH2 involvement in nitrite-mediated cardioprotection.

A representative diagram providing an overview of the mechanisms that will be investigated in the present study is depicted in figure. 1.5.

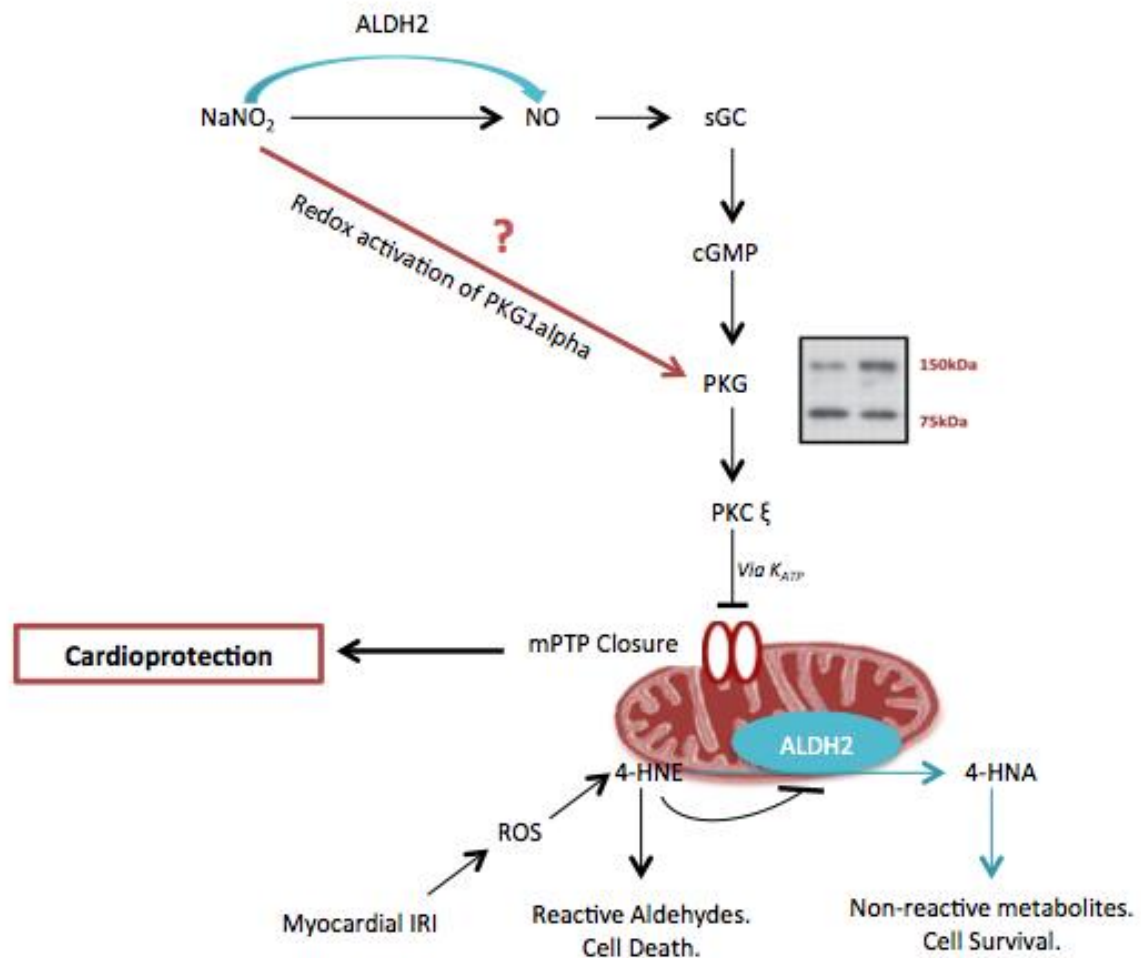


Figure.1.5.: The mechanisms that will be investigated in the present study: the involvement of PKG1alpha oxidation and ALDH2 enzyme in eliciting the cardioprotective effects of nitrite.

The figure provides a schematic illustration, using black arrows, of the established NO-cGMP-PKG pathway and the way in which, NaNO₂ influences the pathway through reduction to NO; usually by nitrite reductases such as XOR and haem globins.

The red arrow represents an alternative mechanism of PKG activation through the oxidation of PKG1 α , which is able to then activate downstream effects of the NO-cGMP-PKG pathway to elicit cardioprotection. This pathway will be explored in the present study.

Blue arrows show an established involvement of ALDH2 enzyme in cytoprotection against reactive aldehydes at the level of the mitochondria and a potential role for ALDH2 in the reduction of nitrite to NO for subsequent signal transduction and cardioprotection via the NO-cGMP-PKG pathway. The present study aims to elucidate ALDH2 involvement in nitrite-mediated cardioprotection.

Figure. 1.5. Adapted with aspects from Budas et al., 2010.

1.11. Hypotheses and Aims

Hypotheses

- Nitrite-mediated cardioprotection, is elicited *via* the PKG1 α oxidation pathway in a murine *in vitro* model of myocardial IRI.
- Nitrite-mediated cardioprotection is positively influenced by the activity of aldehyde dehydrogenase 2 (ALDH2) enzyme in a murine *in vitro* model of IRI.

Aims

- To assess whether sodium nitrite (NaNO₂; 10 μ M and 100 μ M) elicits cardioprotection in a Langendorff mouse (C57/Bl6) model of myocardial IRI and to determine the concentration to be used in subsequent experiments.
- To investigate whether nitrite elicits a cardioprotective effects *via* PKG1 α oxidation pathway in an *in vitro* mouse model of IRI by using PKG1alpha Wild Type (WT) and Knock In (KI) mice.
- To investigate the influence of aldehyde dehydrogenase 2 (ALDH2) enzyme on the cardioprotective effects of nitrite against an *in vitro* model of myocardial IRI by using ALDH2 WT and Knock Out (KO) mice.

2. METHODS

Animals were maintained humanely in compliance with the “Principles of Laboratory Animal Care”, formulated by the National Society for Medical Research. The animal studies were approved by the UK Government Home Office and conducted according to the Animals Scientific Procedures Act 1986 and European Commission guidelines.

All reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated in text.

2.1. Isolated mouse heart Langendorff model

To demonstrate the well-established protective effects of nitrite and to further dissect specific molecular mechanisms by which nitrite mediates cardioprotection, two Langendorff protocols were used:

Protocol A was used to assess the ability of two different sodium nitrite (NaNO_2) concentrations (10 and $100\mu\text{M}$) to elicit cardioprotection in C57/BL6 mice (see figure.2.2.).

Protocol B was used to determine the involvement of specific pathways by which nitrite is able to elicit cardioprotection: (1) PKG1 alpha oxidation and/or (2) the involvement of ALDH2 enzymes (see figure. 2.3.).

Both protocols were carried out as stated by Hayes (2015) under the same inclusion criteria as previously described by Sutherland and colleagues (2003).

2.1.1. Removal of the heart and attachment to the Langendorff system

Mice were anaesthetised by intra-peritoneal (i.p.) 50:50 co-administration of euthatal sodium pentobarbital (40µl/g; Merial, Essex, UK) and anticoagulant heparin sodium (4IU/g; Fannin, Northamptonshire, UK). The pedal reflex was carefully monitored until response ceased, at which point a thoracotomy was performed to rapidly isolate and mount the murine heart onto the Langendorff apparatus (figure.2.1). The delay between excision and cannulation of the heart on the Langendorff apparatus lies optimally below 5 minutes. Following cannulation, isolated mouse hearts were retrogradely perfused at a constant perfusion pressure of approximately 80mmHg with Krebs-Henseleit buffer (KHB; see Table 2.1 for reagent details; all salts were purchased from VWR International (Leicestershire, UK), except for NaHCO₃ and Glucose (Fisher Scientific, Loughborough, UK) and NaCl (Sigma-Aldrich, Dorset, UK)) and equilibrated with 95% O₂-5% CO₂ (pH 7.4) at 37°C.

Reagent	Concentration (mM)
NaCl	118.5
NaHCO ₃	25.0
KCl	4.7
KH ₂ PO ₄	1.2
MgSO ₄	1.2
Glucose	11.0
CaCl ₂	1.4

Table. 2.1. Concentrations (mM) of reagents used to make Krebs-Henseleit Buffer

Reagents were added in succession to distilled water and solution was gassed with 95% O₂- 5% CO₂ for 15 minutes prior to the addition of CaCl₂.

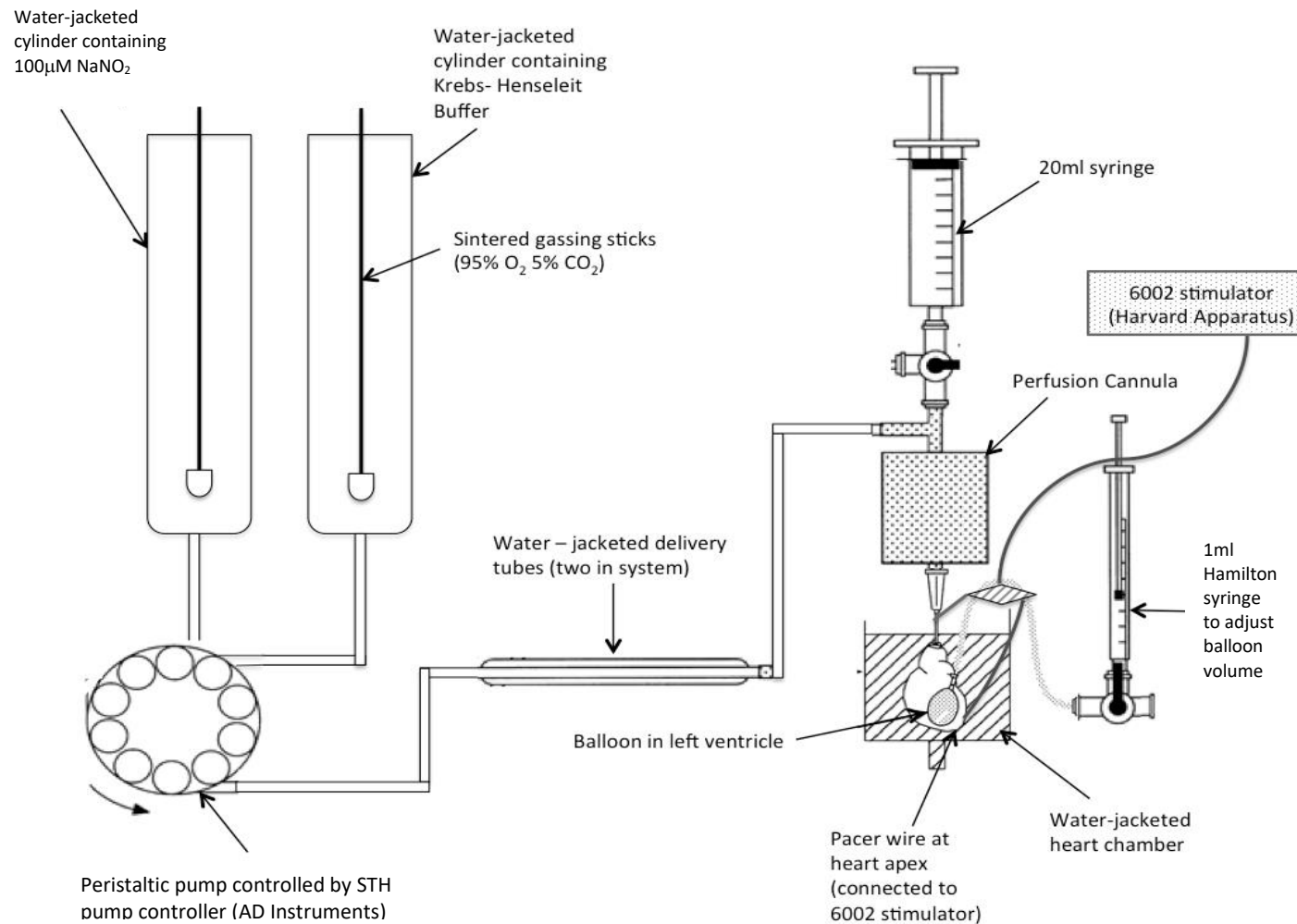


Figure.2.1. Schematic representation of the Langendorff apparatus. Adapted from Sutherland et al., 2003
 Diagram shows a simplified illustration of the murine heart perfusion preparation (not to scale).

2.1.2. Inclusion criteria of the Langendorff apparatus

Cardiac contractile function, including measurements of left ventricular pressure (LVP), left ventricular developed pressure (LVDP) and left ventricular end-diastolic pressure (LVEDP), were monitored through the insertion of a fluid-filled balloon into the left ventricle. The balloon was gradually inflated using the Hamilton syringe until the LVEDP was between 3 and 10mmHg and LVDP >50mmHg as previously described by Sutherland et al. (2003). Also in accordance with these inclusion criteria, and to reflect physiological conditions of murine hearts, the hearts were paced at approximately 600 beats/min (6002 Stimulator, Harvard Apparatus) and coronary flow rate was maintained at 2-5ml/min, which was measured by a feedback circuit controlling the perfusion pump (STH Pump Controller, AD Instruments). The temperature of the Langendorff system was monitored regularly throughout the protocol (manual thermocouple thermometer, Hanna) to ensure that it remained at $37^{\circ}\text{C} \pm 3$. See table 2.2 for the summary of inclusion criteria.

Measured Parameter	Inclusion Criteria
Perfusion Pressure (PP)	80mmHg
Coronary Flow Rate (CFR)	2-5ml/min
Temperature	$37^{\circ}\text{C} \pm 3$
Left Ventricular Developed Pressure (LVDP)	>50mmHg
Left Ventricular End-Diastolic Pressure (LVEDP)	3-10mmHg
Heart Rate	500-600bpm

Table. 2.2. Inclusion criteria applied to the measured parameters of the Langendorff apparatus as stipulated by Sutherland et al., 2003

The inclusion criteria are applied to each individual parameter during the stabilisation period of the Langendorff protocol.

2.2. Assessment of the cardioprotective effects of different concentrations of sodium nitrite against ischaemia-reperfusion injury in C57/BL6 mice

Nitrite has been shown to elicit powerful cardioprotection in the isolated heart [Webb et al., 2004; Duranski et al., 2005]. To demonstrate that such results can be replicated in our hands, hearts isolated from C57/BL6 mice were treated with either 10 or 100 μ M sodium nitrite and subjected to a well-established myocardial ischaemia reperfusion injury Langendorff model [Madhani et al., 2010]. See protocol A (section 2.2.1.) for further details.

2.2.1. Langendorff Protocol A

A schematic illustration of Langendorff protocol A is shown in Figure.2.2. Isolated C57/BL6 mouse hearts (mice were purchased from ██████████, 25-30g) underwent a 40 min period of stabilisation, followed by 30 min global ischaemia and 120 min reperfusion [Madhani et al., 2010] [Hayes, 2015]. Electrical pacing of the heart was stopped 2 min after contraction ceased during ischaemia and was re-started 5 min into reperfusion. As shown in figure. 3.2, hearts received one of three following treatments: (1) Placebo (KHB) for the duration of stabilisation (40 min), (2) 10 μ M or (3) 100 μ M of sodium nitrite (NaNO_2) dissolved in KHB for 30 min during stabilisation, following 10 min perfusion with KHB. 10 μ M Nitrite (a concentration shown to trigger significant protective changes in pressure and contractility data in a study by Pellegrino et al. (2009) using a similar Langendorff model) and 100 μ M Nitrite (a concentration deemed to elicit a protective effect in myocardial IRI in a study by Webb et al. (2004)) were the rationale for the concentrations to be used in this project. All hearts were then subjected to 30 min global ischaemia followed by 120 min reperfusion with KHB. At the end of 120 min reperfusion, the hearts were immediately removed from the Langendorff system and stained for infarct size (see section 2.2.2. for further details).

Haemodynamic data were recorded and extracted from software LabChart 7 and analysed for the following parameters: Left Ventricular Pressure (LVP; the slope of which was analysed to calculate LVdP/dt Max and LVdP/dt Min), Left ventricular Developed Pressure (LVDP), Left Ventricular End-Diastolic Pressure (LVEDP) and Coronary Flow Rate (CFR).

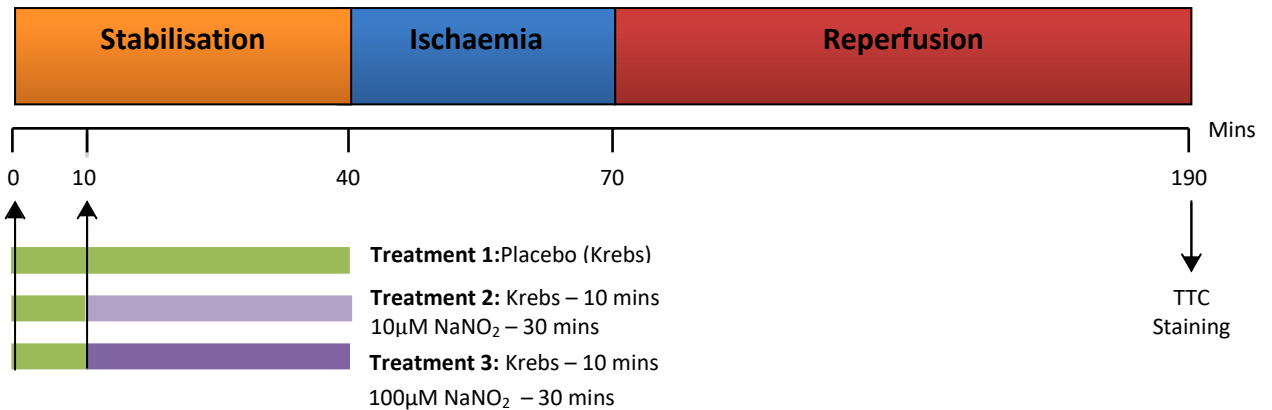


Figure. 2.2. Schematic representation of Langendorff Protocol A.

Hearts underwent a 40 min stabilisation period followed by 30 min ischaemia and 120 min reperfusion. During stabilisation, hearts were perfused with (1) placebo of Krebs-Henseleit buffer, (2) 10µM NaNO₂ or (3) 100µM NaNO₂.

2.3. Investigating the role of PKG1α oxidation and Aldehyde dehydrogenase 2 (ALDH2) in nitrite-mediated cardioprotection.

To elucidate the mechanism(s) by which nitrite mediates cardioprotection in myocardial IRI, we investigated two potential mechanisms:

- (1) PKG 1alpha oxidation
- (2) Aldehyde dehydrogenase 2 (ALDH2) presence/activity.

(1) PKG1 α oxidation pathway

To assess whether nitrite utilises redox signalling and mediates cardioprotection *via* the PKG1 α oxidation pathway in this process, PKG1 α cys42 wild type (WT) and transgenic PKG1 α cys42 knock-in (KI) male mice were used (25-30g; a kind gift from [REDACTED], [REDACTED]). The “redox-dead” PKG1 α cys42 KI mice are unable to sense or transduce oxidant signals via this pathway due to a single atom substitution of serine in place of cysteine 42, which in turn abolishes the ability to form an active PKG disulphide dimer. The mice expressing PKG1 α cys42ser constitutively were produced on a C57/BL6 background by TaconicArtemis [Prysyazhna et al., 2012].

(2) ALDH2 enzymatic pathway

To determine whether the ALDH2 enzymatic pathway contributes to nitrite-mediated cardioprotective effects during myocardial IRI, ALDH2 wild-type (WT) and ALDH2 Knock out (KO) mice, sourced from [REDACTED] [REDACTED], were used. The ALDH2 KO mouse has an inactive ALDH2 enzyme due to the insertion of a stop codon into a vital promotor gene and therefore has severe deficiency in ALDH2 activity. Embryonic stem (ES) cells carrying the mutation were injected into C57/BL6 blastocysts and resulting male chimeras were bred with female C57/BL6 mice. The mutant mice were back-crossed to C57/BL6 more than seven times [Kitagawa et al., 2000] [Riken BRC, 2016].

Mice from both the PKG1 α cys42 and the ALDH2 colonies were subjected to Langendorff protocol B as detailed below in section 2.3.1.

2.3.1. Langendorff Protocol B

Isolated hearts from PKG1 α cys42 WT and KI mice or ALDH2 WT and KO mice (25-30g) underwent a 40 min period of stabilisation, followed by 30 min global ischaemia and 120 min reperfusion [Madhani et al., 2010] [Hayes, 2015]. As previously described in Langendorff Protocol A (section 2.2.1), electrical pacing of the heart was stopped 2 min after the onset of ischaemia and was resumed 5 min into the reperfusion phase. As shown in Figure.2.3, hearts received one of two following treatments: Placebo (KHB) for the duration of stabilisation (40 min) or 100 μ M NaNO₂ in KHB (deemed to elicit cardioprotective effects in C57/Bl6 mice following myocardial IRI in Langendorff Protocol A) for 30 min during stabilisation, following 10 min perfusion with KHB. Following 120 minutes reperfusion, all hearts were perfused immediately with 1% triphenyltetrazolium chloride (TTC) for the assessment of infarction size (see section 2.3.2 for further details). Haemodynamic data (LVP, LVDP, LVEDP and CFR) were also recorded from software LabChart 7.

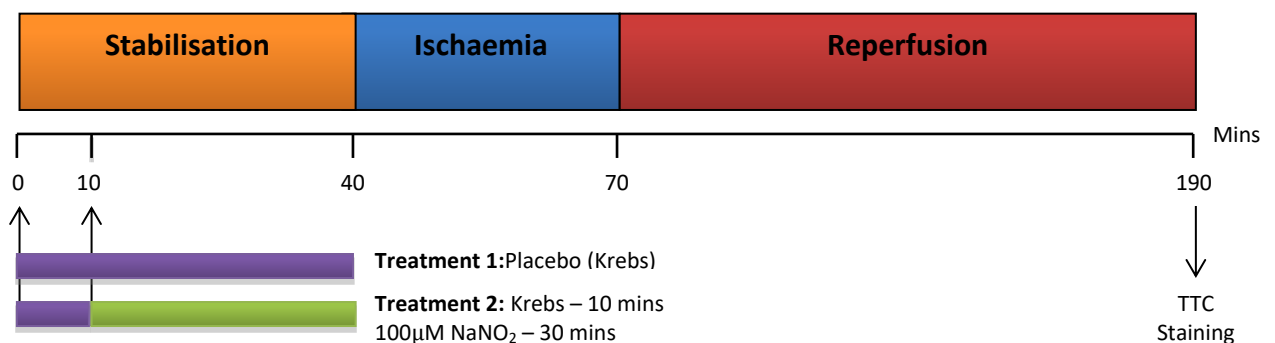


Figure. 2.3. Schematic representation of Langendorff Protocol B.

Hearts underwent a 40 min stabilisation period followed by 30 min ischaemia and 120 min reperfusion. During stabilisation, hearts were perfused with a placebo of Krebs-Henseleit buffer or 100 μ M Nitrite (NaNO₂).

2.3.2. Triphenyltetrazolium chloride staining of hearts following Langendorff protocols A and B

To determine the infarction size following myocardial IRI, we used a well-established technique to stain the isolated mouse hearts with 1% TTC (triphenyltetrazolium chloride in phosphate buffer saline) [Madhani et al 2010; Jacquet et al., 2009]. The colourless TTC solution is reduced by dehydrogenase enzymes, present in mitochondria, to a red formazan [Benedek et al., 2006]. Stain intensity is correlated to the presence of viable cardiomyocytes, which have complete dehydrogenase activity. Infarcted myocardium, lacking dehydrogenase activity, remains unstained and appears white, thus aiding differentiation for analysis. [Khalil et al., 2006]

Preparation of the isolated mouse hearts for infarction size analysis was carried out as previously described [Madhani et al., 2010; Jacquet et al., 2009]. Briefly, at the end of 2 hrs reperfusion in Langendorff Protocol A and B, the hearts were immediately removed from the Langendorff apparatus, with cannulation intact, and retrogradely perfused manually for 5 min at 1ml/min with 1% TTC/PBS (37°C). The hearts were immediately removed from the cannula and placed into a vial of 1% TTC/ PBS incubated in a water bath for 10 min at 37°C. The hearts were then blotted dry, atria were removed and tissue was weighed and stored at -20°C for up to 1 week maximum for heart sectioning.

2.3.3. Preparation and slicing of hearts

Within the week following each Langendorff experiment, the hearts were removed from -20°C freezer and thawed in 2.5% glutaraldehyde (Merck Schuchardt OHG, Hohenbrunn, Germany) for 2 min and then immediately set in 5% agarose (Bioline) blocks. The hearts were then sectioned from apex to base in 0.7mm slices using a Vibratome (Agar Scientific). The hearts slices were immediately placed in a tissue culture plate containing 10% formaldehyde and

stored for approximately 24 hrs at room temperature. The following day, 10% formaldehyde solution was removed from each heart slice and immediately replaced with PBS solution for a further 24 hrs at 4°C. The next day, the heart slices were placed onto a 0.75mm glass plate and scanned at high resolution (Canon CanoScan 5600F). Magnification of individual heart slices was carried out using software Adobe Photoshop 7.1.

2.3.4. Infarction analysis

Figure 2.4 depicts a mouse heart slice stained with 1% TTC. As described above, the viable tissue was stained red (figure 2.4 A), whilst the infarcted area was stained white (figure 2.4 B). To determine the % of infarct size, RBS Image J 1.48 software was used to measure (1) the total area of each heart slice, (2) area of infarcted tissue (TTC-negative area) and (3) area of the viable tissue (TTC-positive area). The infarction was then calculated by using the following mathematical equation:

$$\left(\frac{\text{infarcted tissue area}}{\text{total area}} \right) \times 100\%$$

Infarct size was then calculated as a percentage of heart volume by averaging the infarct size of each individual slice. All heart sections were coded and analysed by two separate investigators, both blinded to genotype and the treatment received by each heart to eliminate bias.

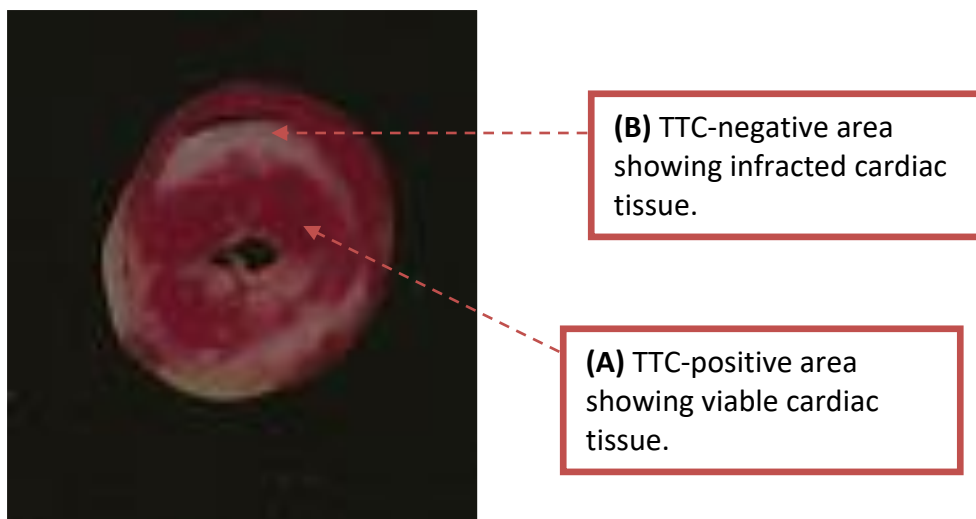


Figure. 2.4. Example of a mouse heart slice stained with TTC.

(A) TTC-positive area showing viable cardiac tissue stained red and (B) TTC-negative area showing the infarcted area stained white.

Viable cardiomyocytes possess a greater TTC stain intensity and appear red when viewed (area A). Infarcted myocardial tissue however appears white (area B). This differentiation aids the analysis process and infarct size is calculated by divided infarcted cardiac tissue area (area B) by total area (areas A and B combined).

2.4. Genotyping of protein kinase G 1 α oxidation and aldehyde dehydrogenase 2 mice

Mice used for Langendorff protocol B (see section 2.3.1.) were genotyped for either (1) PKG 1 α cys42 Wild-Type (WT) or Knock-in (KI), or (2) ALDH2 WT or knock-out (KO) genotypes.

The DNA extraction and PCR were carried out as follows:

2.4.1. DNA extraction

The process of DNA extraction was carried out identically for both the PKG1 α cys42 and the ALDH2 colonies, using the Bioline isolate II DNA kit manufacturer's guidelines (Bioline, London, UK). Briefly, ear clippings from the mice were digested in 180 μ l lysis buffer GL and 25 μ l protein kinase K overnight on a heating block shaker at 56 $^{\circ}$ C and 300rpm (ThermoMixer C, Eppendorff). The following day, DNA extraction and purification were carried out as

described in the manufacturer's guidelines (Bioline isolate II DNA kit). Briefly, samples were incubated with 200µl lysis buffer G3 at 70°C for 10 min and 210µl of ethanol (96-100%) was added to allow DNA binding. Samples were then transferred to an ISOLATE II genomic DNA spin column and underwent 3 times washing and centrifugation process (11,000 rcf at 20°C for 1 min), with flow-through discarded from a collection tube between each step. The DNA was then eluted following the addition of 100µl pre-heated elution buffer G (70°C) and a final 1 min centrifugation at 11,000 rcf (20°C). Once the DNA was eluted, the DNA sample was then used for PCR (see section 2.4.2.). Figure.2.5 provides a schematic diagram of the DNA extraction and purification process.

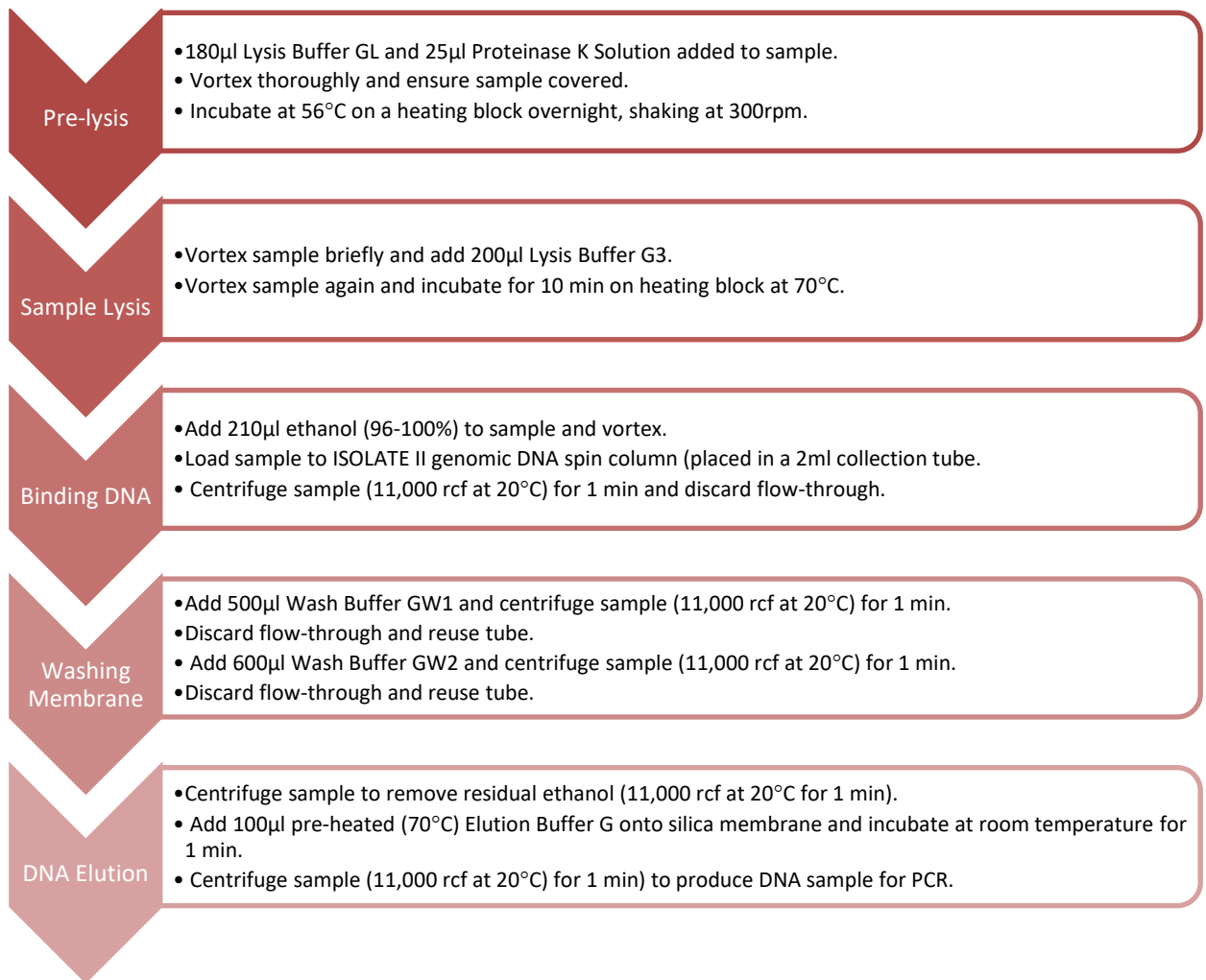


Figure. 2.5. The multistep process of DNA extraction, purification and elution carried out during the genotyping process.

Schematic flow chart to illustrate the DNA extraction, purification and elution processes as detailed in the Bioline isolate II DNA kit manufacturer's guidelines.

2.4.2. PCR

PKG 1alpha cys42ser PCR:

Each DNA sample (5µl) was added to 49µl of a PCR master mix containing PKG1 alpha oligo 1 forward primer (5' CAGTTTAGGGACAGAGTTGG 3'; Sigma), PKG1 alpha oligo 2 reverse primer (5'ACCTGCTTCATGCGCAAGG 3'; Sigma), molecular biology grade water and 2x Reddy Mix containing 1.5mM MgCl₂ (Thermo Scientific). The reaction mixture volumes are listed in table 2.3. One sample containing 49µl master mix only was used as a negative control.

The PCR samples subsequently underwent a 37-cycle PCR programme in a PCR thermocycler (Geneflow Labcycler) as described in figure 2.6.

Reagent	Volume added to Master Mix per sample
PKG1 alpha oligo 1 forward primer	2µl
PKG1 alpha oligo 2 reverse primer	2µl
Molecular biology grade water	18µl
2x Reddy Mix	27µl

Table. 2.3. Volumes of reagents in PCR Master Mix for PKG samples.

Reaction mixture volumes combined to make Master Mix for the PKG 1alpha cys42ser PCR protocol.

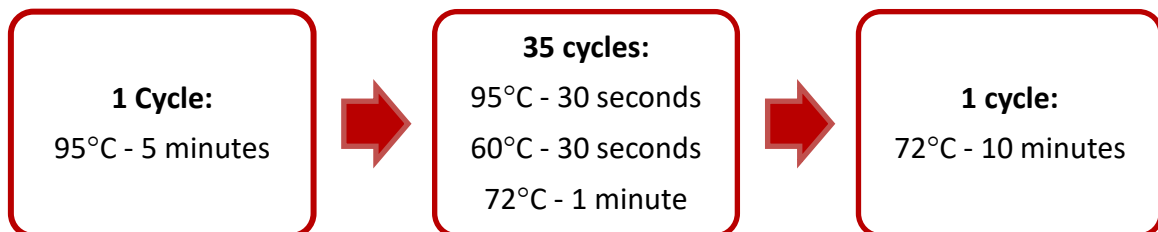


Figure. 2.6. PCR protocol used on PKG samples.

All digested and purified DNA samples were combined with a Master Mix and underwent a 37-cycle PCR programme; the conditions and time constraints of which are detailed in a flow chart.

ALDH2 PCR:

ALDH2 DNA samples (1µl) were added to 18.5µl of master mix containing oligonucleotide primers (ALDH2-F1 forward primer: 5' CCCTTACTGACTGTCCCATCTCAGTTGCT 3', ALDH2 int3-Rv: 5' GTTCACTCTGGTGACCACCATAGAG 3' and ALDH2-R3 reverse primer: 5' GGTGGATGTGGAATGTGTGGTGGAGGC 3'; all purchased from Sigma), molecular biology grade water and 2x Reddy Mix (containing 1.5mM MgCl₂) in the quantities stipulated in table 3.4. One sample containing 18.5µl of master mix only was also prepared as a negative control. The ALDH2 PCR samples were then subjected to a 32-cycle PCR programme, as depicted in figure. 2.7.

Reagent	Volume added to Master Mix per sample
ALDH2-F1 forward primer	1µl
ALDH2 int3-Rv primer	1µl
ALDH2-R3 reverse primer	0.5µl
Molecular biology grade water	6µl
2x Reddy Mix	10µl

Table. 2.4. Volumes of reagents in PCR Master Mix for ALDH2 samples.
Reaction mixture volumes combined to make Master Mix for the ALDH2 PCR protocol.

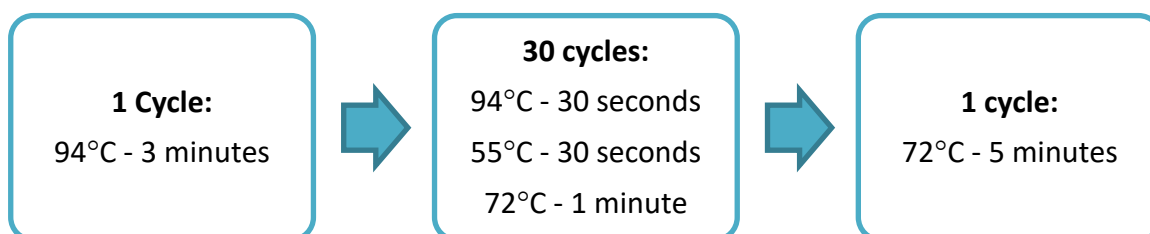


Figure. 2.7. PCR protocol used on ALDH2 samples.

All digested and purified DNA samples were combined with a Master Mix and underwent a 32-cycle PCR programme; the conditions and time constraints of which are detailed in a flow chart.

2.4.3. Gel electrophoresis

2% agarose gel was prepared (Bioline) with 1x Tris Acetate-EDTA buffer (TAE; diluted from 10x TAE stock solution; Sigma). The gel was placed into an electrophoresis tank (kuroGEL Mini Plus 10 Electrophoresis Horizontal; VWR) and covered with 1x TAE buffer. 100bp DNA ladder (Promega; 5 μ l) and the PCR samples (15 μ l) from both colonies were loaded into the wells and ran at 100 volts for 40 minutes. Separation of the DNA samples was checked using an Ultraviolet Transilluminator (UVP, California, United States) and photographed using Gene Genius.

2.4.4. Interpretation of DNA banding

Following gel electrophoresis, the genotype for either PKG1 α cys42 WT or KI mouse was determined by a single band visible at 293bp for a homozygous PKG1 α cys42 WT genotype whilst a single band at 456bp suggests a homozygous PKG1 α cys42 KI genotype. Two bands, one at each respective molecular weight, indicates a heterozygous (HET) mouse. A representative image of DNA genotype bands are shown in figure 2.8.

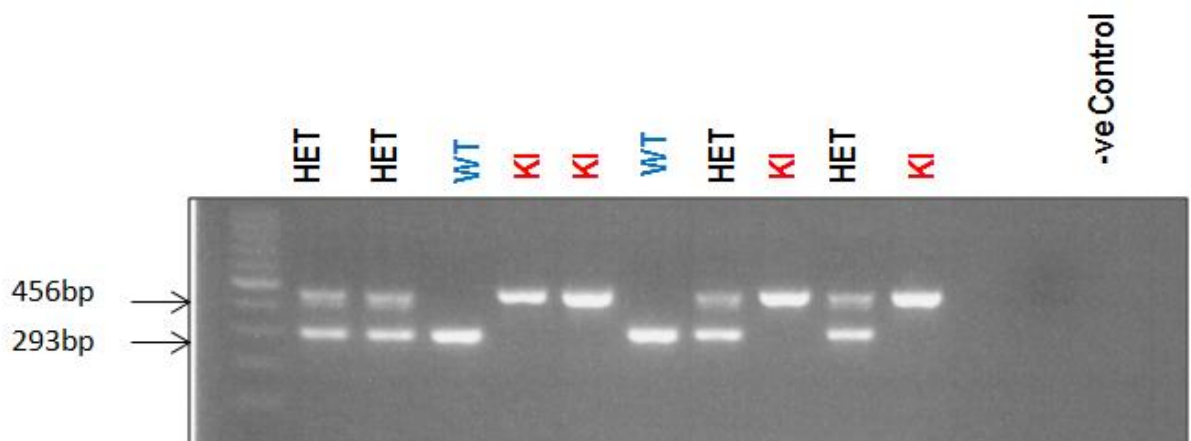


Figure. 2.8. Example of DNA banding observed during genotyping of PKG 1alpha litters. Genotyping of all litters expected to include mice carrying the PKG1 α cys42 wild-type (WT) and the PKG1 α cys42 knock-in (KI) genotype carried out prior to animal use in experimental procedures. In this example, 2 mice were found to carry the WT genotype (single band at 293bp), 4 mice were identified as having the KI genotype (single band 456bp) and a further 4 mice were observed to be heterozygous (HET; a band at each respective molecular weight). There is no contamination of the samples as indicated by the absence of a band in response to a negative control sample.

Expected fragments following separation of ALDH2 DNA samples included a single band at 597bp, for a homozygous ALDH2 WT genotype and a single band at 280bp for a homozygous ALDH2 KO genotype. Two bands at each molecular weight (597 and 280bp) signify an ALDH2 HET mouse. In the present study, mice carrying either HET genotype or female mice carrying any of the listed genotypes above were not used in experimental procedures. An example of these DNA fragments is presented in figure 2.9.

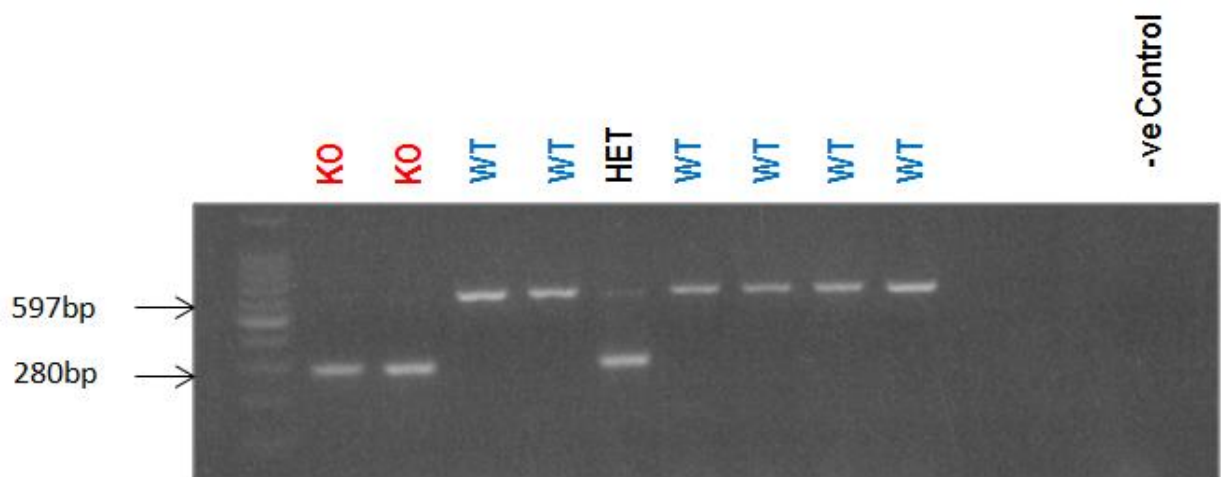


Figure. 2.9. Example of DNA banding observed during genotyping of ALDH2 litters.

Genotyping of all litters expected to include mice carrying the ALDH2 wild-type (WT) and the ALDH2 knock-out (KO) genotype were carried out prior to animal use in experimental procedures. In this example, 6 mice were found to carry the WT genotype (single band at 597bp), 2 mice were identified as having the KO genotype (single band 280bp) and a single mouse was observed to be heterozygous (HET; a band at each respective molecular weight). There is no contamination of the samples as indicated by the absence of a band in response to a negative control sample.

2.5. Data analysis and statistics

All data are presented as means \pm SEM. Comparisons between multiple groups were performed by one-way or two-way ANOVA followed by a Bonferroni post-hoc test. All statistical analysis was performed using GraphPad Prism software 5. The significance of the

data was analysed at a 95% confidence interval and $P < 0.05$ was considered statistically significant.

3. RESULTS:

3.1. Sodium nitrite is cardioprotective against myocardial IRI in C57/BL6 mice

To determine whether sodium nitrite can elicit protection against myocardial IRI, 10 or 100 μ M concentrations were administered prior to 30 minutes global ischaemia in C57/BL6 mice (see Langendorff protocol A; figure 2.2.).

3.1.1. Sodium nitrite improves haemodynamic parameters

As shown in Figure 3.1A sodium nitrite (10 and 100 μ M) increased LVdP/dt min (indicative of cardiac relaxation) during the stabilisation period when compared to the placebo group by 1.2-fold and 1.8-fold, respectively. However, this increase was not statistically significant ($p > 0.05$; $n = 5-7$). In contrast, 100 μ M of nitrite significantly increased LVdP/dt max values (an indicator of cardiac contractility) when compared to 10 μ M nitrite treatment by approximately 1.7-fold (Figure 3.1B; $p < 0.05$; $n = 5-7$). However, although 100 μ M nitrite treatment consistently caused increased LVdP/dt max when compared to the placebo group, this was not statistically significant (1.6-fold when compared to the control; $p > 0.05$; $n = 5-7$).

Haemodynamic parameters (LVDP, LVEDP and CFR) were monitored throughout the Langendorff IRI protocol (Langendorff protocol A; Figure 2.2.). Figure 3.1C shows that 30 min infusion of 10 μ M (12.2 ± 1.5 mmHg at 120 min) or 100 μ M (14.5 ± 3.6 mmHg at 120 min) nitrite prior to ischaemia significantly improved LVDP at reperfusion in C57/BL6 mice when compared to the placebo group (10.3 ± 1.2 mmHg at 120 min; $p < 0.001$; $n = 5-7$). In contrast, treatment with nitrite (10 or 100 μ M) elicited no significant change in the LVEDP at reperfusion when compared to hearts treated with KHB placebo ($p > 0.05$; $n = 5-7$; Figure 3.1D).

However, CFR was significantly improved following treatment with 10 μ M (0.6 ± 0.1 ml/min at 120 min) and 100 μ M nitrite (0.9 ± 0.2 ml/min at 120 min) when compared to the placebo treatment group (0.5 ± 0.1 ml/min at 120 min; $p < 0.001$; $n = 5-7$; Figure 3.1E).

3.1.2. Infarct size is reduced concomitantly with increasing concentrations of sodium nitrite

Figure 3.2A shows representative heart sections taken from each treatment group. As depicted in Figure 3.2B, quantitative analysis of the infarcted tissue (stained white) vs viable tissue (stained red) was compared from all 3 treatment groups. There was a dose-dependent decrease in infarct size following treatment with 10 μ M (40.5 ± 2.6 %) and 100 μ M nitrite (37.9 ± 3.3 %) when compared to the placebo (KHB) treatment group (49.3 ± 0.9 %). However, this was only significant at 100 μ M nitrite concentration vs placebo group ($p < 0.05$; $n = 5-7$ Figure 3.2B).

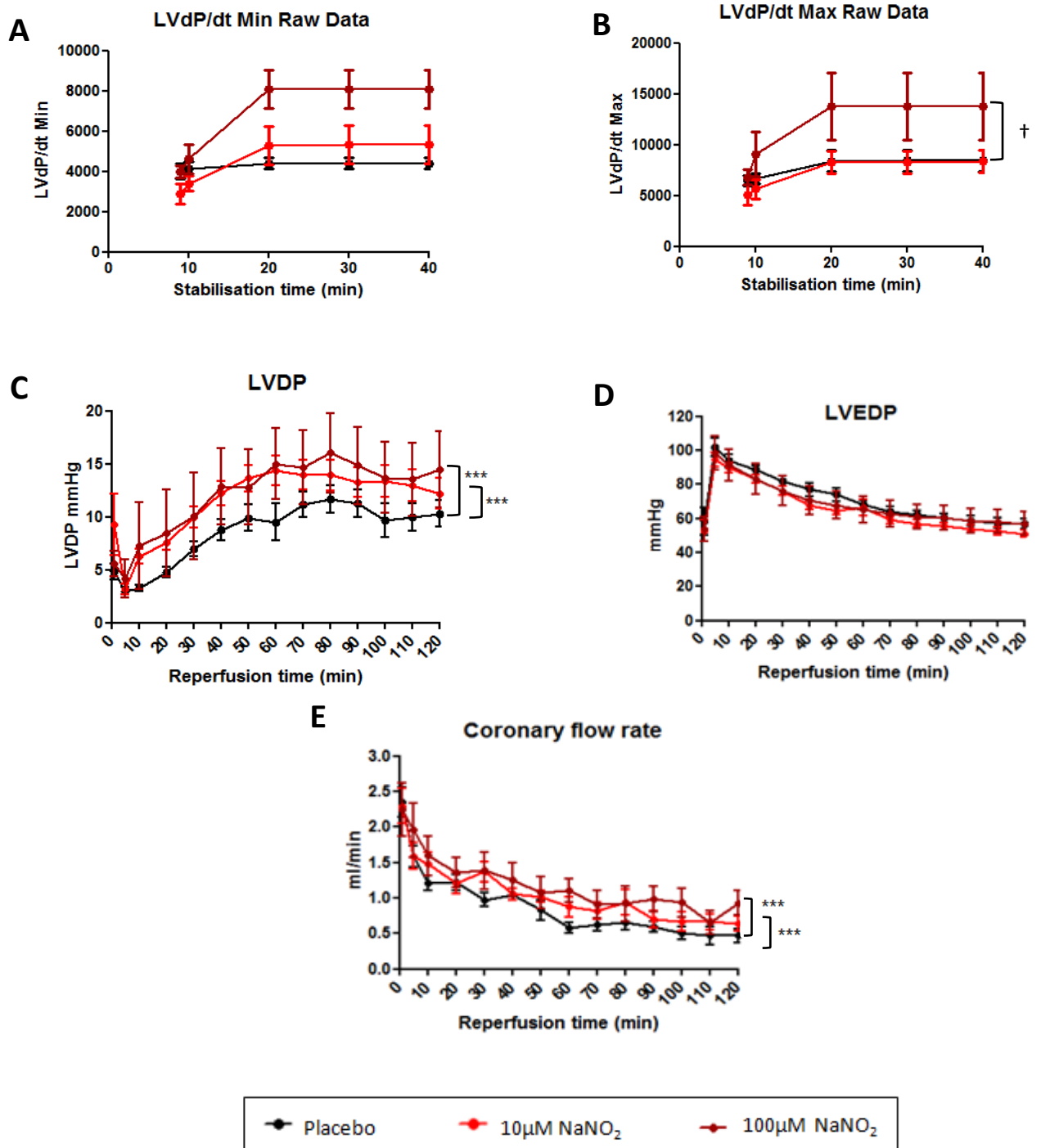


Figure 3.1. Nitrite improves cardiac function in C57/BL6 mice.

Hearts were treated for 30 min pre-ischaemia with (1) Placebo (KHB), (2) 10µM NaNO₂ or (3) 100µM NaNO₂. (A) LVdP/dt min (relaxation) and (B) LVdP/dt max (contractility) were recorded pre-ischaemia. Haemodynamic parameters were also recorded during 120 min reperfusion post-ischaemia (C): LVDP, (D): LVEDP and (E) Coronary Flow Rate. Data are presented as mean ± SEM; n= 5-7 mice. Statistical analysis carried out using 1-way ANOVA followed by Bonferroni post-hoc test; ***p<0.001 vs placebo treatment; †p<0.05 vs 10µM nitrite.

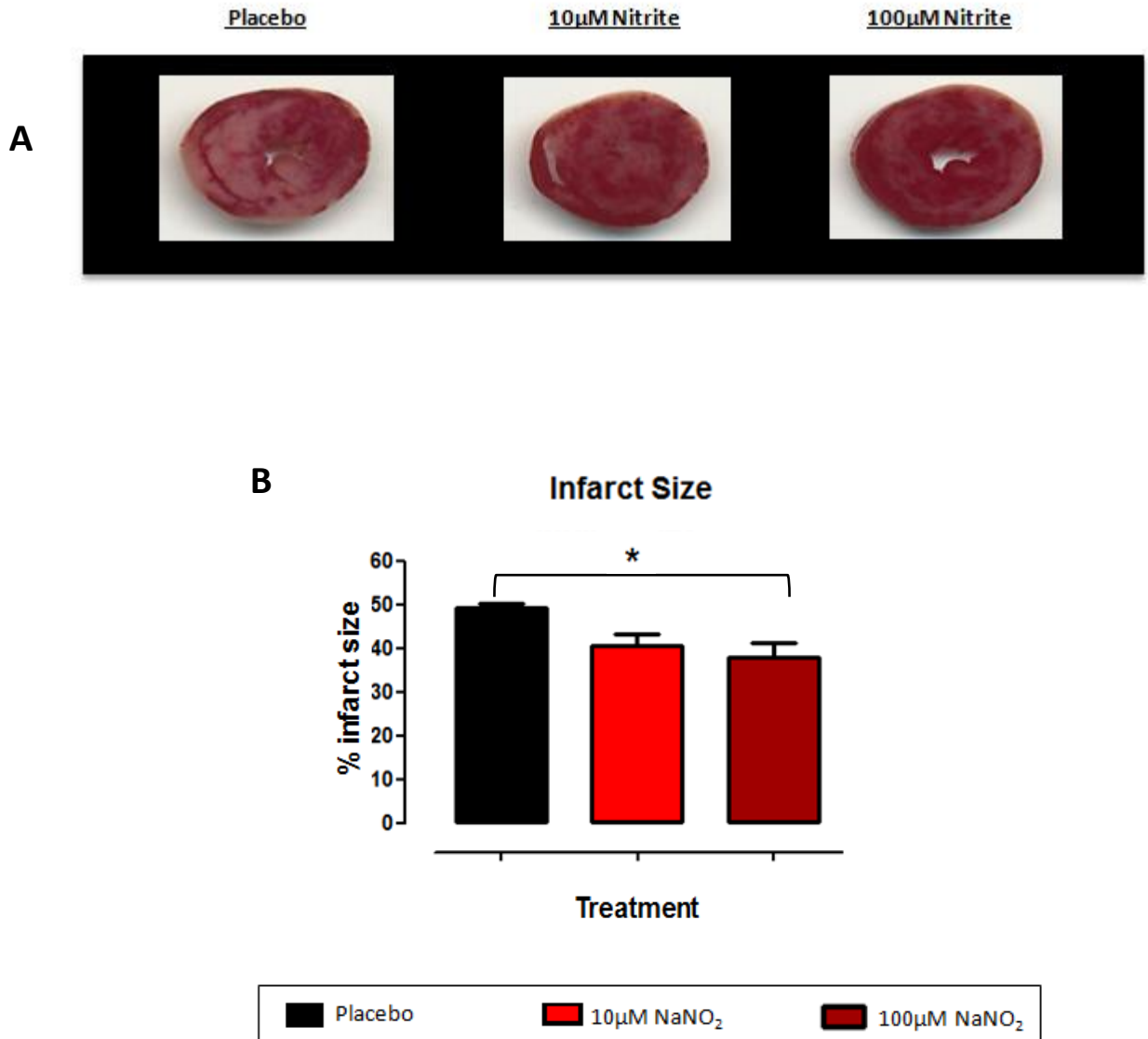


Figure 3.2. Nitrite reduces infarct size in C57/BL6 mice.

Hearts were treated for 30 min pre-ischaemia with (1) Placebo (KHB), (2) 10µM NaNO₂ or (3) 100µM NaNO₂. (A) Representative heart slices from each treatment group. (B): Average % infarct size observed in C57/BL6 mice given nitrite (10 or 100µM). Data are presented as mean ± SEM; n= 5-7 mice. Statistical analysis carried out using 1-way ANOVA followed by Bonferroni post-hoc test; *p<0.05 vs. WT placebo.

3.2. Nitrite-mediated cardioprotection via PKG1 α oxidation following myocardial IRI

As shown in section 3.1, 100 μ M nitrite protected the C57/BL6 mouse hearts against myocardial IRI. To elucidate the mechanism by which nitrite mediates cardioprotection we assessed the role of PKG1 α oxidation in response to 100 μ M nitrite treatment (Langendorff protocol B; see Figure 2.3.). As shown in Figure 3.3, we used PKG 1 α WT and PKG 'redox-dead' Cys42Ser KI (unable to undergo PKG 1 α disulfide dimerisation and therefore PKG 1 α oxidation) mice.

3.2.1. The effects of nitrite on haemodynamics during stabilisation via the PKG1 α oxidation pathway

Both LVdP/dt min and LVdP/dt max values were measured during the stabilisation period. As shown in Figure 3.3A, nitrite (100 μ M) increased LVdP/dt min by approximately 2-fold in the PKG1 α WT mice when compared to the placebo treated WT hearts, but this was not significant ($p > 0.05$; $n = 7-10$). A similar pattern was also observed with the nitrite treated PKG1 α WT mice vs placebo KI littermates (3-fold increase; $p > 0.05$; $n = 7-10$). However, there was a significant difference in the LVdP/dt min from the nitrite treated PKG1 α WT mice when compared to the PKG1 α KI nitrite group (3.4-fold increase; $p < 0.01$; $n = 7-10$). Conversely, there was no significant difference between LVdP/dt max values recorded following nitrite treatment in PKG1 α WT mice and PKG1 α KI mice ($p > 0.05$; $n = 7-10$; Figure.3.3B). Furthermore, nitrite treated PKG1 α WT mice and PKG1 α KI mice showed similar increases in LVdP/dt max values vs placebo treated littermates (1.7-fold increase in WT mice and 1.4-fold increase in KI mice, respectively) but did not reach statistical significance ($p > 0.05$; $n = 7-10$; Figure 3.3B).

As shown in figure 3.3C, there was a clear difference in the LVDP at stabilisation when comparing the placebo PKG1 α WT (65.5 ± 4.4 mmHg at 40 min) vs placebo treated KI ($54.4 \pm$

8.0 mmHg at 40min) mice, but this was not significantly different ($p>0.05$; $n=8-12$). Furthermore, when comparing the LVDP for all four treatment groups at stabilisation, there was no significant difference ($p>0.05$; $n=8-12$). However, we observed a significant increase in the LVEDP following nitrite treatment from the PKG1 α KI mice when compared with the PKG1 α WT hearts (10.2 ± 1.1 vs 8.9 ± 0.7 mmHg respectively; $p<0.05$; $n=8-12$; Figure 3.3D). In contrast, there was no significant difference observed in the LVEDP between nitrite treated PKG1 α WT and KI mice when compared to their respective KHB placebo treated littermates ($p>0.05$; $n=8-12$). Figure 3.3E shows PKG1 α WT hearts also exhibited a consistently higher CFR following nitrite treatment when compared to hearts of the same genotype given placebo treatment (3.7 ± 0.4 vs 3.2 ± 0.3 ml/min; $p>0.05$; $n=8-12$; Figure 4.3E). However, this trend was not observed in PKG1 α KI hearts ($p>0.05$ when comparing PKG1 α KI placebo hearts vs PKG1 α KI nitrite hearts; $n=8-12$).

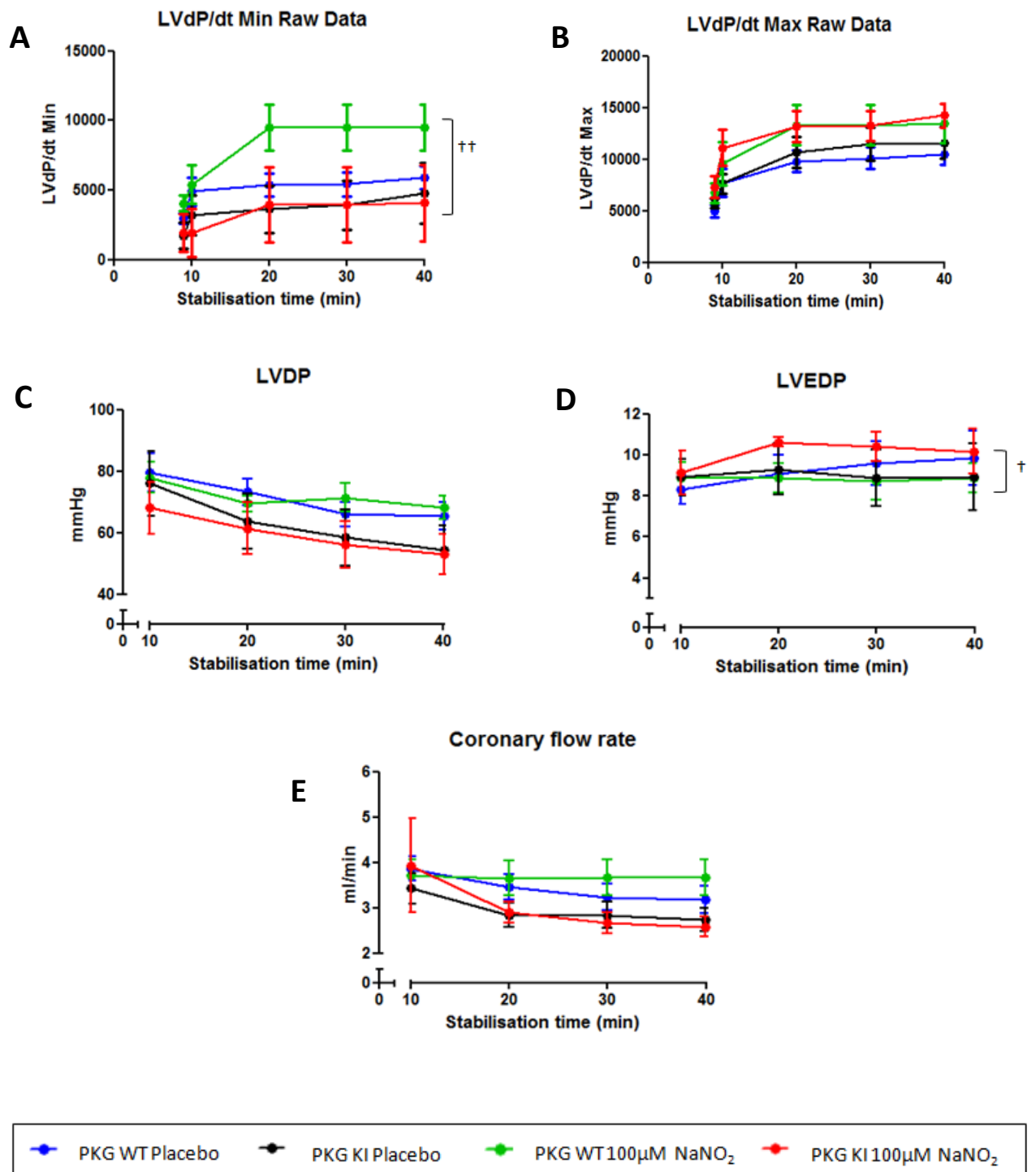


Figure 3.3. Nitrite does not alter cardiac function *via* PKG1 α oxidation during stabilisation period.

PKG1 α WT and PKG1 α KI hearts were treated for 30 min pre-ischaemia with (1) Placebo (KHB) or (2) 100 μ M NaNO₂. All parameters were recorded during 40 min stabilisation period. (A): LVdP/dt min (n=7-10) (B): LVdP/dt max (n=7-10) (C): LVDP (n=8-12) (D): LVEDP (n=8-12) (E): Coronary Flow Rate (n=8-12). Data are presented as mean \pm SEM. Statistical analysis carried out using 1-way ANOVA followed by Bonferroni post-hoc test; ††p<0.01 vs KI nitrite; †p<0.05 vs KI nitrite.

3.2.2. Nitrite protects against IRI via the PKG1 α oxidation pathway

Fig. 3.4A shows that 100 μ M nitrite significantly improved LVDP at reperfusion in the PKG1 α WT hearts when compared to placebo treated PKG1 α WT (19.9 ± 3.6 vs 10.4 ± 1.8 mmHg; $p < 0.01$; $n = 8-12$) and the nitrite treated PKG1 α KI mice (19.9 ± 3.6 vs 13.3 ± 3.8 mmHg; $p < 0.05$; $n = 8-12$). However, there was no significant difference observed between nitrite and placebo treatment groups in their KI littermates (13.3 ± 3.8 vs 12.8 ± 3.0 mmHg; $p > 0.05$; not significant; $n = 8-12$). In contrast, nitrite treatment did not significantly improve LVEDP in PKG1 α WT mice when compared to the placebo control (30.2 ± 4.3 vs 27.3 ± 5.7 mmHg; $p > 0.05$; $n = 8-12$; Figure 3.4B). There was however a significant difference between LVEDP recorded in the WT hearts following nitrite treatment when compared to KI littermates receiving the same treatment (30.2 ± 4.4 vs 16.6 ± 7.5 mmHg; $p < 0.05$; $n = 8-12$).

As depicted in figure 3.4C, coronary flow was also significantly improved at reperfusion following the treatment with 100 μ M nitrite in PKG1 α WT mice when compared to the control WT (2.2 ± 0.4 vs 1.5 ± 0.2 ml/min; $p < 0.001$; $n = 8-12$) and KI nitrite (2.2 ± 0.4 vs 1.1 ± 0.2 ml/min; $p < 0.001$; $n = 8-12$) groups. This trend was absent in the CFR data taken from the KI hearts as there was no significant difference between KI hearts given nitrite or placebo treatments. Furthermore, there was a significant difference between PKG1 α WT and KI genotypes in response to placebo treatment, with WT hearts again exhibiting a higher CFR (1.5 ± 0.2 ml/min) than KI (1.1 ± 0.3 ml/min) hearts given the same treatment ($P < 0.001$; $n = 8-12$).

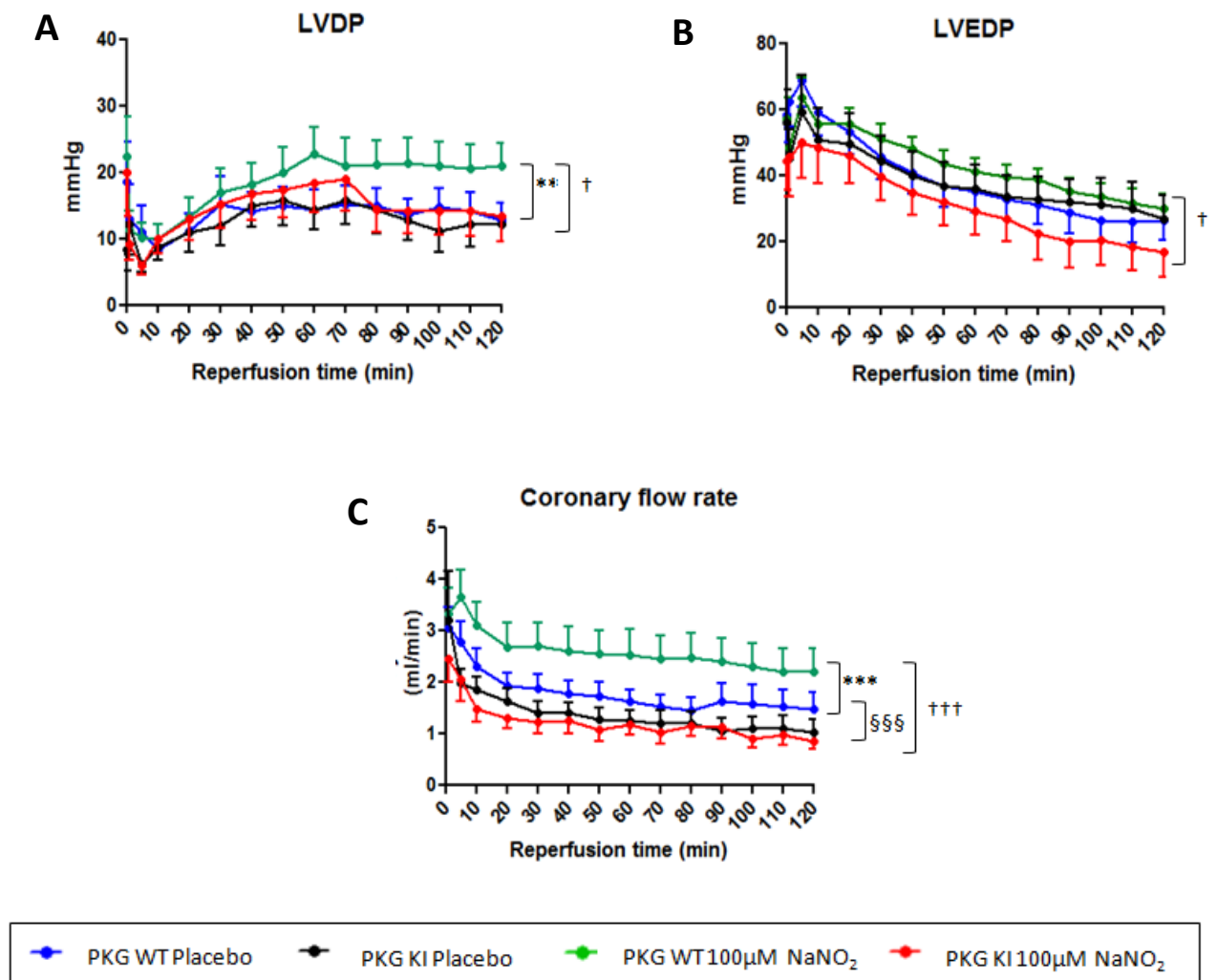


Figure 3.4. Nitrite improves cardiac function via PKG1 α oxidation post-ischaemia.

PKG1 α WT and PKG1 α KI hearts were treated for 30 min pre-ischaemia with (1) Placebo (KHB) or (2) 100µM NaNO₂. All parameters were recorded during 120 min reperfusion period post-ischaemia. **(A)**: LVDP **(B)**: LVEDP **(C)**: Coronary Flow Rate. Data are presented as mean \pm SEM; n=8-12 mice. Statistical analysis carried out using 1-way ANOVA followed by Bonferroni post-hoc test; **P<0.01 vs. WT placebo; ***p<0.001 vs WT placebo; †P<0.05 vs. KI nitrite; †††p<0.001 vs KI nitrite; ††††p<0.001 vs KI placebo.

3.2.3. An alternative pathway may be involved in nitrite-mediated protection of viable cardiomyocytes

Figure 3.5A shows representative heart sections from each treatment group in PKG1 α WT and KI mice. As depicted in figure 3.5B, we observed approximately 36.2% reduction in infarct size from the 100 μ M sodium nitrite treated PKG1 α WT mice when compared to the placebo treated PKG1 α WT group (27.6 ± 2.4 % and 43.3 ± 3.8 % respectively; $p < 0.05$; $n = 8-12$; Figure 3.5B). However, there was no significant difference between both PKG1 α WT treatment groups when compared to their KI littermates ($p > 0.05$; $n = 8-12$).

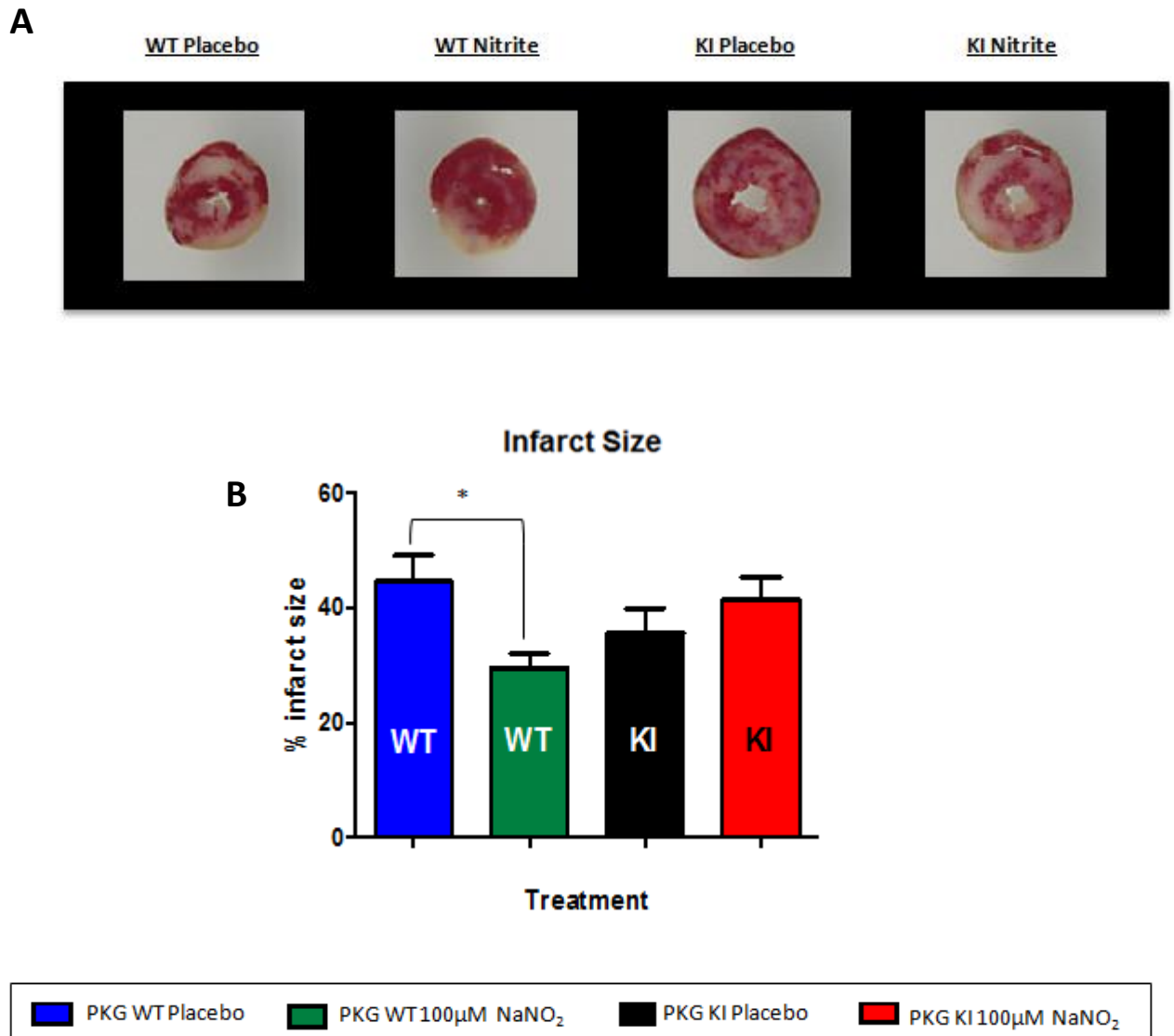


Figure 3.5. Nitrite reduces infarct size *via* PKG1 α oxidation.

PKG1 α WT and PKG1 α KI hearts were treated for 30 min pre-ischaemia with (1) Placebo (KHB) or (2) 100µM NaNO₂. (A) Representative heart slices from each treatment group. (B) Average % infarct size observed in PKG1 α WT and PKG1 α KI hearts receiving placebo or nitrite treatment. Data are presented as mean \pm SEM; n= 8-12 mice. Statistical analysis carried out using 1-way ANOVA followed by Bonferroni post-hoc test; *p<0.05 vs. WT placebo.

3.3. The involvement of ALDH2 in nitrite-mediated cardioprotection against myocardial IRI

To further elucidate the mechanism by which nitrite mediates cardioprotection and in particular, determine the role of ALDH2 enzyme in this process, we used ALDH2 WT and ALDH2 KO (with an inactive ALDH2 enzyme) mice in Langendorff protocol B (see Figure.2.3).

3.3.1. At stabilisation period nitrite improves coronary flow rate via the ALDH2 pathway

As shown in Figure 3.6A, nitrite (100 μ M) significantly increased LVdP/dt min by approximately 2-fold in the ALDH2 WT mice when compared to the placebo treated WT mice ($p < 0.05$; $n = 7-11$). A similar pattern was also observed with the nitrite treated ALDH2 KO littermates vs placebo treated KO mice, but this was not significant (1.6-fold increase with nitrite treatment; $p > 0.05$; $n = 7-11$). An increase in LVdP/dt max was measured following nitrite treatment in both ALDH2 WT and KO mice vs their placebo treated littermates (1.6-fold increase in WT mice and 1.4-fold increase in KO mice respectively; $p > 0.05$; $n = 7-11$; Figure.3.6B). However, changes observed in LVdP/dt max values did not reach statistical significance.

During the stabilisation period, as depicted in figure 3.6C, there is no clear difference in LVDP between all four treatment groups ($p > 0.05$; $n = 7-11$). Furthermore, when comparing the LVEDP measured in nitrite (100 μ M) treated ALDH2 WT mice vs placebo treated ALDH2 WT mice, there was no clear difference between the groups (10.9 ± 3.2 vs 11.751 ± 2.8 mmHg; $p > 0.05$; $n = 7-11$; Figure.3.6D). Although nitrite treated ALDH2 KO hearts showed a trend of a decrease in LVEDP when compared to placebo treated littermates, this data was not statistically significant (9.4 ± 1.5 vs 14.0 ± 3.6 mmHg; $p > 0.05$; $n = 7-11$; Figure.3.6D). Figure 3.6E shows nitrite treated ALDH2 WT hearts exhibited a consistently higher CFR when

compared to KHB placebo treated WT hearts (4.5 ± 0.6 vs 3.5 ± 0.4 ml/min; $p > 0.05$; $n = 7-11$), but this was not significant. However, there was a significant difference in CFR in response to nitrite treated ALDH2 WT vs KO littermates (4.5 ± 0.6 vs 3.2 ± 0.3 ml/min; $p < 0.01$; $n = 7-11$). Conversely, there was no significant difference between ALDH2 KO mice treated with $100\mu\text{M}$ nitrite when compared to placebo treated KO mice (3.2 ± 0.3 vs 3.5 ± 0.2 ml/min; $p > 0.05$; $n = 7-11$).

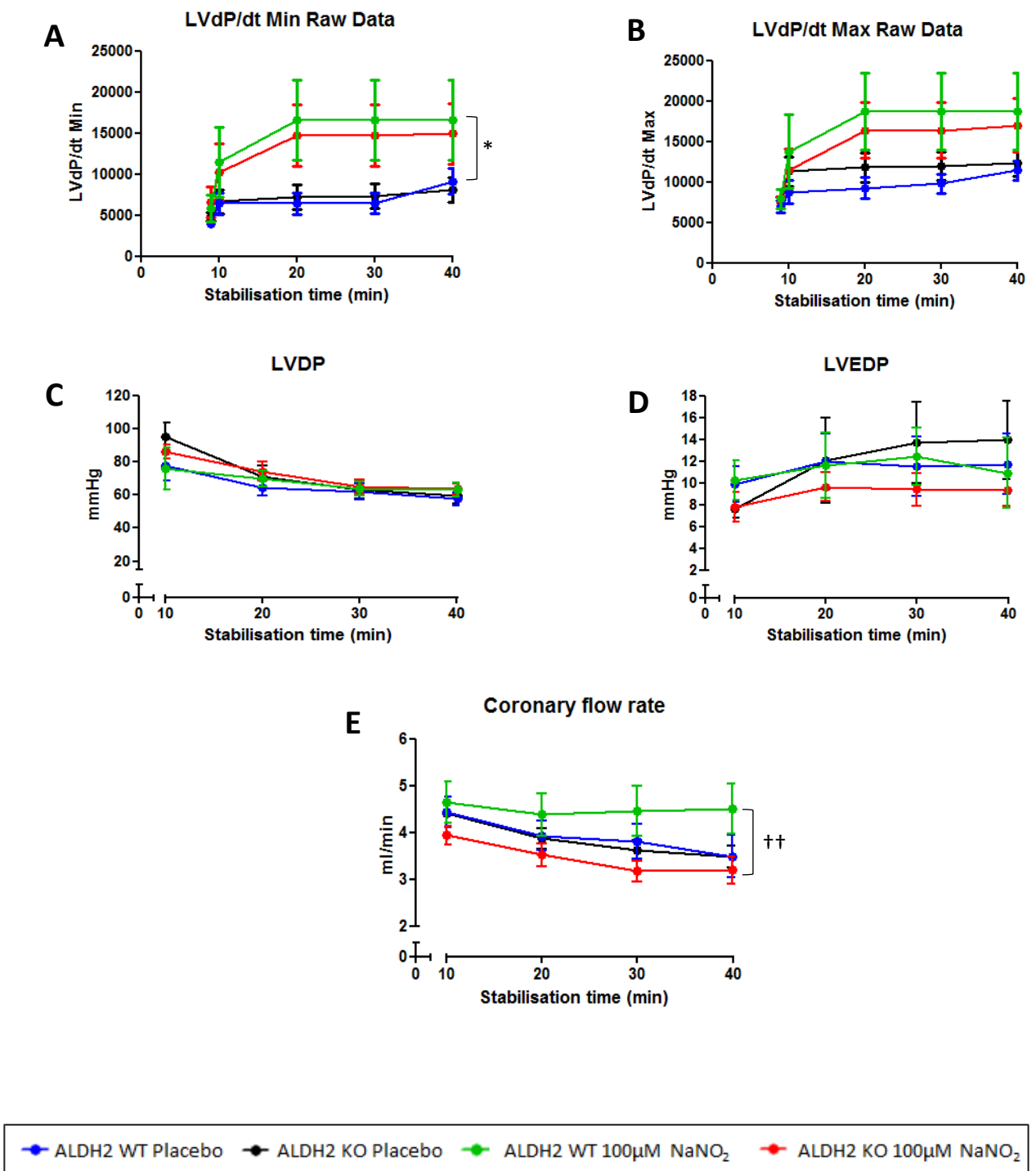


Figure 3.6. Nitrite improves cardiac relaxation and coronary flow *via* ALDH2 during stabilisation period.

ALDH2 WT and ALDH2 KO hearts were treated for 30 min pre-ischaemia with (1) Placebo (KHB), or (2) 100µM NaNO₂. All parameters were recorded during 40 min stabilisation period. (A): LVdP/dt min (B): LVdP/dt max (C): LVDP (D): LVEDP (E): Coronary Flow Rate. Data are presented as mean ± SEM; n=7-11 mice. Statistical analysis carried out using 1-way ANOVA followed by Bonferroni post-hoc test; *p<0.05 vs WT placebo; ††p<0.01 vs KO nitrite.

3.3.2. Nitrite mediates improvements in coronary flow rate via the ALDH2 enzyme following IRI

Figure 3.7A shows LVDP following IRI in ALDH2 WT and KO mice. Nitrite treated ALDH2 WT mice showed improved LVDP when compared to WT mice given the KHB placebo, but this data was not statistically significant (21.6 ± 3.7 vs 16.2 ± 3.2 mmHg; $p > 0.05$; $n = 7-11$). In contrast, in ALDH2 KO littermates, LVDP was decreased in response to nitrite treatment when compared to placebo treated KO hearts (16.0 ± 2.0 vs 20.3 ± 3.1 mmHg; $p > 0.05$; $n = 7-11$). Interestingly, placebo treated ALDH2 KO mice demonstrated an increase in LVDP vs placebo treated WT mice (20.3 ± 3.1 vs 16.2 ± 3.2 mmHg; $p > 0.05$; $n = 7-11$).

ALDH2 WT mice demonstrated a statistically significant increase in LVEDP following treatment with $100\mu\text{M}$ nitrite when compared to the WT control group (31.7 ± 6.9 vs 19.2 ± 4.5 mmHg; $p < 0.01$; $n = 7-11$; Figure.3.7B). In addition, LVEDP was significantly higher in nitrite treated ALDH2 WT hearts vs KO littermates that received the same treatment (31.7 ± 6.9 vs 21.2 ± 5.7 mmHg; $p < 0.001$; $n = 7-11$; Figure.4.7B). Comparing ALDH2 WT vs KO placebo control groups showed no significant difference (19.2 ± 4.5 vs 23.6 ± 5.5 mmHg; $p > 0.05$; $n = 7-11$; Figure.3.7B).

As shown in figure 3.7C, coronary flow rate was significantly improved in ALDH2 WT mice treated with $100\mu\text{M}$ nitrite when compared to WT placebo controls (3.4 ± 0.4 vs 2.2 ± 0.5 ml/min; $p < 0.001$; $n = 7-11$). Furthermore, nitrite treated ALDH2 WT mice exhibited a significant increase in CFR when compared to both nitrite-treated (3.4 ± 0.4 vs 1.4 ± 0.2 ml/min; $p < 0.001$; $n = 7-11$) and placebo-treated (3.4 ± 0.4 vs 2.5 ± 1.0 ml/min; $p < 0.001$; $n = 7-11$) KO littermates. In addition, there was no significant differences observed between nitrite and placebo treatment groups in ALDH2 KO mice ($p > 0.05$; $n = 7-11$).

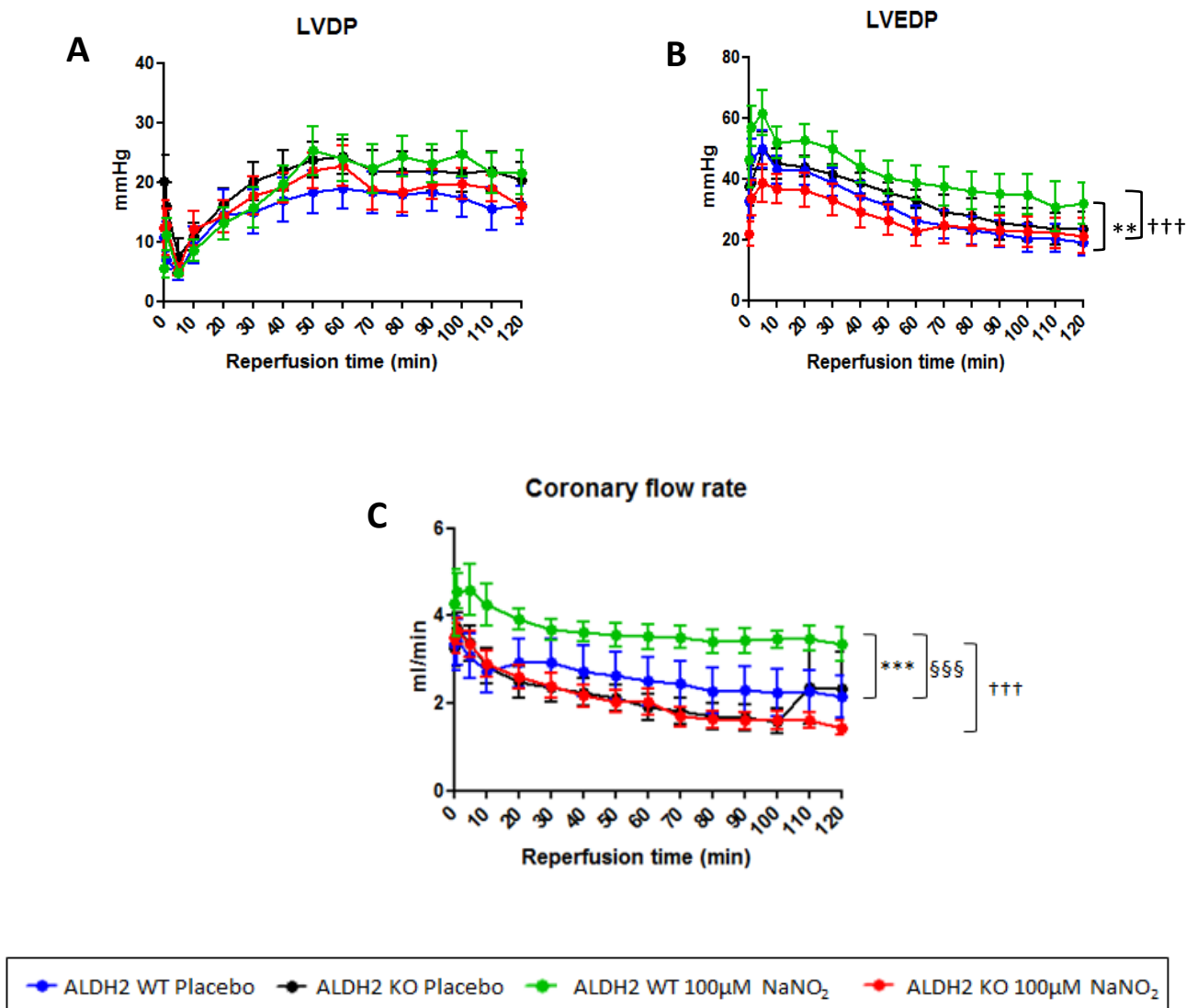


Figure 3.7. Nitrite improves coronary flow *via* ALDH2 post-ischæmia.

ALDH2 WT and ALDH2 KO hearts were treated for 30 min pre-ischæmia with (1) Placebo (KHB) or (2) 100µM NaNO₂. All parameters were recorded during 120 min reperfusion period post-ischæmia. (A): LVDP (B): LVEDP (C): Coronary Flow Rate. Data are presented as mean ± SEM; n=7-11 mice. Statistical analysis carried out using 1-way ANOVA followed by Bonferroni post-hoc test; **p<0.01 vs WT placebo; ***p<0.001 vs WT placebo; †††p<0.001 vs KO nitrite; §§§p<0.001 vs KO placebo.

3.3.3. The ALDH2 enzyme may be involved in nitrite-mediated protection of viable cardiomyocytes

Figure 3.8A displays representative heart sections from each of the four treatment groups in ALDH2 WT and ALDH2 KO mice. As shown in figure 3.8B, we observed approximately 34.3% reduction in infarct size from the nitrite treated ALDH2 WT mice when compared to the placebo treated controls, but this was not significant ($23.1 \pm 3.4\%$ and $35.2 \pm 2.7\%$, respectively; $p > 0.05$; $n = 7-11$). In contrast, following treatment with placebo or $100\mu\text{M}$ sodium nitrite in the ALDH2 KO mice, there was no clear change in infarct size and this data was also not statistically significant ($33.2 \pm 4.6\%$ and $34.8 \pm 4.9\%$, respectively; $p > 0.05$; $n = 7-11$).

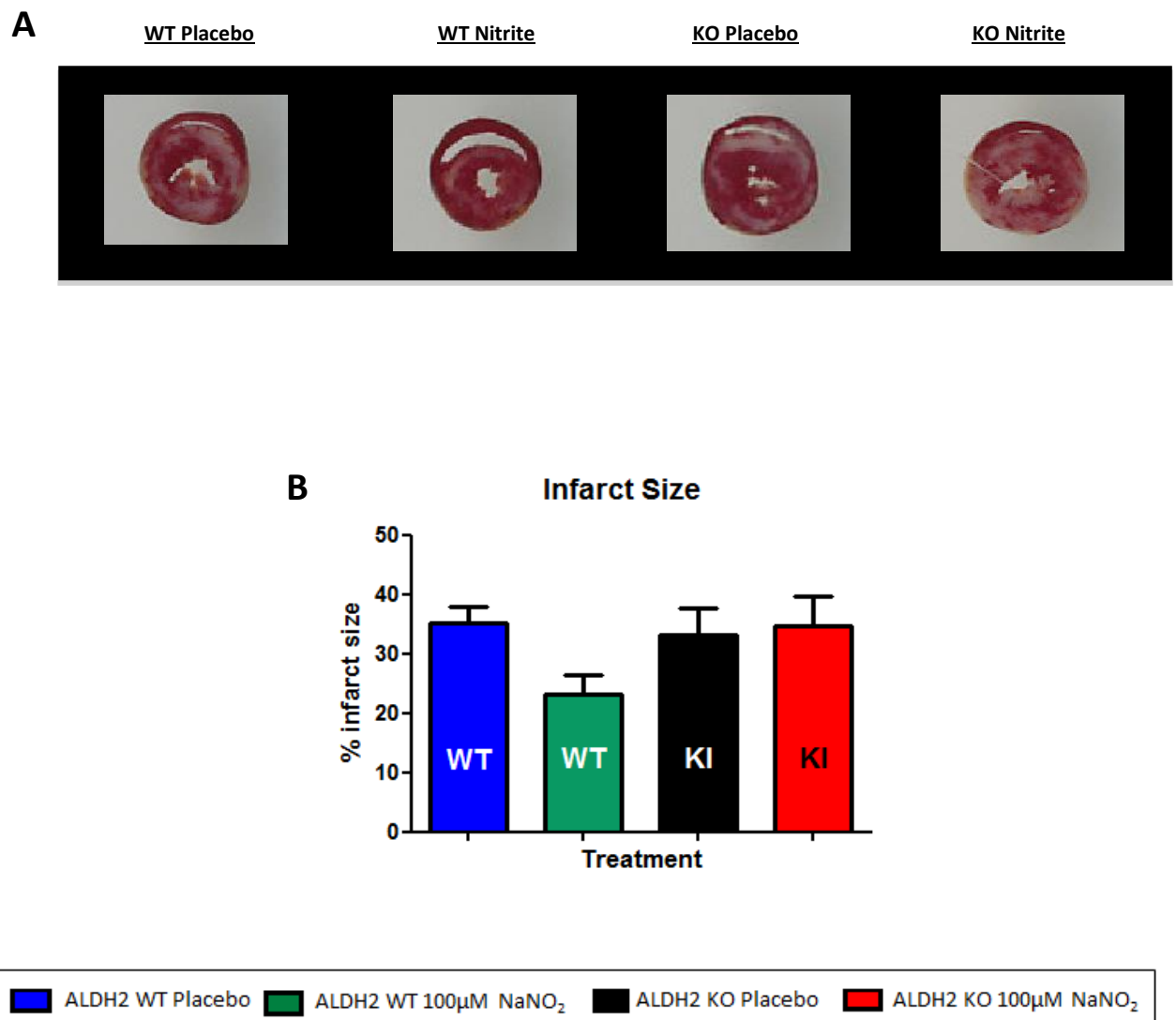


Figure. 3.8. The effects of nitrite on infarct size in ALDH2 WT and ALDH2 KO mice.

ALDH2 WT and ALDH2 KO hearts were treated for 30 min pre-ischaemia with (1) Placebo (KHB) or (2) 100µM NaNO₂. (A) Representative heart slices from each treatment group. (B) Average % infarct size observed in ALDH2 WT and ALDH2 KO hearts receiving placebo or nitrite treatment. Data are presented as mean ± SEM; n= 7-11 mice. Statistical analysis carried out using 1-way ANOVA followed by Bonferroni post-hoc test.

4. DISCUSSION

The ability of nitrite to elicit cardioprotection is well established. [Webb et al., 2004] [Ingram et al., 2013] However, the mechanism underlying this cardioprotection remains unclear. In the present study we focused on two potential mediators: (1) PKG1 α oxidation and (2) ALDH2 enzymatic action. Our data shows that sodium nitrite is able to elicit cardioprotection in C57/BL6 mice subjected to a murine model of myocardial IRI. Furthermore, we demonstrate that nitrite (1) improves cardiac function *via* the PKG1 α oxidation pathway and (2) increases coronary flow rate at reperfusion through the ALDH2 enzyme following myocardial IRI.

4.1. Cardioprotective effects of nitrite in C57/BL6 mice

4.1.1. Nitrite mediates inotropic effects during normoxic conditions

Stabilisation data was analysed for LVdP/dt min and LVdP/dt max values as indicators of cardiac contraction (inotropic effects) and relaxation (negative inotropy), respectively. It has previously been suggested that nitrite acts as a physiological reservoir of NO and is able to elicit potent negative inotropic effects [Pellegrino et al., 2009]. In the present study, isolated C57/BL6 mouse hearts were perfused with either 10 or 100 μ M of nitrite, which resulted in a dose-dependent increase in LVdP/dt min when compared to placebo controls, but this was not significant. Conversely, 100 μ M nitrite treatment significantly increased inotropic effects when compared to 10 μ M nitrite, thus suggesting that nitrite is indeed able to influence cardiac contractility in a dose-dependent manner.

4.1.2. Nitrite elicits cardioprotection against myocardial IRI in C57/BL6 hearts

Webb et al. (2004) reported improvements in cardiac function (LVDP) and infarct size following treatment with either 10 or 100 μ M concentrations of nitrite in rat Langendorff model of myocardial IRI. Confirming that these results are transferable to a mouse model of myocardial IRI, our results corroborates Webb and colleagues' findings as we observed a significant improvement in LVDP and CFR at reperfusion (post-ischaemia) when compared to the placebo group [Webb et al., 2004]. Furthermore, although not statistically significant, there was a trend of a decrease in LVEDP following treatment with nitrite (10 and 100 μ M), which suggests that nitrite improves diastolic function [Pourmoghaddas et al., 2011]. Additionally, we demonstrate a significant reduction in infarct size with 100 μ M sodium nitrite when compared to the control group, which corroborates with previous studies [Shiva et al., 2007] [Bryan et al., 2007] [Duranski et al., 2005].

From our experiments using hearts isolated from C57/BL6 mice we were able to conclude that a 100 μ M concentration of sodium nitrite was able to elicit cardioprotection against myocardial IRI. We therefore use this concentration to probe for candidate enzymes/proteins involved.

4.2. Nitrite-mediated cardioprotection via PKG1 α oxidation in myocardial IRI

Hydrogen peroxide, an oxidative stress mediator, was reported by Burgoyne and colleagues to induce vasorelaxation *via* PKG1 α dimerisation in the resistance vessels and the heart [Burgoyne et al., 2007]. In addition, Pryszyzhna et al. (2012) demonstrated that redox-dead Cys42Ser PKG1 α knock-in (KI) mice showed less vasodilatation in response to H₂O₂ when compared to their WT littermates. PKG1 α oxidation has also been implicated in glyceryl trinitrate (GTN) tolerance and has been shown to attenuate vasorelaxation responses in

PKG1 α KI mice [Rudyk et al., 2012]. To our knowledge, whether nitrite elicits protection against myocardial IRI *via* the PKG1 α oxidation pathway has not yet been investigated. As such, in the present study we assessed whether the PKG1 α oxidation pathway plays an essential role in nitrite-mediated cardioprotection by using PKG1 α WT and KI mice.

4.2.1. Nitrite mediates negative inotropy in normoxia via PKG1 α oxidation

Previous studies using animal models have confirmed nitrite to have negative inotropic effects and has been proposed to mediate via the classical nitrite-NO-cGMP-PKG pathway [Cerra et al., 2009] [Pellegrino et al., 2009]. In parallel studies, an alternative novel PKG1 α oxidation pathway has recently been identified [Prysyazhna et al., 2012] [Rudyk et al., 2012]. PKG1 α oxidation has been implicated in the maintenance of myocardial relaxation [Prysyazhna and Eaton, 2015], with PKG1 α KI mice showing reduced diastolic volume and slower ventricular filling velocities, thus suggesting impaired relaxation and increased myocardial stiffness [Prysyazhna et al., 2012a] [Prysyazhna et al., 2012b]. Our data conforms to these ideas as we demonstrate an increase in cardiac relaxation (increased LVdP/dt min values), and therefore negative inotropy in PKG1 α WT hearts treated with 100 μ M nitrite when compared to placebo controls. This nitrite-mediated effect was not replicated in the PKG1 α KI mice, therefore highlighting PKG1 α oxidation as a novel pathway by which cardiac contractility is modulated. Nitrite-treated PKG1 α WT mice also show significantly increased LVdP/dt min when compared to nitrite-treated PKG1 α KI mice, thus further supporting the importance of the PKG1 α oxidation pathway.

In contrast, LVdP/dt max values were increased in both PKG1 α WT and KI mice in response to nitrite treatment when compared to control groups, but this was not significant.

4.2.2. Nitrite-mediated cardioprotection following IRI via PKG1 α oxidation

A normoxia-dependent action of nitrite was demonstrated in a recent study by Omar and colleagues in healthy subjects [Omar et al., 2015]. Nitrite treatment resulted in a reduction in central haemodynamics (taken to suggest a reduction in the probability of experiencing adverse cardiovascular events) and a marked increase in radial dilatation [Omar et al., 2015]. Previous studies have also demonstrated that nitrite potentiates vasodilatory effects in animal and human vessels under hypoxic conditions [Arif et al., 2015] [Dautov et al., 2014] and improves cardiac and pulmonary haemodynamics in heart failure patients [Omerod et al., 2015]. However, the mechanism(s) underlying these beneficial effects remains unclear. In the present study, we investigated whether nitrite improves cardiac function *via* the PKG1 α pathway. At stabilisation period (e.g. normoxic conditions) we observed a trend whereby nitrite improved cardiac function (with an increase in LVDP and CFR and a decrease in LVEDP) in the PKG1 α WT mice when compared to the control group. However, this was not statistically significant. In contrast, at reperfusion phase (post-ischaemia), there was a significant improvement in LVDP and CFR in PKG1 α WT mice treated with 100 μ M sodium nitrite when compared to placebo controls. Conversely, an increase in these haemodynamic parameters was not present in PKG1 α KI littermates, thus suggesting the involvement of the novel PKG1 α oxidation pathway in nitrite-mediated vasodilatation of the cardiac microvasculature.

4.2.3. Nitrite elicits a reduction in infarct size via an alternative mechanism

We observed a significant decrease (36.2%) in infarct size in the nitrite treated PKG1 α WT mice *vs* placebo WT control, which corroborates with the protective effects of nitrite demonstrated by the present study (C57/BL6) and others [Webb et al., 2004] [Duranski et al., 2005] [Shiva et al., 2007]. However interestingly, there was no difference between both

PKG1 α WT treatment groups when compared to their KI littermates. Our results therefore implicate an alternative mechanism by which nitrite may attenuate cardiomyocyte apoptosis, independently of the PKG1 α oxidation pathway. Prior animal studies have demonstrated attenuation of the cytoprotective effects of nitrite in the heart in the presence of NO scavengers [Webb et al., 2004] [Duranski et al., 2005] and have therefore suggested that enzymatic reduction to NO plays an important role [Dezfulian et al., 2007]. Furthermore, it has been suggested that nitrite-mediated cardiovascular effects are dependent upon specific components of the classical pathway such as cGMP [Pellegrino et al., 2009] [Francis et al., 2010]. It might be plausible, therefore, that the increase in LVDP and the reduction in infarct size observed could be attributed to the bioconversion of nitrite to the NO-cGMP-PKG pathway during the hypoxic conditions of IRI [Zweier et al., 1995].

As such, our data suggest a potential dual mechanism by which nitrite mediates cardioprotection. This includes (1) the classical nitrite-NO-cGMP-PKG to attenuate infarct size and the (2) PKG1 α oxidation pathway to mediate vasodilatory effects, but further studies are warranted to confirm these findings.

4.3. ALDH2 involvement in nitrite-mediated cardioprotection in myocardial IRI

ALDH2 has a vital role in the detoxification of reactive aldehydes produced in response to ROS generation during myocardial IRI. ALDH2 activation has been linked to improvements in ventricular function in myocardial IRI and heart failure. Additional studies have demonstrated a vital role for ALDH2 in the vasodilatory response induced by GTN [Nossaman et al., 2012] and sodium nitrite [Chen et al., 2002] [Arif et al., 2015], therefore suggesting that this enzyme is involved in the bioactivation of inorganic nitrite to gaseous radical NO. Thus far however, no studies have investigated whether nitrite mediates cardioprotection via ALDH2. In the

present study, we therefore aimed to elucidate ALDH2 involvement in nitrite-mediated cardioprotection in our Langendorff model of myocardial IRI using ALDH2 WT and KO mice.

At basal conditions, ALDH2 KO mice have been shown to display no differences in body weight, blood pressure, heart rate or echocardiographic parameters (e.g. Left Ventricular pressure) when compared to WT mice [Sun et al., 2014]. However, post-MI they have been shown to exhibit greater left-ventricular dysfunction, manifested in lower ejection fraction and increased LVEDP recordings, in addition to greater cardiomyocyte damage [Endo et al., 2009].

4.3.1. The effect of nitrite on cardiac contractility in ALDH2 WT and KO mice during stabilisation

ALDH2 has been implicated in improvements in contractility (both maximal velocity lengthening and shortening) in a previous study by Ma and colleagues, in which they used a Langendorff model to analyse ethanol-induced changes in ALDH2 transgenic mice [Ma et al., 2009]. In the present study we observed a significant increase in LVdP/dt min values in ALDH2 WT hearts treated with 100 μ M nitrite when compared to placebo controls. Although a smaller increase in LVdP/dt min was observed in nitrite treated ALDH2 KO littermates vs placebo controls, this change was not significant. Furthermore, similar trends were observed in LVdP/dt max data. Nitrite treated ALDH2 WT hearts exhibited an increase in LVdP/dt max vs control groups when compared KO littermates receiving the same treatment, but this data was not statistically significant.

4.3.2. ALDH2 activity influences cardiac function post-IRI

In the present study, we did not observe any statistically significant differences in LVDP or LVEDP between all four treatment groups during stabilisation period. However, differences in

cardiac function (LVDP and LVEDP) were observed between ALDH2 WT and ALDH2 KO mice post-ischaemia. A trend was observed in LVDP during reperfusion phase in which nitrite treatment elicited an increase in LVDP in ALDH2 WT hearts but conversely elicited a decrease in LVDP in ALDH2 KO littermates vs control groups. This data was not statistically significant but ALDH2 activation has previously been implicated in LVDP recovery following IRI in an *in vitro* rat model [Wang et al., 2012]. Interestingly, following IRI, the placebo treated ALDH2 KO mice exhibited increased LVDP when compared to placebo treated WT mice. This data is consistent with findings by Endo and colleagues who were able to demonstrate that untreated ALDH2 KO mice showed better recovery of LVDP when compared to untreated WT mice subjected to the same Langendorff protocol [Endo et al., 2009].

LVEDP was observed to be significantly higher in ALDH2 WT mice following 100µM nitrite treatment when compared to WT controls and ALDH2 KO littermates also given nitrite treatment. These findings are contrary to those published in a cardiotoxicity study by Gao et al. (2015) in which the ALDH2 antagonist Daidzin was found to increase LVEDP, whilst the ALDH2 activator Alda-1 lowered LVEDP, in mice exposed to the cardiotoxic drug Doxorubicin. LVEDP was not significantly different in nitrite treated ALDH2 KO mice vs control group.

4.3.3. Nitrite-mediated improvements in coronary flow in normoxia and following IRI via ALDH2

Our research group previously demonstrated a dose-dependent increase in vasorelaxation in rat aortae and human resistance vessels in response to nitrite in conditions of both normoxia and hypoxia, which were attenuated by ALDH2 inhibitor cyanamide. However, the effects of nitrite were more pronounced in conditions of hypoxia [Arif et al., 2015]. These results may offer an explanation for our results in the present study, as we observe a statistically significant increase in CFR in nitrite treated ALDH2 WT hearts vs nitrite treated KO hearts during both stabilisation (normoxia) and reperfusion (following a period of hypoxia).

Similarly, the nitrite-mediated increase in CFR in WT mice vs KO littermates occurs to a greater extent during reperfusion. In addition, we observed CFR to be significantly higher in nitrite treated ALDH2 WT hearts vs WT placebo controls post-IRI. Conversely, there was no significant difference between CFR in ALDH2 KO nitrite and ALDH2 KO placebo groups during reperfusion phase. Overall, these results corroborate with previous studies [Arif et al., 2015] [Nossaman et al., 2012] and suggest nitrite is able to elicit vasorelaxation of the cardiac microvasculature *via* an activity of the ALDH2 enzyme.

4.3.4. Nitrite-mediated cardioprotection may involve ALDH2 enzymatic activity

Chen and colleagues found an inverse correlation between ALDH2 activity and infarct size using the ALDH2 activator Alda-1 in an *in vivo* rat model of AMI [Chen et al., 2008]. Additionally, ALDH2 phosphorylation has been implicated in the bioactivation of nitrite [Perlman et al., 2009]. In nitrite treated ALDH2 WT hearts we demonstrated a 34.3% decrease in infarct size when compared to their placebo controls. In contrast, we did not observe any clear change between nitrite treated ALDH2 KO mice placebo treated controls. Although, not statistically significant, our findings corroborate with the work of previous studies and suggest nitrite-mediated cardioprotection is elicited *via* a pathway involving the ALDH2 enzyme.

Overall our data suggests that ALDH2 enzymatic activity is able to influence nitrite-mediated effects in the heart and vasculature. This includes (1) vasorelaxation, and thus CFR, at the level of the cardiac microvasculature and (2) cardioprotection of cardiomyocytes. However, due to issues with the ALDH2 breeding colony, further experiments are required and will need to be undertaken in the laboratory to increase n numbers and to confirm these results in future studies.

4.4. Limitations of the Study

As with any experimental animal model, there is the unpreventable limitation of heterogeneity. Although the application of strict inclusion criteria stipulated by Sutherland and colleagues to minimise heterogeneity between mice, differences are still likely to be present [Sutherland et al., 2003]. Heterogeneity may also be abated by ensuring n numbers lie above the prerequisite threshold of n=10. The majority of data sets in the present study satisfy this figure, however ALDH2 experiments are currently being continued in the laboratory in order to meet the appropriate power calculations for the study.

In the present study, we utilised a Langendorff model which subjects the isolated heart to global ischaemia. This is not clinically relevant as AMI subjects the heart to a regional ischaemia. Therefore, further studies are warranted to confirm our findings in a clinically relevant model such as the *in vivo* LAD mouse model of IRI [Lygate, 2006]. For instance, Doganci and colleagues have demonstrated in a porcine model of regional ischaemia that an intracoronary infusion of sodium nitrite is able to confer cardioprotection against myocardial IRI [Doganci et al., 2012]. Other larger animal studies have also demonstrated similarly positive results [Tripathi et al., 1997] [Gonzalez et al., 2008].

A further limitation of our Langendorff model is the use of a heart from a healthy animal, which is then subjected to IRI. As such, this cannot be seamlessly translated to human studies in a clinical setting as it does not account for a plethora of co-morbidities, such as age, smoking, hypertension and diabetes mellitus, prevalent in individuals at risk of myocardial infarction [Siddiqi et al., 2014] [Ferdinandy et al., 2014]. It is plausible that these risk factors could impact nitrite signalling in the preconditioning paradigm. However, a recent study has successfully translated nitrite-mediated protection to humans with improvements in

parameters such as post-operative cardiac events and myocardial salvage index [Jones et al., 2015].

Finally, the full effects of knocking out the ALDH2 enzyme have not been completely established. A plethora of nitrite reductases have been implicated alone or in combination in the bioactivation of nitrite [Arif et al., 2015]. It is conceivable that the loss of function of a particular enzyme could in fact result in alterations in the activity of other enzymes such as upregulation/increased activity.

4.5. Further Research

As previously mentioned, work is ongoing in the lab to increase n numbers for specific data sets as required to ensure all data satisfies the predetermined sample size of n=10 per treatment group, therefore providing the appropriate power calculations for the study.

To further dissect the mechanisms underlying nitrite-mediated cardioprotection *via* PKG1 α oxidation, it would be beneficial to abolish the effects of the classical NO activation pathway (NO-sGC-cGMP-PKG). An NO scavenger such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (CPTIO) or sGC inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one) could be infused as a co-treatment with nitrite in the current Langendorff protocol [Webb et al., 2004] [Moro et al., 1996].

Regarding the ALDH2 study, our group has recently demonstrated that nitrite-mediated dilatation in rat aorta and human (heart failure) resistance vessels was diminished in the presence of an ALDH2 inhibitor (cyanamide) [Arif et al., 2015]. In addition, our current data implicates that ALDH2 plays a role in nitrite-mediated coronary vasorelaxation following IRI.

Therefore, to elucidate the coronary microvascular implications of ALDH2 activity, the assessment of both isolated conduit and resistance vessels via myography could be conducted in the absence and/or presence of nitrite during normoxic or hypoxic conditions. Interestingly, our group have recently conducted studies in isolated vessels from PKG WT and KI mice and have shown that nitrite predominantly mediates vasorelaxation via the PKG1 α oxidation pathway in the resistance vessels when compared to the conduit vessels (unpublished data). In contrast, in the conduit vessels, nitrite mediates via the classical NO pathway. Therefore, to determine whether nitrite has specific effects on different vascular beds *via* the ALDH2 pathway, and if it mediates *via* nitrite-dependent or -independent mechanisms, further studies could be carried out using ALDH2 deficient mice with various NO/sGC scavengers/inhibitors, respectively.

In the present study, LVdP/dt min and LVdp/dt max values were calculated from measurements of left ventricular pressure (LVP) and utilised as indicators of cardiac contraction and relaxation, respectively [Angelone et al., 2012]. However, it is possible to utilise data collected during Langendorff protocols to complete alternative measurements of cardiac work and contraction and relaxation of the left ventricle such as those carried out by Pellegrino and colleagues: rate pressure product (RPP; HR x LVP), an index of cardiac work; maximal value of the first derivative of LVP, representative of maximum left ventricular contraction; and time to peak tension of isometric twitch, used as a measure of inotropism [Pellegrino et al., 2009]. Such values will allow further assessment of heart performance and the extent to which this performance is protected by preconditioning treatment with nitrite. Furthermore, indicators of cardiac contraction and relaxation have only been analysed during the stabilisation phase of the Langendorff protocol. To gain a clearer perspective of the effect nitrite has on these parameters in the pathological setting of myocardial IRI, additional measurements could be taken at the end of reperfusion and compared to a pre-ischaemic

value to generate a percentage recovery value; a method employed by Webb and colleagues when investigating the protective effects of nitrite in a similar model [Webb et al., 2004].

Many clinical trials, focusing on the translation of novel cardioprotective agents observed in animal models to patients undergoing heart surgery, utilise a variety of clinical end-points. Creatine kinase (an indicator of cardiac injury) is often used in the clinical arena and has shown good correlation with infarct size [Busmann et al., 1981] [Lang et al., 2013] [Singh et al., 2016]. Measurements of this biomarker is often from serum samples taken from experimental studies during a LAD ligation model of IRI [Doganci et al., 2012] [Lang et al., 2013] [Wang et al., 2015] or from patients in the clinical setting [Piot et al., 2008] [Thielmann et al., 2013]. However, analysis can be completed using cardiac effluent samples [Asayama et al., 1992]. Commonly, in addition to or as an alternative to changes in CK levels, troponin I release is monitored as an indicator of infarction size and cardiac injury in the clinical setting [Piot et al., 2008] [Jones et al., 2015]. To provide additional measurements of infarct size in the current study, which may then produce results more easily comparable to the results of clinical trials, coronary effluent samples collected during the Langendorff protocol could be assayed for these biomarkers. [Zunping et al., 2015] Furthermore, in several studies focusing on the impact of ALDH2 activity on cardioprotection, lactate dehydrogenase (LDH) is another alternative biomarker of tissue damage [Lang et al., 2013] [Li and Lang, 2015]. Therefore, this could be considered in the present study as an analytical tool for the effluents taken from the isolated Langendorff experiments from the ALDH2 WT and KO hearts.

Finally, the present study was designed to determine the involvement of the PKG1 α oxidation pathway and/or the enzyme ALDH2 in eliciting the protective effects of nitrite in a murine Langendorff model of myocardial IRI. To elucidate additional mechanisms that may be

involved in nitrite-mediated cardioprotection, PKG1 α KI and ALDH2 KO mice could undergo cross-breeding to produce mice devoid of both pathways. Further experiments using the same Langendorff protocol could then confirm the protective effects of nitrite that occur independently of PKG1 α oxidation and ALDH2 enzymes, with pharmacological inhibitors employed to dissect potential novel mechanisms.

4.6. Conclusion

Nitrite has been shown in numerous animal studies, healthy subjects and patients with STEMI to be a promising preconditioning agent [Ashmore et al., 2014] [Raaf et al., 2009] [Ingram et al., 2013] [Jones et al., 2015]. Our results in C57/BL6 mice in the present study corroborate with these findings and support the use of nitrite in the clinical arena. We may infer that the cardioprotective nature of nitrite can be exploited only when an ischaemic incident can be premeditated, during surgical procedures such as CABG (coronary artery bypass graft) surgery or PPCI. However, it has also been demonstrated in a mouse model that a pre-treatment regime using dietary sources of nitrite over a relatively short time scale (7 days) is able to reduce infarct size following myocardial IRI. This finding supports the notion of a preventative use for nitrite in individuals deemed to be 'at-risk' of myocardial infarction [Bryan et al., 2007] [Kapil et al., 2010].

Mechanistically, we provide evidence for a potential dual mechanism by which nitrite elicits changes in the cardiovascular system through PKG. Analysis of haemodynamic parameters throughout a well-established Langendorff model of IRI showed that nitrite-mediated improvements in LVDP and CFR post-IRI occurred via the PKG1 α oxidation pathway. In contrast, nitrite-mediated cardioprotection, as demonstrated through a decrease in infarct size, is not dependent upon this pathway. Considering evidence from previous studies, we

propose that nitrite may elicit its cardioprotective effects through the classical pathway of PKG activation i.e. bioconversion of nitrite to the NO-cGMP-PKG pathway during the hypoxic conditions of IRI. This does not entirely corroborate with our original hypothesis, in which we expected nitrite-mediated cardioprotection to be afforded via the PKG1 α oxidation pathway alone.

In addition, we considered the involvement of ALDH2 enzyme in nitrite-mediated cardiovascular effects using the same model of IRI. We observed significant increases in CFR during the stabilisation period and post-IRI in WT mice with functional ALDH2 enzyme, thus suggesting ALDH2 is integral in nitrite-mediated changes at the level of the cardiac microvasculature. Furthermore, there was a trend of a decrease in infarct size in nitrite treated WT mice, which was not observed in KO littermates. This data suggests that ALDH2 may play an important role in nitrite-mediated cardioprotection and would support our original hypothesis. However, it was not statistically significant and further experiments are warranted to confirm our results.

Overall, the present study offers novel evidence to support the involvement of the PKG1 α oxidation pathway (potentially as part of a dual mechanism) and ALDH2 enzyme in nitrite-mediated changes at the level of the cardiac microvasculature and the cardiomyocytes. However, additional experiments are warranted in order to confirm our results and further dissect the mechanism(s) underlying nitrite-mediated cardioprotection following myocardial IRI.

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