MECHANISMS OF ISCHAEMIC PROTECTION IN HUMANS

Thesis presented for the degree of Doctor of Philosophy

in the Faculty of Medicine, University College London

Dr Michael Okorie

Acknowledgements

I am very grateful to Professor Raymond MacAllister for his supervision, support and for giving me the opportunity to work with him. A special thanks to Professor Patrick Vallance for believing in me and fully supporting my British Heart Foundation (BHF) PhD fellowship application.

I am indebted to the BHF for the funding, and to all the volunteers who participated in the study. This thesis would not have been possible without their assistance. I also wish to acknowledge the support of Dr Stavros Loukogeorgakis who, from the outset, encouraged me with his drive and enthusiasm. I am also grateful to Professor John Deanfield for his assistance.

Dr Adrian Hobbs of UCL performed chemiluminescence for nitrate and nitrites and Dr Roy Sherwood and Ms Tracey Drew of KCH supervised my ELISA for beta endorphins. I am sincerely grateful for their contribution.

An extra special thanks to my wife (Pepe) and children (Michelle and Muna) who have stood by me and remained a loving family throughout.

Finally, I give thanks to God for guiding me, especially through some very trying times.

Abstract

Reperfusion limits ischaemic tissue damage. Paradoxically, reperfusion can cause additional tissue injury and contribute to a composite phenomenon known as ischaemia reperfusion (IR) injury. Therapeutic interventions aimed at reducing IR injury have the potential to improve outcomes in the management of ischaemic conditions. Protective procedures such as ischaemic preconditioning (IPC), ischaemic postconditioning (PostC), remote preconditioning (RIPC) and remote postconditioning (RPostC) have all been shown in animals and humans to be effective in reducing IR injury. Experiments in this thesis sought to determine tractable aspects of the mechanisms underlying these protective phenomena with a view to validating potential pharmacological targets in humans. IR induced endothelial dysfunction in the forearm of healthy volunteers was characterised by vascular ultrasound and venous occlusion plethysmography.

IPC, PostC, RIPC and RPostC all protected against IR-induced endothelial dysfunction. Oral inorganic nitrates in the form of beetroot juice or potassium nitrate (KNO_3) also protected against endothelial IR injury. The magnitude of protection from IR injury was similar.

The mechanism of PostC was investigated in detail. Protection by PostC was blocked by glibenclamide, a non selective K_{ATP} channel blocker, suggesting that activation of these potassium channels was necessary for PostC-induced ischaemic protection. Selectivity of K_{ATP} channels was evident because glimepiride (a selective K_{ATP} channel blocker) did not affect the protective effect of PostC. A role for the mitochondrial permeability transition pore (mPTP) was suggested by the effect of ciclosporin (blocker of the mPTP) to mimic PostC-induced protection. These aspects

of the mechanism of PostC resemble previously identified mechanisms of IPC and RIPC in the human forearm.

Studies were undertaken to explore the mechanism whereby protection spreads systemically. Systemic protection by RIPC from ischaemic injury to the endothelium was blocked by the opioid receptor antagonist, naloxone without any effect on protection conferred by IPC or RPostC These data implicate the opioid receptor pathway in the facilitation of RIPC, and is likely to involve a haematogenous mechanism. Conversely, the alpha adrenergic receptor antagonist phentolamine, blocked systemic protection from RPostC but had no effect on RIPC. This highlights a role of a component of the autonomic nervous system in the mediation of RPostC.

Ischaemic protection in humans is mechanistically a complex process but results in this thesis contribute to the validation of pharmacological targets as a prelude to drug development.

Table of contents

List of Tables

List of figures

Abbreviations

ACh	acetylcholine
ANOVA	analysis of variance
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AUC	area under curve
BP	blood pressure
CV	coefficient of variation
cAMP	cyclic AMP
cGMP	cyclic GMP
EF	endothelial function
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FMD	flow mediated dilatation
ICAM	Inter-Cellular Adhesion Molecule
IPC	ischaemic preconditioning
IR	ischaemia- reperfusion
K_{ATP} channel	ATP dependent potassium channel
KCH	Kings College Hospital
MAPK	mitogen-activated protein kinase
mPTP	mitochondrial permeability transition pore
NO	nitric oxide

PostC	postconditioning
PI3K	phosphatidylinositol 3-kinase
PKG	protein kinase G
PKC	protein kinase C
PTEN	phosphatase and tensin homolog deleted on chromosome 10
RIPC	remote ischaemic preconditioning
RISK	reperfusion injury salvage kinases
ROS	reactive oxygen species
ROCK	rho-dependent protein kinases
RPostC	remote ischaemic postconditioning
SUR	sulfonylurea receptor
UCL	University College London
VCAM	vascular cell adhesion molecule

Publications arising from work in this thesis

1. Okorie MI, Bhavsar DD, Ridout D, Charakida M, Deanfield JE, Loukogeorgakis SP, MacAllisterRJ. Postconditioning protects against human endothelial ischaemia-reperfusion injury via subtype-specific KATP channel activation and is mimicked by inhibition of the mitochondrial permeability transition pore. Eur Heart J. 2011; 32:1266-74

2. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, Macallister R, Hobbs AJ, Webb AJ, Ahluwalia A. Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. Hypertension. 2010; 56:274-8

3. Webb A.J, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs A, Ahluwalia A Acute Blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension. 2008; 51:784-90

Published abstracts

1. Michael I Okorie, Miriam Berger, Farihah Akram, Rupert Williams, John E Deanfield, Stavros P Loukogeorgakis, and Raymond J MacAllister Role of Endogenous Opioids in Remote Ischemic Preconditioning in Humans. Circulation. 2009; 120**:** S1464.

2. M.Okorie, F. Akram, S. Loukogeorgakis, A. Webb, J. Deanfield, N. Benjamin, A. Hobbs, R. MacAllister, A. Ahluwalia. Oral inorganic nitrate protects against endothelial ischaemia-reperfusion injury in humans. Basic and Clinical Pharmacology and Toxicology. 2009; 105: 26.

3. Kapil, V and Okorie, M and Akram, F and Rehman, F and Milsom, A and Arghandawi, S and Pearl, V and Deanfield, J and Benjamin, N and Loukogeorgakis, S and Hobbs, A and Macallister, R and Webb, A and Ahluwalia, A. Oral inorganic nitrate lowers blood pressure and protects against endothelial ischaemia-reperfusion injury in humans. Nitric Oxide-Biology and Chemistry. 2009**;** 20: S37 - S37.

4. Rupert P Williams, Michael I Okorie, Harminder Gill, John E Deanfield, Raymond J MacAllister, Stavros P Loukogeorgakis. Role of the Autonomic Nervous System in Remote Postconditioning in Humans. Circulation. 2008;118: S1473

5. M.I.Okorie, R.P. Williams, D.D. Bhavsar, J.E. Deanfield, S.P. Loukogeorgakis, R.J. Macallister. Inhibition of the mitochondrial permeability transition pore (mPTP) mimics postconditioning and protects against endothelial ischaemia reperfusion injury in humans European Heart Journal 2008; 29 (Abstract Supplement), 9.

6. Okorie, Michael; Loukogeorgakis, Stavros; MacAllister, Raymond Postconditioning protects against endothelial ischaemia-reperfusion injury in humans (Abstract) British Journal of Clinical Pharmacology 2008; 65(6):976-977.

Chapter 1

Introduction

1.1 Introduction

Cardiovascular disease remains the major cause of death globally amounting to 17 million deaths a year (1). Ischaemia accounts for the majority of these deaths and results from arterial occlusion mainly due to athero-thrombosis. Most commonly this presents clinically as coronary heart disease (CHD) and stroke which are projected to remain the leading causes of death worldwide over the next two decades (1). Timely reperfusion improves outcomes of CHD and stroke but paradoxically this causes an injury to tissues. The resulting injury is a composite of ischaemia and reperfusion – *ischaemia-reperfusion* (IR) injury. Reducing reperfusion injury might further improve the clinical effectiveness of existing reperfusion strategies.

This thesis aims to use an *in vivo* model of IR injury in the human forearm to investigate the mechanisms of protective strategies against IR injury. These are *postconditioning (PostC), remote preconditioning (RIPC), remote postconditioning (RPostC)* and *inorganic nitrate*.

1.2 Athero-thrombosis

1.2.1 Definition and relevance to ischaemia

Atherosclerosis is the most frequent underlying cause of human arterial thrombosis. It has been defined as “a multifocal, smouldering, immunoinflammatory disease of medium–sized and larger arteries fuelled by lipid” (2). This inflammatory process can ultimately lead to the development of complex lesions, or plaques, that protrude into

the arterial lumen and cause vascular obstruction and occlusion (3). This is manifested clinically as acute or chronic ischaemic syndromes involving the heart, brain, leg and other tissues. The main risk factors for the development of atherosclerosis are elevated plasma cholesterol, age, hypertension, diabetes, smoking and male gender (2).

Pre-clinical atherosclerosis begins in early childhood and progresses during adolescence and adulthood (4, 5). In susceptible individuals under the influence of risk factors, clinical disease processes caused by atherogenesis become evident later in life.

1.2.2 Pathology of atherosclerosis

Arterial wall morphological changes, including thickening and reorganisation of the tunica intima, excess synthesis of collagenous matrix (fibroblastic intimal thickening) and permanent or dynamic deposition of lipids (fatty streaks) already occur in childhood or adolescence (6). A fatty streak is an accumulation of subendothelial lipid laden cells that are prevalent in young people and never cause symptoms (7, 8). In the presence of risk factors, endothelial dysfunction occurs and this is thought to be the promoter of the atherothrombotic disease process.

Endothelial dysfunction and leakage of the endothelial barrier increases the expression of two classes of adhesion molecules, the selectins and the immunoglobulin gene superfamily [VCAM-1 and ICAM-1] (6, 9). This leads to an accumulation of monocytes, T-lymphocytes and lipids in the subendothelial space where potential atherogenic lipoproteins are retained and modified to become

cytotoxic, proinflammatory, chemotaxic and proatherogenic (2, 6). The imbalance in lipoprotein influx and efflux, intraplaque haemorrhage and the development of the extracellular matrix promotes progression of early atherosclerotic lesions which are known as plaques (9). An advanced coronary plaque is composed of a necrotic lipid-rich core and hypocellular fibrous cap (2). Rupture of the cap exposes the prothrombotic core to circulating blood and leads to vascular occlusion. Atherogenesis may have a genetic basis, making some individuals more susceptible to the effects of hyperlipidaemia (10). It has been proposed that microalbuminuria reflects a state of generalised transendothelial leakiness for plasma proteins, an important event in atherogenesis (10). Risk factor modification has an important influence on the progression of atherosclerotic disease, particularly blood pressure and cholesterol reduction.

1.3 Ischaemia

Ischaemia is the result of vascular occlusion and leads to deprivation of oxygen and nutrients in a tissue or organ. Research has focused mainly on myocardial ischaemia in view of its substantial impact on morbidity and mortality. The pathophysiologic mechanisms of ischaemia in the heart, however, also apply to other tissues and organs.

Acute ischaemia is characterised by (a) cessation of aerobic metabolism, (b) depletion of creatine phosphate (high-energy phosphate), (c) onset of anaerobic glycolysis and (d) accumulation of glycolytic products such as lactate and catabolites of the nucleotide pools in tissues (11). These processes, which commence within

seconds of ischaemia, are consequent upon hypoxia and subsequent loss of aerobic adenosine triphosphate (ATP) production and if prolonged may lead to irreversible cell damage. The shift to an anaerobic source of ATP is compensatory but still only comprises about one fourth of the myocardial aerobic glycolytic rate which eventually ceases after about 60 minutes of ischaemia (11).

1.3.1 ATP metabolism during ischaemia

The high-energy bond of ATP is the main source of energy for myocardial function and ATP concentration has been identified as an important correlate of myocardial function following ischaemia (12, 13). Reduction in oxidative phosphorylation through the citric acid cycle causes myocardial ATP levels to decrease progressively during ischaemia, resulting in decreasing levels of intracellular creatine phosphate and increasing levels of intracellular phosphate (demand for high energy phosphate exceeds supply) [Figure 1.1]. As the ATP is metabolised, adenosine diphosphate (ADP) begins to accumulate. ADP is in turn converted to ATP and adenosine monophosphate (AMP) by the action of adenylate kinase (11). The ATP formed from ADP by adenylate kinase is re-used as a source of energy while the AMP is converted to adenosine and inorganic phosphate (Pi) by 5' nucleotidase (5'ND). The adenosine is deaminated to inosine via adenosine deaminase (14). Adenosine and inosine are nucleosides and in contrast to nucleotides, can diffuse from the myocyte to the extracellular space where the inosine is degraded to hypoxanthine and xanthine by the action of nucleoside phosphorylase and xanthine oxidase respectively (11). As the duration of ischaemia increases, further breakdown to

diffusable metabolites occurs resulting in decreasing levels of ADP and AMP which invariably affect supplementary ATP production (12). In addition, further oxidation of xanthine by xanthine oxidase is a source of generation of reactive oxygen species (ROS) (15, 16). Both processes may lead to cell death.

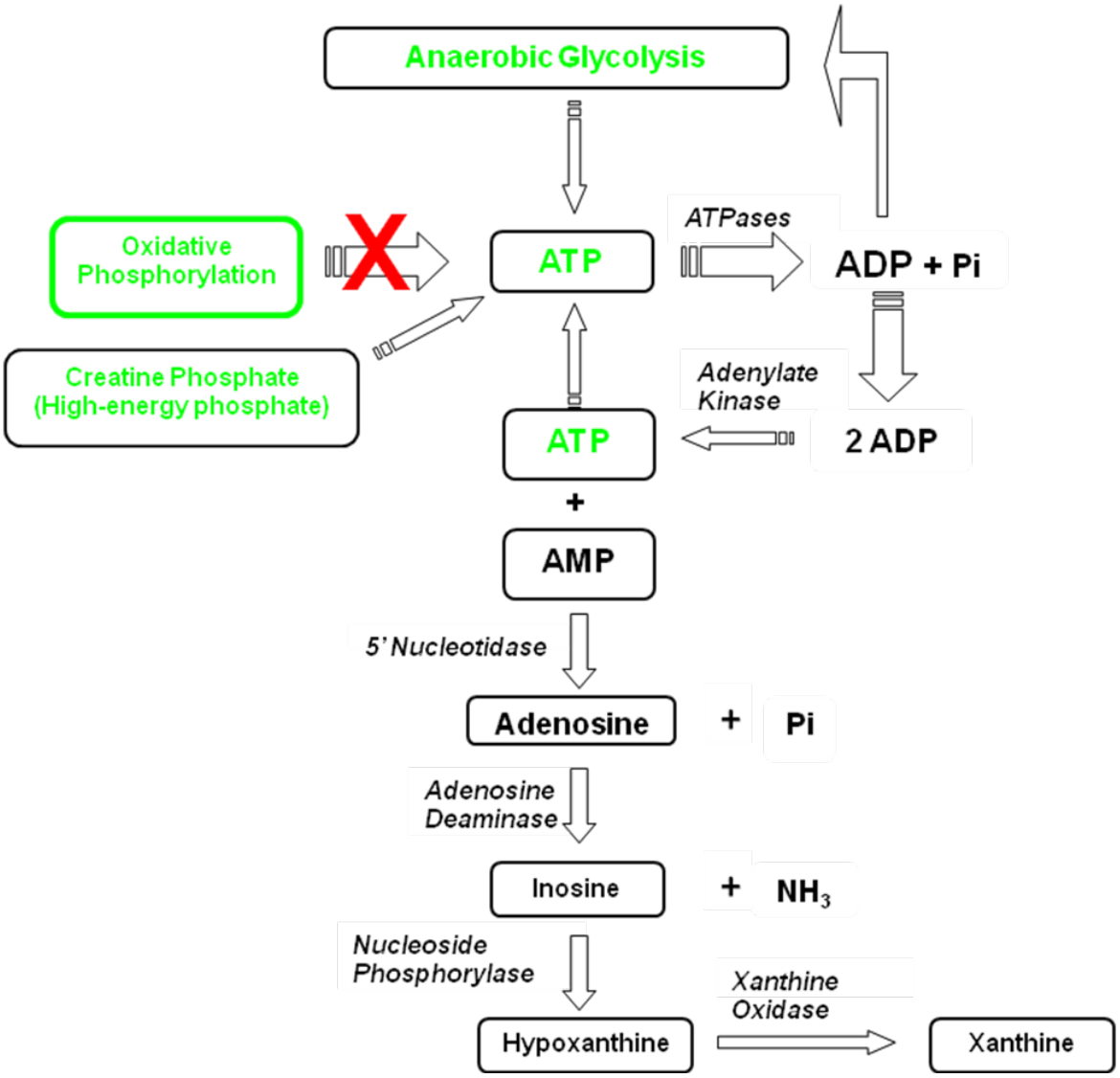


Figure 1.1: ***Changes in ATP metabolism during ischaemia.*** *Tissue hypoperfusion causes a reduction in oxidative phosphorylation which is the main cellular source of ATP (aerobic pathway). The cells resort to other sources of ATP (anaerobic pathway) which provide amounts of ATP insufficient for normal cellular function. If this process is prolonged irreversible cell damage will occur. (Adapted from Jennings RB, 1982)*

1.3.2 Ionic homeostasis during ischaemia

Energy supply in the ischaemic myocardium is determined by the rate of ATP generation and the declining tissue ATP concentration (rate of high energy phosphate utilisation, or demand) (11). With prolonged ischaemia, anaerobic metabolism leads to the abnormal accumulation of metabolites and lactic acid, a decrease in intracellular pH and K^+ and an increase in intracellular Na^+ and Ca^{2+}. These changes are deleterious to myocardial function (15, 17).

The reduction in intracellular pH activates the Na^+/H^+ exchanger in an attempt to restore the pH_i. Together with the reduction of ATP production and inhibition of the Na^+/K^+ -ATPase there is an increase in the intracellular Na^+ , Cl^- and water, which leads to cell swelling (15, 17). Inhibition of the Na^+/Ca^{2+} exchanger and both sarcolemmal and plasmalemmal Ca^{2+} ATPases, which usually pump Ca^{2+} out of the cell, increases intracellular Ca^{2+} (18). This may result in activation of degradation enzymes such as phospholipases, proteases and nucleases that can lead to irreversible cell damage characterised by disruption of the plasma membrane (15, 17) [Figure 1.2].

1.3.3 Mitochondrial function during ischaemia

Mitochondrial integrity is important in cell survival. An impermeable inner mitochondrial membrane is essential to maintain the membrane potential and pH gradient that enables ATP synthesis through oxidative phosphorylation. If the permeability barrier of the inner membrane is disrupted, mitochondria become uncoupled, and thus, can neither synthesise ATP by oxidative phosphorylation nor

separate cytosolic and mitochondrial pools of metabolites (15). These mitochondrial changes, which occur during ischaemia, have been attributed to an increase in intracellular phosphate and disruption in cell Ca^{2+} homeostasis and may ultimately lead to cell death (15, 19).

1.3.4 Vascular injury during ischaemia

Endothelial cells maintain vascular homeostasis and are vulnerable to ischaemic damage. Prolonged hypoxia reduces endothelial cell production of certain bioactive agents (prostacyclin, nitric oxide) and stimulates the production of other agents (endothelin, thromboxane A2) (20). However, overt microvascular damage in the myocardium does not appear until after 60 minutes or more of severe *in vivo* ischaemia, at which time endothelial disruption is believed to contribute to microvascular obstruction. (11).

1.3.5 Irreversible ischaemic injury

The hallmarks of early phase irreversible injury include: a) ATP<10% of control; b) high concentrations of H^+, AMP, inosine, and hypoxanthine; c) cessation of anaerobic glycolysis; d) high lactate and low glycogen; e) mitochondrial swelling with amorphous matrix densities and f) focally disrupted sarcolemma which seems to be the final event (11). Reperfusion prevents progression from reversible to irreversible injury by restoration of oxidative phosphorylation and washout of harmful metabolites of glycolysis such as lactate.

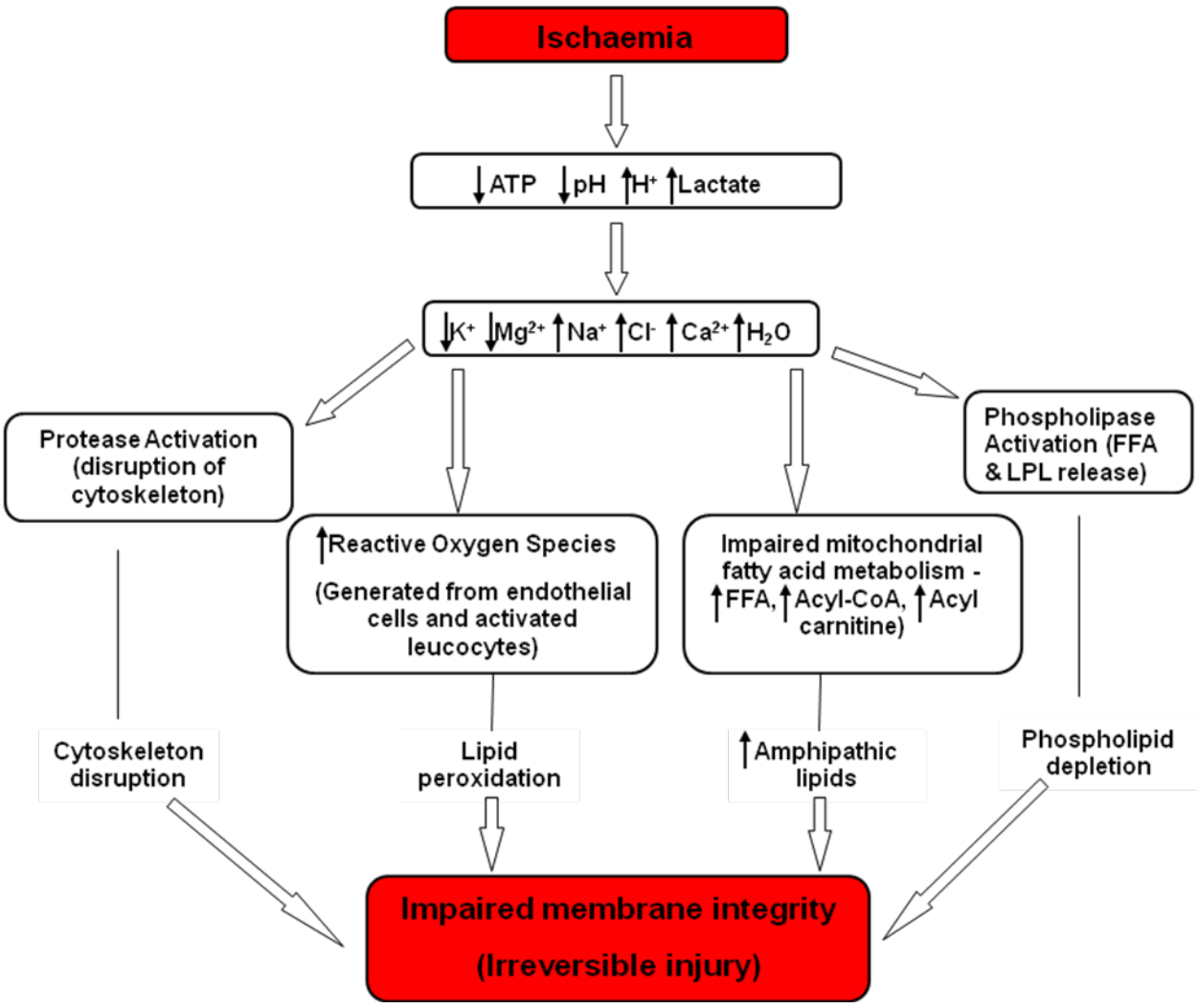


Figure 1.2: ***Cellular ionic homeostasis during ischaemia****. Tissue hypoperfusion results in untilisation of anaerobic sources of ATP production. The formation of acidic cellular metabolites leads to a disruption of ionic homeostasis which is deleterious to cellular membrane integrity. This culminates in cellular injury and death. (Adapted from Buja LM, 2005)*

1.4 Reperfusion injury

Reperfusion is essential for tissue salvage but paradoxically has deleterious effects on tissues. Reperfusion injury does not occur independently of ischaemia and this composite is often referred to as ischaemia-reperfusion (IR) injury.

A period of prolonged ischaemia causes the cells to resort to the glycolytic source of ATP production. This pathway does not provide sufficient amounts of ATP. Furthermore, the anaerobic state leads to the accumulation of lactic acid, a decrease of the intracellular pH, and an increase in intracellular Na^+ concentration. Further depletion of ATP leads to dysfunction of the Na^+/K^+ -ATPase which causes a reversal of the Na^+/Ca^{2+} antiporter and intracellular Ca^{2+} overload. The details of this process have been discussed above. With reperfusion the resupply of oxygen causes an abundance of oxygen free radicals which in combination with intracellular Ca^{2+} overload have a deleterious effect on cellular function. The duration of ischaemia correlates with the amount of tissue damage and without reperfusion there will be no tissue salvage. However, timely reperfusion of salvageable tissue may itself account for up to 50% of the final myocardial infarct size in experimental studies (21).

1.4.1 Mechanism of reperfusion injury

Recent research has demonstrated a key role for mitochondria as an end effector in the mechanism of IR injury. Myocardial reperfusion leads to a number changes which include: the generation of reactive oxygen species (ROS), intracellular calcium overload, the rapid restoration of physiologic pH, and inflammation (21). These

changes lead to disruption of mitochondrial membranes (permeabilisation) which plays a crucial role in cell death (15, 22 - 24).

Cell death may occur via a variety of pathways. Apoptosis is a programmed cell death and results from permeabilisation of the outer mitochondrial membrane (OMM) which leads to release of cytochrome c and other pro-apoptotic factors (25, 26). The details of this process remain unclear but it is thought to be a cause of naturally occurring cell death in response to developmental, homeostatic or internal damage signals. Autophagy occurs in cellular nutrient deprivation and entails recruitment of proteins such as $p19^{ARF}$ to the mitochondria predisposing them to engulfment by autophagic vacuoles and transfer to lysosomes (26). The process of necrosis involves opening of the non-specific pore in the inner mitochondrial membrane (IMM), known as the mitochondrial permeability transition pore (mPTP) (25, 26) [Figure 1.3]. Necrosis results from deleterious cellular conditions such as the presence of toxins or during reperfusion injury.

1.4.2 The mitochondrial permeability transition pore

The mPTP is a non-specific pore in the inner mitochondrial membrane that normally remains closed but under conditions of cellular stress can open and lead to cell death. There are two major consequences of opening of the pore (24): a) There is free passage of molecules of <1.5kDa across the inner mitochondrial membrane, but not proteins. This creates a colloidal osmotic pressure that causes the mitochondria to swell and eventually there is rupture of the outer membrane leading to release of proteins such as cytochrome c into the intermembrane space. The result is cell

death. b) The inner membrane becomes freely permeable to protons and this uncouples oxidative phosphorylation. The resulting depletion of intracellular ATP levels leads to disruption of ionic and metabolic homeostasis and activation of degradation enzymes. If pore closure does not occur then these changes will lead to irreversible cell damage (22-24).

The exact molecular structure of the mPTP is not yet known, but it is thought to comprise two candidate proteins - adenine nucleotide translocase (ANT) and the mitochondrial phosphate carrier (PiC) which occur in abundance in the IMM and are susceptible to damage by oxidant stress (26). Another mitochondrial protein known as cyclophilin D (Cyp-D) possesses peptidyl-propyl *cis-trans* isomerase (PPIase) activity and binds to PiC under the influence of increasing Ca^{2+} concentration and oxidative stress. This leads to a conformational change in either PiC or ANT that promotes pore formation/opening (24, 26) [Figure 1.4]. Evidence of a role for Cyp-D in pore formation is supported by studies in which pharmacological inhibition or genetic ablation of Cyp-D conferred resistance to acute IR injury (24, 27-31). By preventing Cyp-D binding to PiC or ANT, ciclosporin acts as a potent inhibitor of the mPTP. Another potent inhibitor of the mPTP is Sangliferin A (SfA), which inhibits PPIase activity (24). Other proteins thought to be implicated in mPTP formation include the voltage dependent anion channel (VDAC), the peripheral benzodiazepine receptor (PBDR), hexokinase, and Bcl-2, but their role is not well defined (26). Experimental evidence indicates that the mPTP is closed during ischaemia and only opens during reperfusion (32). This makes the early phase of reperfusion an attractive therapeutic target.

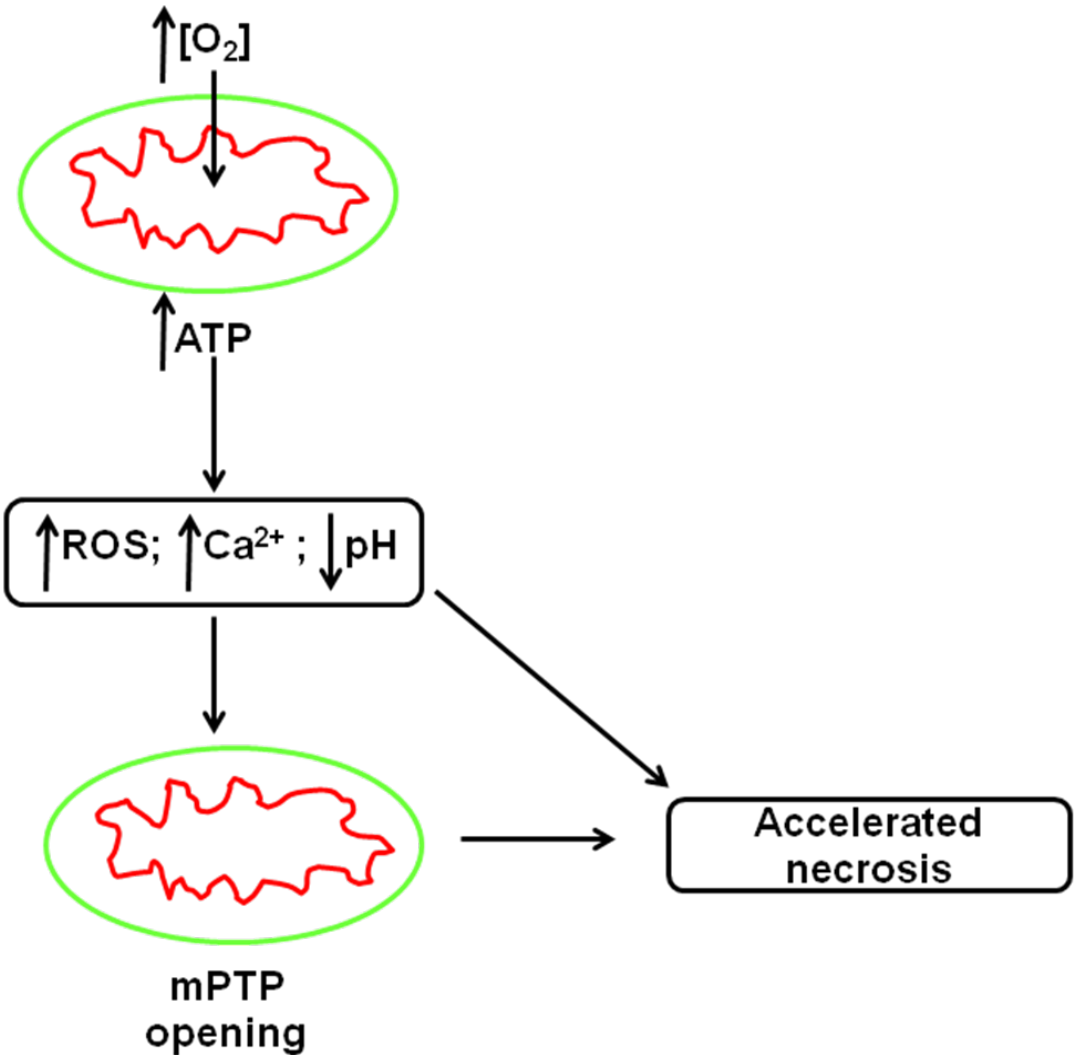


Figure 1.3: mPTP: the final common pathway of reperfusion injury. *With reperfusion, the rapid replacement of oxygen causes mitochondrial re-energisation which leads to generation of ROS, a further increase in intracellular calcium concentration (due to a combination of dysfunction of the sarcoplasmic reticulum calcium uptake mechanism and rapid restoration of the Na^+/Ca^{2+} exchanger activity) and a reduction in pH. These processes lead to opening of the mPTP resulting in cell death.*

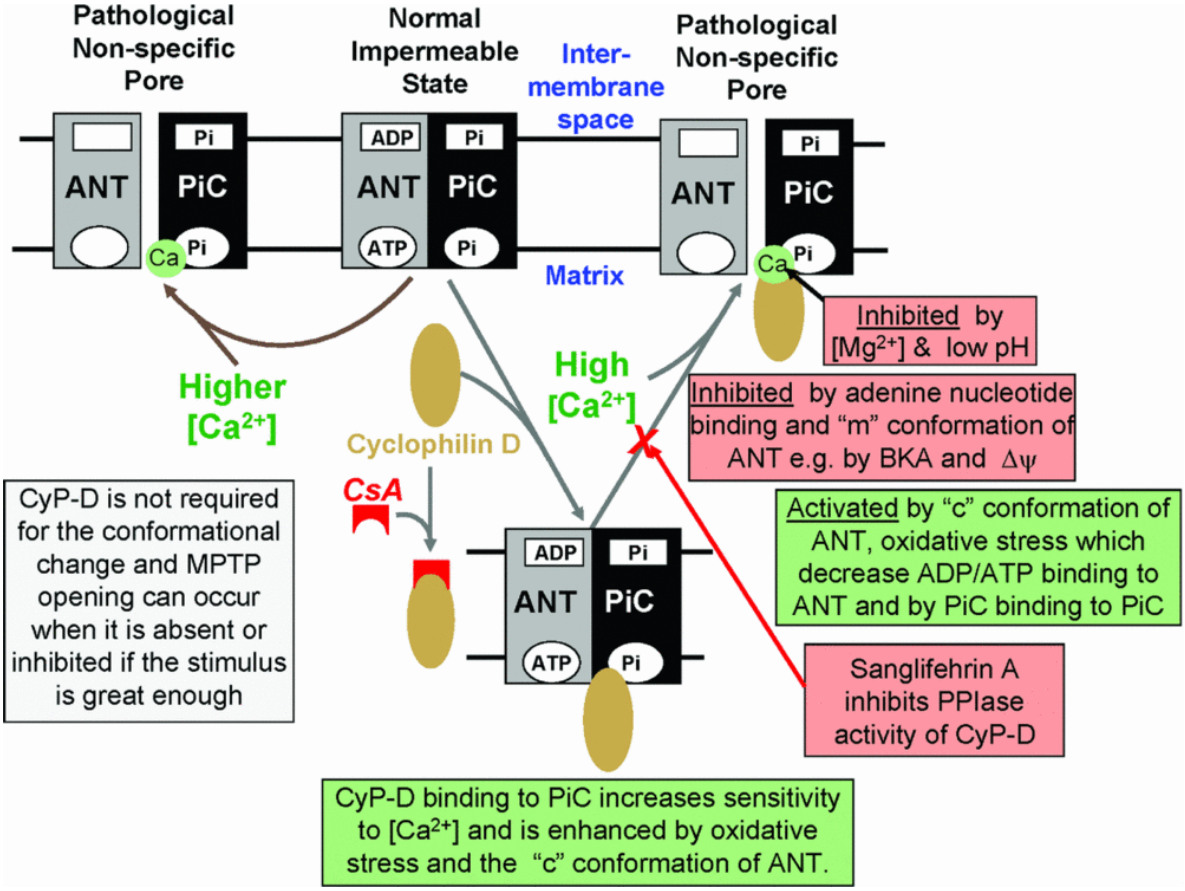


Figure 1.4: ***A proposed working model of the mPTP (26).***

1.4.3 Reactive oxygen species

Free radicals are molecules that contain one or more unpaired electrons and so are chemically reactive. Oxygen free radicals are formed continuously in minute quantities during normal metabolism of mammalian cells and these are inactivated by free radical scavenging systems such as superoxide dismutase, catalase and glutathione peroxidase (33-35). During IR injury increased production overwhelms these protective mechanisms, and increased concentration can be detected by using chemiluminescence, fluorescent detection and electron paramagnetic resonance

spectroscopy (36-38). In addition, the reduction of IR injury by ROS scavengers in experimental models implicates their role in IR injury (39-42).

ROS generation in reperfused myocardium occurs within the endothelial cells and myocytes. Enzymatic sources include activation of leucocyte NADPH oxidase, xanthine oxidase, mitochondrial oxidative phosphorylation, cycloxygenase mediated unsaturated fatty acid oxidation, catecholamine oxidation, P450-mediated oxidation, uncoupling of eNOS, and iron release and redox cycling (43, 44). The overall burden of oxidative stress is further exacerbated by chemotaxis of leucocytes resulting from ROS produced by endothelial cells and myocytes.

NADPH oxidase (also called Nox) is a major source of superoxide and is found mainly in phagocytes (neutrophils, eosinophils, monocytes and macrophages) (45). The enzyme is inactive in resting phagocytes but is activated by contact with microbes or inflammatory mediators (45, 46). Structurally, NADPH oxidase consists of the membrane bound catalytic subunit (*Nox 1 – 5*) that transfers electrons from NADPH to molecular oxygen to form superoxide, a smaller membrane-bound protein (p22phox) that stabilizes the Nox subunit within the membrane and cytosolic regulatory subunits – p47phox, p40phox, p67phox and GTPase RAC (45, 46).

IR injury results in neutrophil activation which promotes NADPH oxidase activity and increased ROS production (47). The role of NADPH oxidase in the pathophysiology of IR injury is suggested by upregulation of Nox 2 in ischaemic human cardiomyocytes (48) and protection against IR injury in NADPH oxidase knockout mice in a variety of organs (49-52). These findings are supported by evidence from a recent study, using a human *in vivo* model of IR injury. Patients with chronic

granulomatous disease who have mutations in genes encoding for specific NADPH oxidase with disruption of oxidase activity, exhibit reduced endothelial IR injury (53).

Xanthine oxidoreductase is an another important contributor to the total cellular ROS load and exists in two interconvertible forms – xanthine dehydrogenase (XDH) and xanthine oxidase (XO), both of which catalyse the conversion of hypoxanthine and xanthine (54). XDH utilises mainly NAD^+ to accept electrons yielding NADH and uric acid whereas XO has a greater affinity for oxygen forming superoxide and hydrogen peroxide. The relative availability of these two forms is therefore important in determining the amount of ROS produced by these enzymes.

ROS are therefore recognised to contribute to cell death during IR injury in experimental studies using animal and human models of IR injury. However, reducing ROS activity has proved to be an intractable therapeutic target to date. A number of clinical studies of antioxidants in acute ischaemia have demonstrated a null therapeutic effect (55). The reasons for this might be variability in clinical characteristics, dose and timing of interventions in experimental and clinical studies.

1.4.4 Neutrophil activation

IR injury activates the innate immune mechanisms to induce an inflammatory reaction by increasing the expression of cytokines (TNF, IL-1β, IL-6, IL-8), complement fragments (C5a), ROS, leukotriene B4, thromboxane A2, platelet activation factor, ICAM and P-selectin (56). This promotes chemotactic recruitment of neutrophils and their adhesion to the endothelium. This causes occlusion of the microvasculature and the release of neutrophil-derived mediators of IR injury

(proteolytic enzymes, ROS, CD 11/CD18). Evidence for a pathogenic role for neutrophils during IR injury is mainly from animal studies in which protection against IR injury was achieved with neutrophil depletion or specific blockade of neutrophil adhesion molecules (57-61).

However, the significance of neutrophil activation remains contentious given that IR injury occurs in neutrophil-free systems such as isolated heart preparations (56). In addition, results from clinical studies of anti-neutrophil and anti-inflammatory therapies have not been successful in reducing IR injury (55, 56).

1.5 IR injury and the vasculature

Vascular endothelial cells appear to be particularly susceptible to injury as a result of both ischaemia and reperfusion. This manifests as endothelial dysfunction that affects the arterioles, capillaries and venules (20).

1.5.1 Physiology of endothelium

The endothelium is a single layer of cells that lines the inner surface of blood vessels. They were initially thought to be inert, acting as a barrier between blood and vascular smooth muscle. However, vascular endothelial cells have been demonstrated to possess paracrine functions which regulate a number of vascular processes including vascular tone, cell adhesiveness and coagulation (62). The endothelial effect on blood vessel function is achieved by the local production of vasodilators (NO, prostacyclin and endothelium derived hyperpolarisation factor,

EDHF) and vasoconstrictors (thromboxane and endothelin) (62). During normal vascular function the endothelial release of vasodilators predominates and any changes in this balance may lead to altered vascular tone. The release of vasodilators by endothelial cells can be potentiated by a number of chemical agonists (acetylcholine, substance P, bradykinin) and physical factors such as sheer stress generated by an increased blood flow.

1.5.2 Endothelial mediators

Furchgott and Zawadzki demonstrated that vascular smooth muscle relaxation occurred in response to acetylcholine and this was dependent on an intact endothelial layer (63). They named the mediator responsible for this effect endothelium derived relaxing factor (EDRF) and this was later identified as NO.

NO is a free radical gas derived in the endothelium mainly by the conversion of the precursor amino acid L-arginine in the presence of molecular oxygen and co-factors such as BH4, NADPH and flavin adenine dinucleotide (FAD) (64). This process is catalysed by the constitutively expressed endothelial nitric oxide synthase (eNOS). However other isoforms of NOS may contribute to NO bioavailability and include neuronal (n) NOS and inducible (i) NOS. The nomenclature for the NOS isoforms reflects the tissues of origin for the original protein and DNA isolates and their level of expression might vary under different physiological conditions (64, 65). NO is freely diffusible and its vasodilator properties of NO arise from its effect on vascular smooth muscle cells by activating soluble guanylate cyclase (sGC). This leads to increased production of cyclic 3',5' guanosine monophosphate (cGMP) and a

reduction in intra-cellular calcium within the smooth muscle cell, causing smooth muscle relaxation (66). The biosynthesis of NO is regulated by endogenous NOS inhibitors [asymmetrical dimethylarginine (ADMA) and NG-monomethyl-L-arginine (L-NMMA)]. An alternative pathway of NO generation (endothelium independent), thought to be activated during ischaemia, will be discussed in section 1.10 of chapter 1 of this thesis.

Prostacyclin and thromboxane A2 are endothelium derived molecules synthesized from the precursor compound arachidonic acid (AA) which is released from cell membrane phospholipids (67, 68). Cyclo-oxygenases (constitutive COX-1 and inducible COX-2) act on AA to generate prostaglandin endoperoxides which are the substrate for prostacyclin and thromboxane synthases (67, 68). Prostacyclin causes vasodilatation via activation of adenylate cyclase leading to increased levels of cyclic adenosine monophosphate (cAMP) which causes smooth muscle relaxation. Furthermore, prostacyclin is a potent inhibitor of platelet aggregation. Thromboxane A2, on the other hand, produces vasoconstriction by causing smooth muscle contraction.

EDHF is an endothelial mediator that complements the vasodilator effects of NO and prostacyclin. A number of candidiates have been proposed to be EDHFs, including prostanoids, potassium ions, and C-type natriuretic peptide. Hyperpolarisation of the vascular smooth muscle decreases calcium influx and this leads to relaxation. The vasodilator effect of EDHF increases as the blood vessel size decreases such that their effect is thought to predominate in resistance vessels whereas NO is the principal vasodilator in conduit vessels(69). However, it is postulated that the EDHF-mediated response might become more prominent when NO production is

compromised since mesenteric arteries for eNOS knockout mice showed an upregulation of EDHF (70).

The endothelins are peptides that possess potent vasoconstrictor properties. Endothelial cells in humans produce endothelin-1(ET-1) and increased circulating levels of these have been correlated with the development of atherosclerosis and coronary endothelial dysfunction (71-73). ET-1 acts via two major receptors; endothelin – A (ET_A) which is present on vascular smooth muscle cells and endothelin – B (ET_B) which is located on both vascular smooth muscle and endothelial cells. ET_B receptors which are expressed on the endothelial cells mediate the release of NO and promote pulmonary clearance and endothelial reuptake of ET-1. This has been the justification for investigating the potential role of selective ET_A antagonists in the treatment of coronary artery endothelial dysfunction and hypertension (74-76).

1.5.3 IR induced endothelial dysfunction

Vascular endothelial cells are particularly susceptible to the effects of IR injury. Ku et al demonstrated that coronary artery endothelial dysfunction occurred after 90 minutes of ischaemia and 1-2 hours of reperfusion in the canine heart (77). IR-induced endothelial dysfunction was seen within the first few minutes of reperfusion whereas with ischaemia alone, significant impairment in endothelial response to vasodilators is only detected after 2 hours.

Endothelial cells produce NO (via NOS) and superoxide (from a number of sources including NADPH oxidase). Under normal conditions, the rate of production of NO exceeds that of superoxide production. This allows for NO a) to effectively scavenge

the low intracellular levels of superoxide; b) to modulate arteriolar tone via the guanylate cyclase activation in smooth muscle; c) to inhibit platelet aggregation and thrombus formation and d) to minimise the adhesive interactions between leucocytes and the endothelial cell surface (20). After IR injury, this balance is reversed such that accumulation of superoxide occurs and NO production is impaired. NO synthesis depends on the availability of molecular oxygen, which is reduced during ischaemia. Superoxide production increases as a result of the mechanisms described in section 1.4.3. The relatively low levels of NO react with the abundant supply of superoxide further reducing NO levels. The net effect is a reduction of endothelium-dependent vasodilatation and the production of other reactive oxygen species such as H_2O_2 and HOCl, which further impair endothelial function (20). In addition to impairment of endothelial function, reactive oxygen species promote inflammation and apoptosis which contribute to the process of cellular damage during IR.

1.5.4 The vascular “no-reflow” phenomenon

Another vascular manifestation of IR injury is the “no-reflow” phenomenon. This has been defined as incomplete and non-uniform reperfusion at the microvascular level despite adequate re-opening of the proximal artery after a period of transient ischaemia (78). The importance of this phenomenon lies in the fact that it correlates with infarct size and provides useful prognostic information (79). Reperfusion is thought to cause microthromboemboli and particles of plaque to be showered downstream after plaque rupture, leading to obstruction of small arteries and arterioles (79). The major determinants of the degree of no-reflow are the duration of

occlusion, infarct size and length of reperfusion and no-reflow tends to persist over a period of at least 4 weeks (78).

1.6 Protection against IR injury

Over the last two decades, interventions that are protective against IR injury have emerged. Various models of IR injury (cell cultures, isolated perfused hearts and animal models *in vivo*) have been used to investigate strategies to reduce cellular and tissue damage. Some of these interventions have been translated to clinical studies in patients and could potentially lead to a significant reduction of reperfusion injury in the clinical setting. There are 4 types of intervention that are considered in this thesis; ischaemic preconditioning, ischaemic postconditioning, remote conditioning and administration of oral inorganic nitrate, each of which is discussed in turn below.

1.7 Ischaemic preconditioning

This was first described in 1986 when Murry et al demonstrated in an anaesthetized dog, that 5 minute periods of circumflex artery occlusion alternating with 5 minute periods of reperfusion prior to a 40 minute total occlusion of the same artery, reduced myocardial infarct size (80) [Figure 1.5]. The protective effects of preconditioning (IPC) have since been reproduced in animal and human models. The IPC stimulus is applied prior to the onset of index ischaemia and causes two phases of protection; a “classic” or “early” or “first window of protection (FWOP)”

phase and the “delayed” or “late” or “second window of protection (SWOP)” phase. Classic IPC, as described by Murry and colleagues, in which protection by IPC was lost when the interval between the IPC stimulus and the infarct protocol was extended beyond 60 minutes. Delayed IPC was based on a discovery that even though initial protection by IPC was lost after 60 minutes, protection re-emerged at 24 hours, lasting for up to 72 hours (81-83).

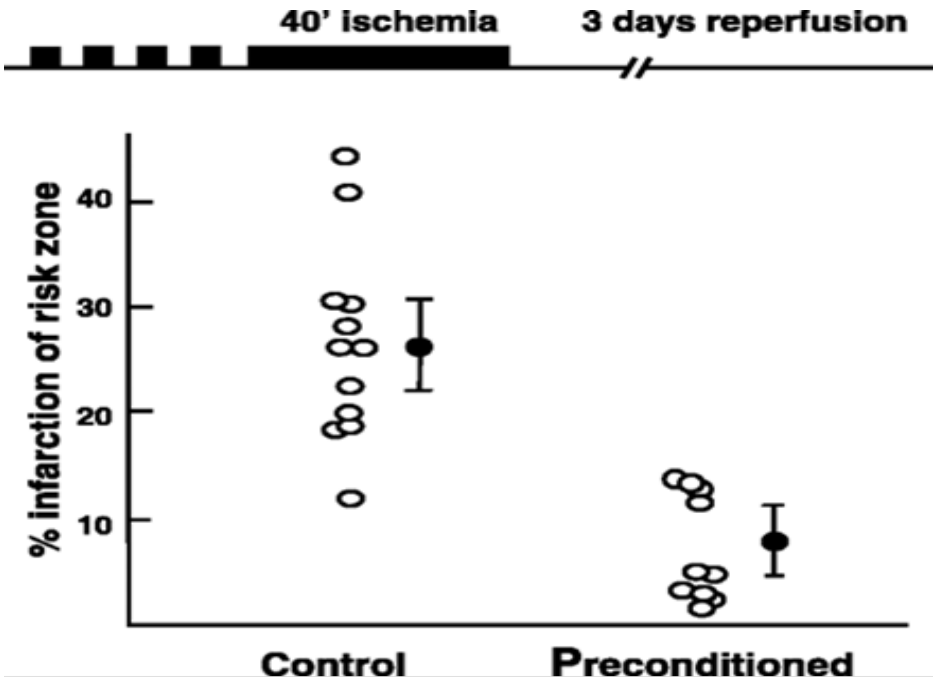


Figure 1.5: *Original description of ischaemic preconditioning canine model of myocardial ischaemia reperfusion injury showing reduction in infarct size with episodes of circumflex artery occlusion-reperfusion prior to the injurious ischaemia in dogs (80).*

1.7.1 Mechanisms of ischaemic preconditioning

IPC promotes the accumulation of protective ligands (triggers) which activate a number of mediators and through a complex process of cell signalling protection against IR injury is conferred by end effectors. In delayed IPC there is prominence of

gene transcription and synthesis of new proteins rather than activation of existing proteins. This enables a sustained period of protection after the FWOP. However the mechanisms of early and delayed IPC remain similar [Figure 1.6a & b].

1.7.1.1 Triggers of ischaemic preconditioning

The IPC stimulus promotes the release and accumulation of triggers which initiate the process of ischaemic protection. Adenosine (84, 85), bradykinin (86, 87), opioids (88-90), NO (91) and acetylcholine (92, 93) have all been identified as triggers and act via cell surface G-coupled receptors. Evidence for involvement of these ligands in IPC stems from studies that show that the protective effects of IPC are abolished in receptor knockout animals or with pharmacological blockade of their respective receptors. The receptors are proposed to act in parallel such that pharmacological antagonism of an individual receptor raises the ischaemic threshold required to trigger protection by IPC (94). Other autacoids implicated as triggers of IPC include free radicals (95, 96), norepinephrine (97) and CGRP (98).

1.7.1.2 Mediators of ischaemic preconditioning

The triggers of IPC activate second messengers including protein kinase C epsilon (PKCε), tyrosine kinases, phophatidylinositol 3-kinase (PI3K), Akt, mitogen-activated protein kinase (MAPK), extracelluar receptor kinase (ERK), JAK/STAT and nuclear factor κB (94). In addition, some downstream proapoptotic proteins are inactivated. These include glycogen synthase-3*β* (GSK-3*β*) and the Bcl-2 proteins – Bad and

Bax (99-101). eNOS activation leads to generation of NO and this activates protein kinase G via elevation of intracellular cGMP. Some downstream consequences of PKCε include K_{ATP} channel opening which further enhances PKCε production and generates ROS which is thought to be an essential part of the signalling cascade (Figure 1.6a).

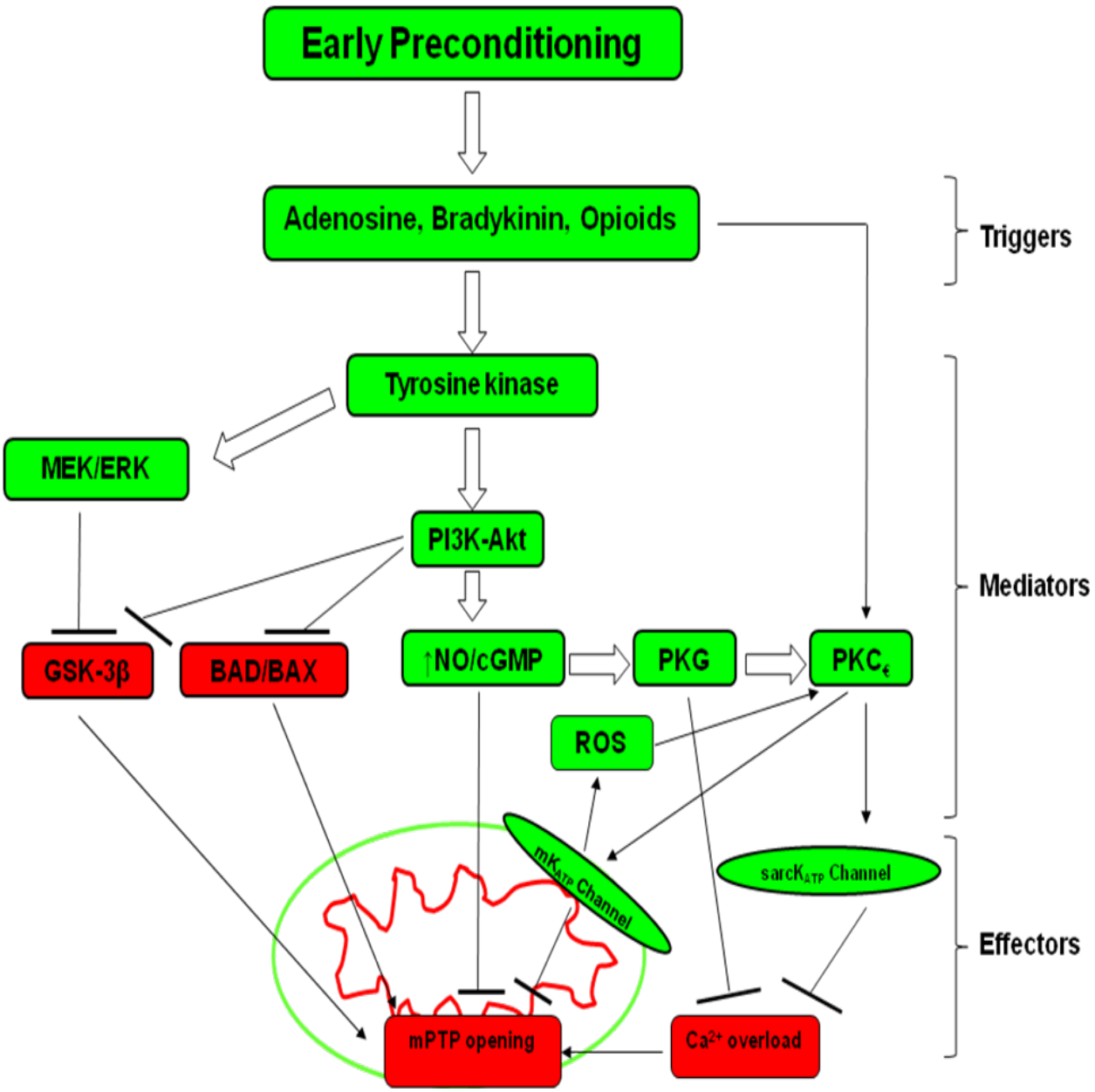
The transcriptional regulator, nuclear factor kappa B (NF-κB) plays a prominent role in the modulation of several genes during delayed IPC. This was evident in studies which showed that delayed IPC induced NF-κB activation and the NF-κB inhibitor DDTC blocked the protective effect of delayed IPC in a rabbit model of IR Injury (102). This group also demonstrated that delayed IPC-induced activation of NF-κB and ischaemic protection was blocked by pre-treatment with the NOS inhibitor N^G-nitro-L-arginine (L-NA), the ROS scavenger *N*-2-mercaptopropionyl glycine, the PKC inhibitor chelerythrine and the tyrosine kinase inhibitor lavendustin A (102). The results highlighted the importance of activation of these pathways during delayed IPC and the possible role of NF-κB as the common distal pathway of delayed IPC. NO has often been considered to act as both a trigger and a mediator during delayed IPC. eNOS releases NO following the IPC stimulus and iNOS mediates the formation of NO which confers protection 24-72 hours later. This is indicated by a biphasic response in measured NOS activity, though there appears to be a degree of overlap (103). The loss of the delayed IPC-induced ischaemic protection with a pharmacological inhibition of NOS or in iNOS knockout mice implicates a role for NO and specifically iNOS in delayed IPC (104, 105).

1.7.1.3 Effectors of ischaemic preconditioning

K_{ATP} channels are proteins that play a key role as effectors of the IPC stimulus. K_{ATP} channels consist of inward rectifier potassium channels (K_{IR}) and sulfonylurea receptor (SUR) subunits which form functional units (106, 107). Isoforms of K_{IR} ($K_{IR}6.1$ and $K_{IR}6.2$) and SUR (1, 2A, 2B) result in heterogeneous populations of K_{ATP} channels, with differing tissue specificity (108, 109). K_{ATP} channels are present the plasma membrane (sarcolemmal K_{ATP} channels) and mitochondria (mitochondrial K_{ATP} channels). Both populations are thought to have a similar structure and are implicated in IPC (110).

Evidence of involvement of K_{ATP} channels arises from studies in which the pharmacological antagonists (glibenclamide – nonselective; HMR1098 – selective for sarcolemmal K_{ATP} channels; 5-hydroxydecanoate – selective for mitochondrial KATP channels) abolished the protection by IPC and pharmacological K_{ATP} channels openers such as diazoxide and nicorandil mimicked protection by IPC (94). In addition, the loss of protective effect of IPC in K_{ATP} channel knockout mice models of IR injury has implicated their role in the mechanism of IPC (111). Evidence suggests that the mitochondrial K_{ATP} channels play a more prominent role in IPC, activation of which prevents opening of the mPTP, thereby reducing IR injury (see section 1.4.2) (112, 113). Suggested mechanisms by which K_{ATP} channel activation inhibits the mPTP include reduction in mitochondrial calcium load (promoting efflux or reducing entry), improvement in mitochondrial energy production by enhancing oxidative phosphorylation and reduction of ROS levels (113). Possible end effectors that play a role specific to delayed IPC includes heat shock proteins (HSP), iNOS, COX and antioxidant enzymes (94, 114).

a.


Early Preconditioning
Adenosine, Bradykinin, Opioids
Triggers
Tyrosine kinase
MEK/ERK
PI3K-Akt
Mediators
GSK-3β
BAD/BAX
↑NO/cGMP
PKG
PKCε
ROS
mKATP Channel
sarcKATP Channel
Effectors
mPTP opening
Ca2+ overload

b.

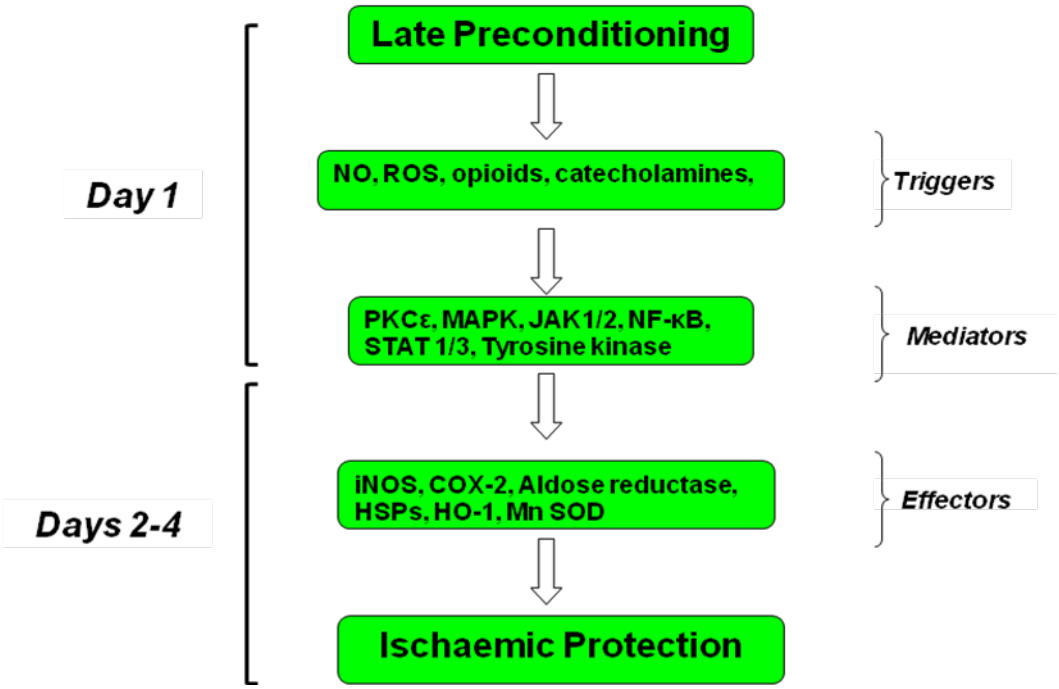


Figure 1.6: ***Major mechanisms of ischaemic preconditioning (IPC).*** *(a) Early IPC promotes the accumulation of protective ligands (green panels) which activates a complex cascade of intracellular events. Interestingly the opening of the mK_{ATP} channels by PKC activation generates ROS which further activation of PKC. Inhibition of processes which facilitate mPTP opening (red panels) forms an essential part of the mechanism of early IPC. (b)Similarly, the triggers of late IPC activate a signal transduction process that entails activation of kinases and transcription factors and gene transcription. These processes facilitate the formation of new proteins which serve as effectors (Adapted from Stein et al 2004).*

1.7.2 Clinical application of ischaemic preconditioning

Since the description of IPC, it has consistently produced significant reduction in IR injury in several experimental models. Yellon and colleagues demonstrated the reduction of myocardial injury in patients undergoing coronary artery bypass surgery using IPC which comprised cross clamping of the aorta (115). Following this, a number of studies demonstrating cardioprotection with IPC protocols have been performed in patients undergoing elective procedures such as aortic and mitral valve replacements and coronary artery by-pass surgery(116, 117). Furthermore, studies have shown that pre-infarct angina might serve as an IPC stimulus and

confer cardioprotection in patients who present with acute myocardial infarction (118-120). One major concern has been the safety of the IPC stimulus which often involves intermittent clamping and unclamping of the aorta or brief coronary artery occlusion and reperfusion in minimally invasive coronary artery by-pass surgery. In addition, some organs, such as the brain, are at a risk of IR injury but not accessible. This created the need to develop preconditioning mimetic drugs which could be administered prior to elective procedures or prophylactically in high risk patients. Based on the knowledge of the molecular mechanisms of IPC, mimetics such as adenosine, nicorandil and nitroglycerin have been tested in humans but have produced mixed results (21,121). Another hindrance to the clinical development of IPC is that it is not applicable during unplanned ischaemic syndromes.

In summary, IPC is an established method of ischaemic protection but two issues limit its clinical application. Firstly, there are risks associated with brief periods of ischaemia to vital organs. Secondly, most cardiovascular events are unpredictable, making it impossible to schedule any such prior preconditioning events. It has therefore been necessary to explore other strategies which might be more clinically applicable.

1.8 Ischaemic postconditioning

Another form of ischaemic protection, discovered more recently, is known as ischaemic postconditioning (PostC). Zhao and colleagues showed in a canine model, that after a 45 minute episode of sustained myocardial ischaemia, the interruption of myocardial reperfusion with three 30 second cycles of alternating

reperfusion/ischaemia reduced the myocardial infarct size by almost 50% (122). Furthermore, this schedule of interrupted reperfusion (PostC) also prevented coronary artery endothelial dysfunction, and neutrophil accumulation in the area at risk. Results of this study not only identified a novel method of ischaemic protection but also demonstrated that its protective effect was comparable to IPC.

The application of the PostC stimulus early in reperfusion is crucial. This is evident in the loss of protection with as little as a one minute delay in application PostC (123,124). Different schedules of PostC have been demonstrated to be protective and include (3-6) cycles of brief (10-30 seconds) alternating ischaemia/reperfusion (125,126).

PostC clearly influences specifically the reperfusion phase of IR injury, yet in general it has a similar degree of ischaemic protection as IPC (122, 127,128). This suggests that much of the reversible tissue injury following arterial occlusion happens early in the reperfusion phase. An additive cardioprotective effect when both stimuli are applied has been observed in the rat, but results have not been consistent in rats and across species (127-131). One interpretation of this is that in most species there is mechanistic overlap between IPC and PostC. The additive effect seen in the some rat models is consistent with a smaller degree of protection by PostC or a larger component of ischaemic injury in this species.

1.8.1 Mechanisms of postconditioning

The mechanisms of PostC resemble that of IPC and can be considered in similar terms: *triggers, mediators, effectors* [Figure 1.7].

1.8.1.1 Triggers

PostC promotes the accumulation or delays the washout of cardioprotective ligands which activate G-protein coupled receptors (132), many of which have been implicated in IPC. Adenosine is involved as non-selective pharmacological antagonism (8-ρ-sulfophenyl theophylline) of adenosine receptors during reperfusion abolished the protective effect of PostC (133, 134). Studies with selective adenosine receptor ligands have suggested a role for A_2 receptor subtypes (A_{2A} in mouse and A_{2B} in rabbits) in PostC (133, 135). Bradykinin B2 receptors have also been implicated in PostC, because 5 cycles of 10 seconds intermittent bradykinin infusion triggered PostC-like protection and the non-peptidic bradykinin B2 receptor antagonist - WIN64338 blocked PostC (136). Endogenous opioids appear to have a role because the protective effects of PostC are blocked by the non-selective opioid receptor antagonist naloxone and the selective [delta]-opioid receptor antagonist naltrindone (137,138). Erythropoietin (EPO) receptor expression in hypoxic tissues is increased and higher endogenous EPO levels have been associated with smaller infarct sizes in patients undergoing PCI for acute myocardial infarction (139). Indeed administration of EPO at reperfusion also reduces infarct size in several animal models but this protective effect is yet to be reproduced in humans (140-142).

1.8.1.2 Mediators

The endogenous protective ligands described above are thought to cause the activation of intracellular signalling molecules and prosurvival kinases, and similar to IPC.

Prosurvival kinases (reperfusion injury salvage kinases, RISK) activated during IPC have been implicated in PostC (143, 144). They include PI3/ Akt, ERK 1/2, JNK, Protein kinase C, Protein kinase G and p70S6K. Using a rat isolated heart model, PI3K/Akt activation has been identified as a mediator of PostC because the administration of PI3K inhibitors (LY294002 or wortmannin) in the first 15 minutes of reperfusion inhibited PostC induced protection (131). Similarly, PD98059, a MAPK/ERK inhibitor aborted the protection afforded by PostC in rabbit myocardium, thus identifying a role for MAPK/ERK in PostC (145). PostC has also been found to be dependent on protein kinase C (PKC) signalling in that the infarct-sparing effect of PostC was abrogated by the non-selective PKC inhibitor, chelerythrine and PKCepsilon inhibitor, KIE1-1 (146). Administration of the PKCdelta inhibitor, rottlerin seemed to mimic the protective effect of PostC. These data suggest that there is an increase in PKCepsilon activity and a reduction in PKCdelta activity in PostC as is the case for IPC. Acidosis might have a direct co-stimulating effect on these kinases; phosphorylation of Akt and ERK induced by postconditioning was blunted by the co-treatment with sodium bicarbonate (147).

Several interventions have been shown, experimentally, to elicit cardioprotection when administered at the time of reperfusion through activation of the RISK pathway and include insulin, IGF-1, erythropoetin, G-CSF, leptin, atorvastatin, pioglitazone,

atrial natriuretic peptides (ANP), Rho kinase inhibitors (Fasudil) and oestrogen (144). This highlights the importance of the RISK pathway in cardioprotection and creates the potential for use of some of these agents clinically. In addition to the up-regulation of antiapoptotic kinases, PostC causes downstream inhibition of proapoptotic proteins such as GSK-3*β* and members of the Bcl-2 protein family (Bad, Bax) (148, 149).

The demonstration of increased eNOS-ser1177 phosphorylation after PostC and the loss of protection by PostC with a selective soluble guanylyl cyclase (sGC) inhibitor (1H-[1, 2, 4]oxadiazolo[4,3-α] quinoxalin-1-one, ODQ) is evidence that GC/NO/cGMP pathway is involved in PostC (131, 134). In addition, in a rabbit heart model of IR injury, infusion of the NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME) before the onset of reperfusion caused a loss of protection by PostC (145). Subsequently Penna and colleagues have demonstrated, in rat isolated hearts, that NOS and sGC play different roles in PostC. Their study revealed that the protection afforded by PostC was only blunted by the NOS inhibitor, L-NAME but fully abolished by the sGC inhibitor, ODQ suggesting an additional route of activation of sGC by PostC which is NOS-independent (150).

Signaling, via NO, leads to accumulation of cGMP which causes activation of PKG. During rapid re-oxygenation (simulated IR injury) of adult rat cardiomyocytes, the presence of the PKG activator – 8-pCPT-cGMP or the cGMP analogue – 8-bromo-cGMP, increased sarcoplasmic Ca^{2+} - ATPase (SERCA) activity which reduced the peak intracellular calcium concentration and cardiomyocyte hypercontracture (151). These effects were abrogated by KT5283, a specific inhibitor of PKG. Some investigators have proposed that PKG is the terminal cytosolic component of the

trigger pathway and exerts its protective effect on the inner mitochondrial membrane via a signaling mechanism that involves K_{ATP} channels and PKC (152).

PostC is also thought to be associated with the reduction of ROS generation which contributes to reperfusion injury (123, 153). In contrast, administration of the ROS scavenger N-acetylcysteine (NAC) before or during PostC abolished protection (154). This was not the case when NAC was administered after PostC. This suggests that ROS generation has complex effects, and may play a role in signaling cardioprotection.

1.8.1.3 Effectors

As with IPC, K_{ATP} channels have been implicated in the mechanism of PostC. In a rabbit model of myocardial IR injury, the non-selective K_{ATP} channel blocker, glibenclamide and the mK_{ATP} channel blocker 5-Hydroxydecanoate (5-HD) administered at the onset of reperfusion, abrogated the protective effect of PostC (145). Recently, Myktenko and colleagues demonstrated that the infarct sparing effect of PostC, in a canine model of IR injury, was abolished by the administration of 5-HD, a mK_{ATP} channel blocker. However, in the presence of HMR1098 - a $sarcK_{ATP}$ channel blocker, the protective effect of PostC was not affected (155). In the same study, PostC up-regulated expression of mK_{ATP} channel Kir6.1 protein. These data suggest a specific role for mK_{ATP} channels in PostC. Activation of mK_{ATP} channels is thought to mediate inhibition of mPTP opening as described above.

A role for the mPTP in PostC was first reported by Argaud and colleagues who demonstrated that the specific inhibitor of the mPTP, NIM811, administered around

the onset of reperfusion, limited infarct size in rabbit hearts to a degree comparable to IPC and PostC (156). In addition, they showed that mitochondria isolated from postconditioned myocardium displayed an increased resistance to Ca^{2+} loading and this was similar to the hearts that underwent preconditioning or treatment with NIM811.

Cardioprotection by inhibition of the mPTP around the time of reperfusion has been explored in an acute angioplasty model in humans (157). Intravenous administration of a bolus dose of the mPTP inhibitor, ciclosporin, immediately before percutaneous coronary intervention (PCI) caused a significant reduction of creatine kinase compared with the control group.

The exact mechanism by which PostC inhibits opening of the mPTP is yet unknown. Recently, it has been suggested that PostC is critically dependent on both maintenance of myocardial acidosis during the initial 2 minutes of reperfusion and the simultaneous supply of oxygen (158). Using an rabbit isolated heart model, it was demonstrated that protection by PostC was lost when the heart was reperfused with an alkaline perfusate, even when the cardioprotective signalling cascade was triggered by activators of PKC (phobol 12-myristate 13-acetate [PMA]) or GSK-3β (SB216763), the latter being a downstream molecule. However the protection by PostC was restored with the addition of ciclosporin suggesting that ciclosporin is a more potent inhibitor of mPTP. It was also observed that in the presence of a hypoxic acidic perfusate, PostC was ineffective. It was postulated that an oxygenated acidic environment was necessary to block the mPTP opening long enough so that signalling could be triggered leading to endogenous attenuation of mPTP opening even after the correction of myocardial pH (158). Other mechanisms

by which PostC inhibits mPTP opening have been proposed and can be divided into indirect (intracellular calcium regulation, ATP preservation, oxidative stress correction) and direct (the phosphorylation and translocation of various protein kinases such as Akt, ERK1/2, GSK-3β, PKG, and PKC-ε) (159).

1.8.2 Clinical application of ischaemic postconditioning

Ischaemic protection by PostC is an attractive protective strategy especially considering its applicability and effectiveness in the acute ischaemic setting. Since first demonstration in humans, PostC has been successfully applied to patients in the setting of acute coronary angioplasty and has been associated with reduction of infarct size, improved endothelial function and improved resolution of ST-segments (160-162). PostC has also been protective during elective valve replacement, when the adult myocardium undergoes cold blood cardioplegic arrest (163). In addition, PostC has been protective during surgical correction of congenital heart disease in children (164).

The long term clinical effect of PostC in patients is yet to be established. In a recent acute coronary angioplasty study, not only did the postconditioned group of patients exhibit a reduced infarct size but also, at one year, a 7% increase in left ventricular ejection fraction compared with controls was observed (165). The limitation of this study is the small number of patients, and much larger studies with long term follow-up will be needed to define its clinical usefulness.

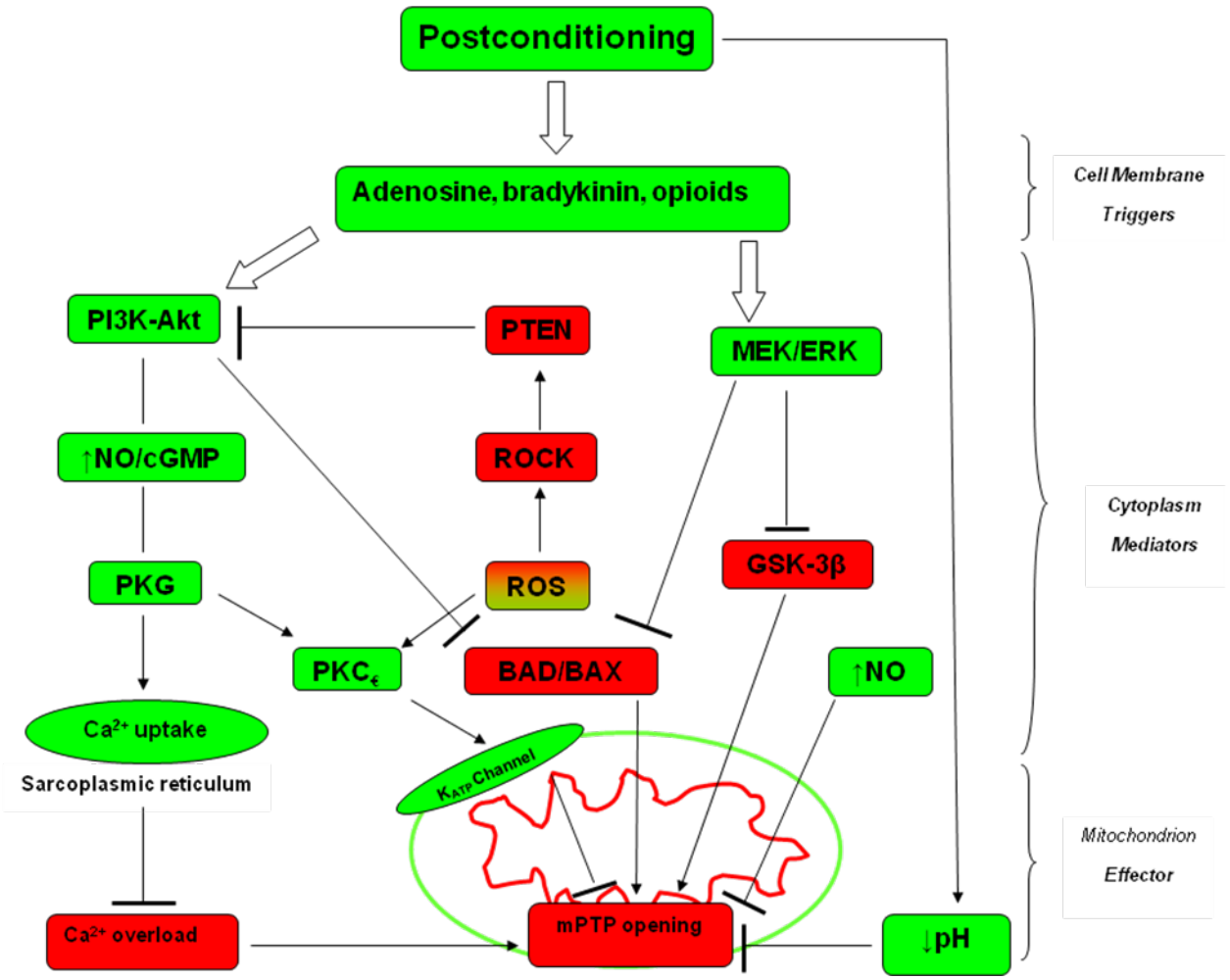


Figure 1.7: ***Mechanisms of postconditioning.*** *PostC is thought to promote the accumulation or delay the wash out of protective ligands (adenosine, bradykinin, opioids etc.) which activate a complex cascade of intracellular events. These processes are protective (green panels) in themselves or lead to inhibition of deleterious products of IR that exacerbate injury (red panels). Ultimately, these intracellular events seem to cause protection by preventing opening of the mPTP.*

1.9 Remote ischaemic conditioning

IPC also has protective effects at sites remote from those exposed to the ischaemic preconditioning stimulus, and this facet of IPC has been termed remote ischaemic preconditioning (RIPC). This was first reported in anaesthetised dogs when regional ischaemic 'preconditioning' protected remote virgin myocardium from subsequent sustained coronary occlusion (166). This study hinted that IPC had systemic

protective effects that were confirmed when it was shown that preconditioning the kidney (167, 178), intestine (168, 178) or limb (169) provided protection against IR injury in the heart and other tissues. This form of protection entailed application of the remote stimulus in advance of the ischaemic insult and is termed remote ischaemic preconditioning (RIPC). More recently, remote postconditioning (RPostC), a variant of RIPC, has also been described. In RPostC, the conditioning stimulus is applied during the ischaemic insult making it a more convenient remote stimulus in the acute ischaemic setting (170, 171). In this thesis I will use the term remote ischaemic conditioning as a blanket term for the two types of remote ischaemic protection.

RIPC shares similar signaling mechanisms to those of IPC and experimental models have implicated similar triggers, mediators and effectors (167, 170-173). This has been discussed in section 1.6.1 of this thesis.

1.9.1 Mechanisms of transfer of remote protection

One of the most intriguing aspects of remote protection is the mechanism of systemic spread of protection from the site undergoing conditioning. Humoral and neurogenic pathways have been proposed, with the potential for a degree of overlap between them [Figure 1.8].

1.9.1.1 Humoral mechanism

Coronary effluent from a preconditioned heart induces myocardial protection in non-preconditioned hearts, an effect that was blocked by administration of the non-specific opioid receptor blocker, naloxone to the recipient (174). This implicated the opioid receptor pathway in the spread of remote protection and highlighted the importance of circulating opioids. Furthermore it was shown that in the rat isolated heart, infarct size was reduced by plasma and dialysate of plasma (obtained using a 15 kDa cut-off dialysis membrane) from donor rabbits subjected to RIPC (174). In addition, the dialysate of plasma from rabbits and humans subjected to RIPC, reduced necrosis in an isolated fresh cardiomyocyte model of simulated ischaemia and reperfusion. Interestingly, these protective effects were abrogated by naloxone, suggesting that cardioprotection by RIPC occurred across species, required opioid receptor activation but did not require an intact nervous system. More recently however, the same group has demonstrated that the intra-arterial injection of adenosine into the femoral artery or limb RIPC released dialysable cardioprotective factor(s) in a manner that was dependent on an intact femoral nerve (175). With prior femoral nerve transection in the donor rabbit, the dialysate was no longer protective in the Langendorff perfused rabbit heart. This suggests a plausible interaction between a neurogenic and humoral mode of transfer. Opiates are not the only implicated humoral factor, with evidence for prostaglandins (176) and unidentified hydrophobic compounds (177).

1.9.1.2 Neurogenic mechanism

Neurogenic mechanisms have also been explored using autonomic ganglionic blockade. In a rat myocardial infarction model (60 minutes coronary artery occlusion with 3 hours of reperfusion), Gho *et al* showed that the ganglion blocker hexamethonium abolished protection by RIPC achieved by 15 minutes of mesenteric artery occlusion-reperfusion (MAO) but had no effect on local myocardial IPC achieved by 15 minutes of coronary artery occlusion-reperfusion(178). Cardioprotection was absent when MAO was sustained throughout the study, indicating that reperfusion in the small intestine was essential to activate the neurogenic pathway. In a similar study, using a rabbit model of myocardial IR injury, an intramesenteric artery infusion of bradykinin (BK) at a dose that stimulates sensory nerves without systemic effects mimicked these protective effects of RIPC. Protection was abolished by both the bradykinin 2 (BK2) receptor antagonist, HOE-140 and the ganglion blocker, hexamethonium (179). Pre-treatment with HOE-140 did not have an effect on infarct size of non-preconditioned rabbits. The researchers propose that the BK2 receptor activates local afferent nerves and lead to protection via the autonomic nervous system. These data corroborate a previous study in which HOE-140 abolished ischaemic protection by IPC in an intact rabbit heart but failed to block protection by IPC in an isolated rabbit heart (180). This suggested that protection by bradykinin was dependent on an intact autonomic nervous system. An increase in the release of the neurotransmitter glutamate and an increased expression of bradykinin receptors in cultured rat dorsal route ganglia sensory neurons, in response to increasing bradykinin concentration has been demonstrated in *in vitro* studies (181, 182).

In an in vivo rabbit model of myocardial infarction, RIPC by renal artery occlusion reduced infarct size by 46% (183). This protection was abolished by intravenous pretreatment with the nonselective adenosine receptor antagonist; 8-SPT. During renal RIPC, the renal afferent nerve discharge increased but this was attenuated by administration of intravenous 8-SPT. Furthermore, renal nerve resection abolished the protective effects of RIPC. These data suggest that the renal sympathetic nerve responds to adenosine receptor activation and triggers spread of protection beyond the kidney.

There is also experimental evidence that calcitonin gene-related peptide (CGRP), a neurotransmitter in capsaicin sensitive sensory nerves (CSSN), is implicated in the mediation of the delayed phase of remote organ protection. In a rabbit model of myocardial infarction RIPC induced by transient ischaemia of the small intestine, caused a reduction in infarct size and creatine kinase with an increase in plasma levels of CGRP (184). In this study, the protective effect of intestinal RIPC was abrogated by pre-treatment with capsaicin administered systemically, which selectively deletes neurotransmitters in the CSSN. Capsaicin also prevented an increase in the plasma level of CGRP. In another study using a pig model of myocardial ischaemia reperfusion injury, administration of the CGRP antagonist – $CGRP_{(8-37)}$ locally or systemically by intravenous infusion, did not influence infarct size. (185). Other studies have shown that the release of CGRP occurs in response to transient ischaemia, hyperthermia or endogenous ligands such as bradykinin (186). These data support the thesis that release of endogenous ligands in the remote organ activates afferent nerves which mediate the transfer of protective effects of RIPC.

The role of the nervous system in remote conditioning has also been demonstrated in humans using an *in vivo* model of vascular IR injury. The autonomic ganglion blocker, trimetaphan, had no effect on endothelial IR injury but abolished the effect of early and late RIPC to prevent such injury (187).

1.9.2 Clinical application of remote conditioning

RIPC obviates the need for complex and invasive IPC protocols because the protective stimulus can be applied non-invasively to a limb), without risking the blood supply to a vital organ. The first study to associate limb ischaemia with remote protection used a composite of electrical stimulation of skeletal muscle and arterial obstruction, to reduce myocardial infarct size in rabbits (188). This study was followed by the demonstration in humans, using an in vivo model of endothelial IR injury, that remote transient limb ischaemia was protective (169). The protective effect of RIPC by transient limb ischaemia has since been reproduced in a number of studies in patients (189). However, in the acute ischaemic setting RIPC is not applicable. RPostC which entails the application of the RIPC stimulus after the onset of injurious ischaemia has been described and in a clinical study of patients with ST-elevation myocardial infarction, who received RPostC in the ambulance prior to PCI, there was a significant myocardial salvage (190). Further studies to harness the full potential of this form of protection are on-going.

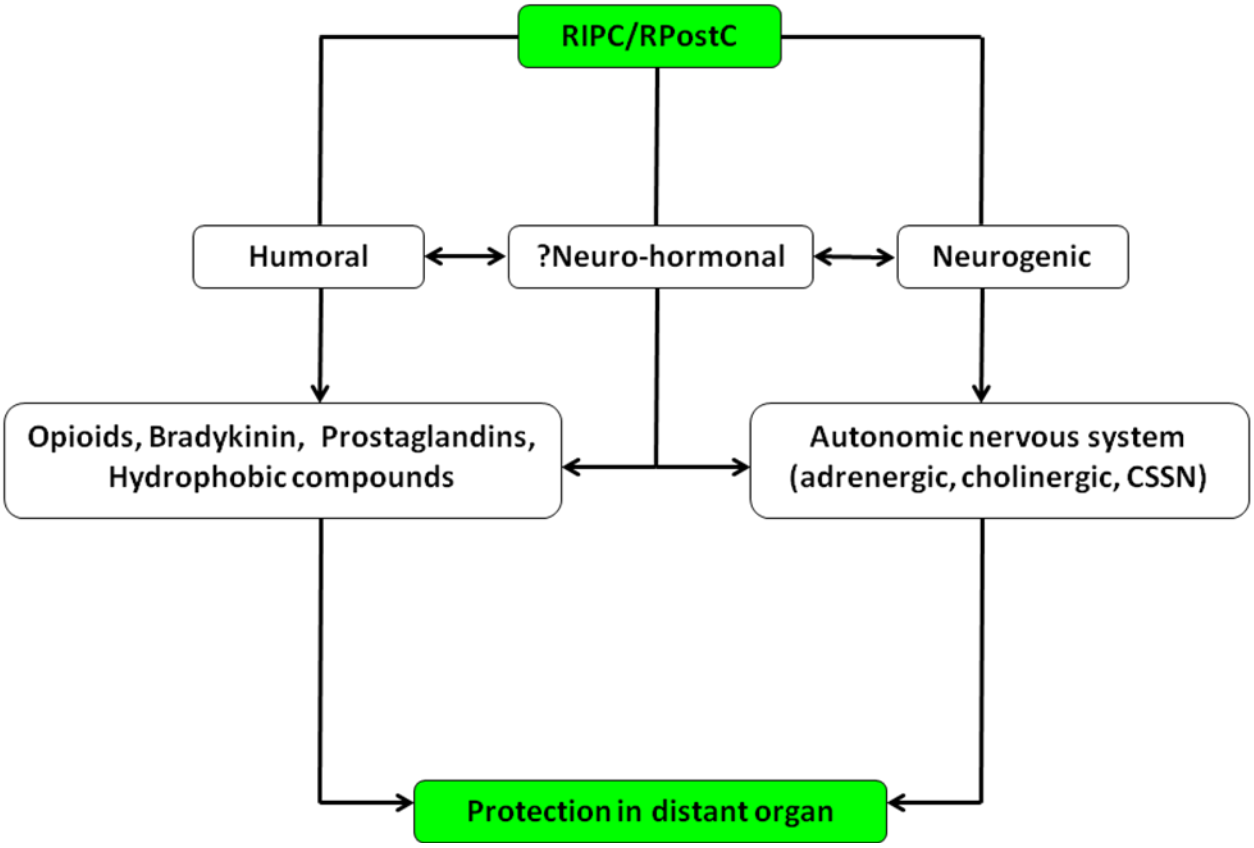


Figure 1.8: ***Mechanisms of transfer of protection by remote conditioning.*** *Protection by RIPC and RPostC is thought to be transferred to the site of injurious ischaemia via a neurogenic pathway or circulating substances (humoral pathway). An interaction between the two pathways is plausible but their relative contribution to overall protection merits further investigation.*

1.10 Ischaemic protection by inorganic nitrates and nitrites

As stated above (section 1.5.2) reduction in the NO bioavailability is a key event during IR injury resulting mainly from disruption to normal oxygen dependent endothelial production of NO. In addition, NO-cGMP signalling has been implicated in the mechanism of ischaemic preconditioning and postconditioning. NO donors reduce IR injury in a variety of experimental models, though clinical trials of NO donors to reduce IR injury have been negative (222). Recently, interest in a parallel pathway for NO generation that is activated at times of oxygen depletion, has led to

a reappraisal of the role of exogenous NO-supplementation as a potential treatment for IR injury. This pathway is the nitrate-nitrite-NO pathway [Figure 1.9] and is described in the next section.

1.10.1 NO generation – the alternative pathway

Within the cardiovascular system NO is generated largely via the activity of the eNOS enzyme (191-193). By this mechanism, NO is made available for physiological functions which may contribute to ischaemic protection as described in sections 1.7.1.2 and 1.8.1.2. Under the influence of the oxyhaemoglobin, a significant proportion of the circulating NO is rapidly oxidised to NO_2^- (nitrite), which is itself oxidised to the more stable NO_3^- (nitrate) (194, 195). Previously, nitrite and nitrate were considered to be inert end products of NO metabolism but current research findings are changing this view. The hypoxic environment promotes the reduction of nitrites and nitrates to produce NO. This alternative (NOS-independent) pathway for NO generation predominates over the NOS-dependent pathway under conditions of hypoxia and acidosis such as occurs with IR injury (196). The implication is that endogenous nitrite and nitrate stores act as a backup source of NO which may be beneficial during IR injury.

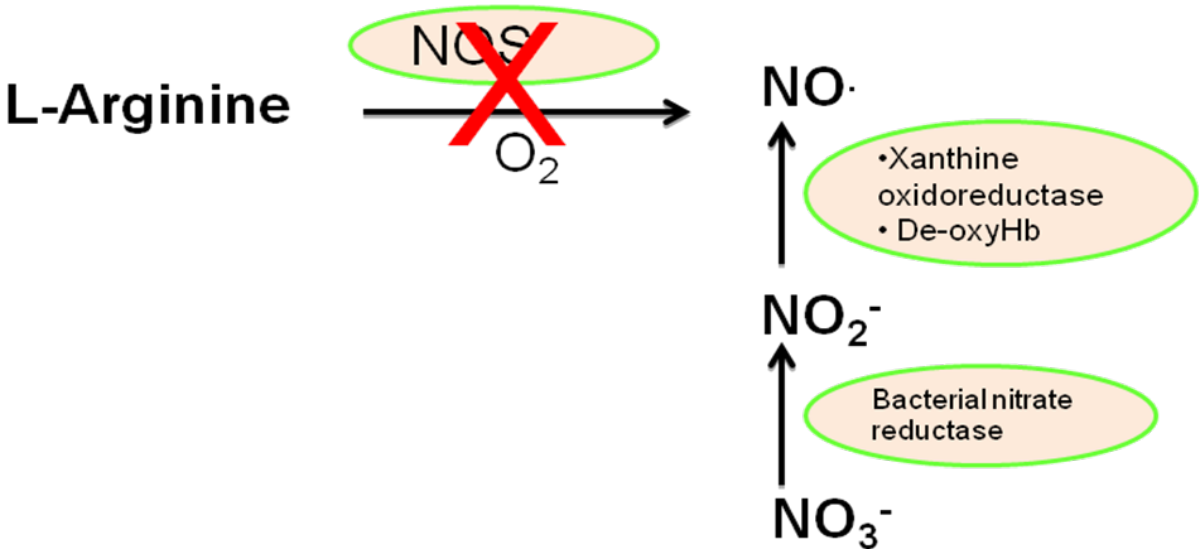


Figure 1.9: ***The nitrate-nitrite-NO pathway.*** *An alternative pathway for NO generation which is activated hypoxic and acidotic conditions when the NOS-dependent pathway is dysfunctional.*

1.10.2 Enterosalivary circulation of nitrates in humans

Ingested nitrates are rapidly absorbed in the small intestine and distributed in the blood to other parts of the body, and whilst up to 75% is eventually excreted in the urine, 25% is taken up by the salivary gland [Figure 1.10] (197). This nitrate is then secreted into the saliva and reduced to nitrite by bacterial nitrate reductases on the dorsum of the tongue [Figure 1.10] (198). The nitrite-rich saliva is swallowed and enters the stomach where under the acidic conditions, some of this nitrite is converted by simple chemical acidification to NO (198, 199). However, it is thought that at least some of this nitrite enters the circulation where it may then be converted to NO by nitrite reductases (200, 201). The importance of the enterosalivary production of nitrite has been highlighted in studies that show that a disruption of enterosalivary pathway, by preventing swallowing of nitrite-rich saliva or use of anti

bacterial mouthwash, results in a lack of a corresponding increase in circulating nitrite levels after ingestion of an oral inorganic nitrate load (202, 203).

Reduction of nitrite to NO in the circulation has been shown to be facilitated by a number of different candidates including deoxyhaemoglobin, xanthine oxidoreductase and mitochondrial enzymes, activities of which are enhanced in ischaemic environments, i.e. nitrite reduction to NO increases with decreasing pH and pO_2 (198). In addition, reduction of nitrite to NO via eNOS has been described (204). In particular it has been suggested that eNOS might act as a nitrite reductase when conventional eNOS activity (ie. L-arginine conversion to NO) is impaired such as in low O_2 conditions (205, 206). This enterosalivary circuit and intravascular processing enables oral inorganic nitrates and nitrites to serve as an intravascular reservoir for NO under hypoxic and acidotic conditions.

Systemic nitrite is derived from oxidation of NO in the plasma, reduction of salivary inorganic nitrate and from dietary sources such as meat, vegetables and drinking water (207). Accordingly under fasting conditions, the majority of nitrite is thought to be from oxidation of NOS derived NO. The reduction of circulating nitrite to NO under hypoxic and acidotic conditions that occurs during ischaemia, might have biological effects to limit tissue injury.

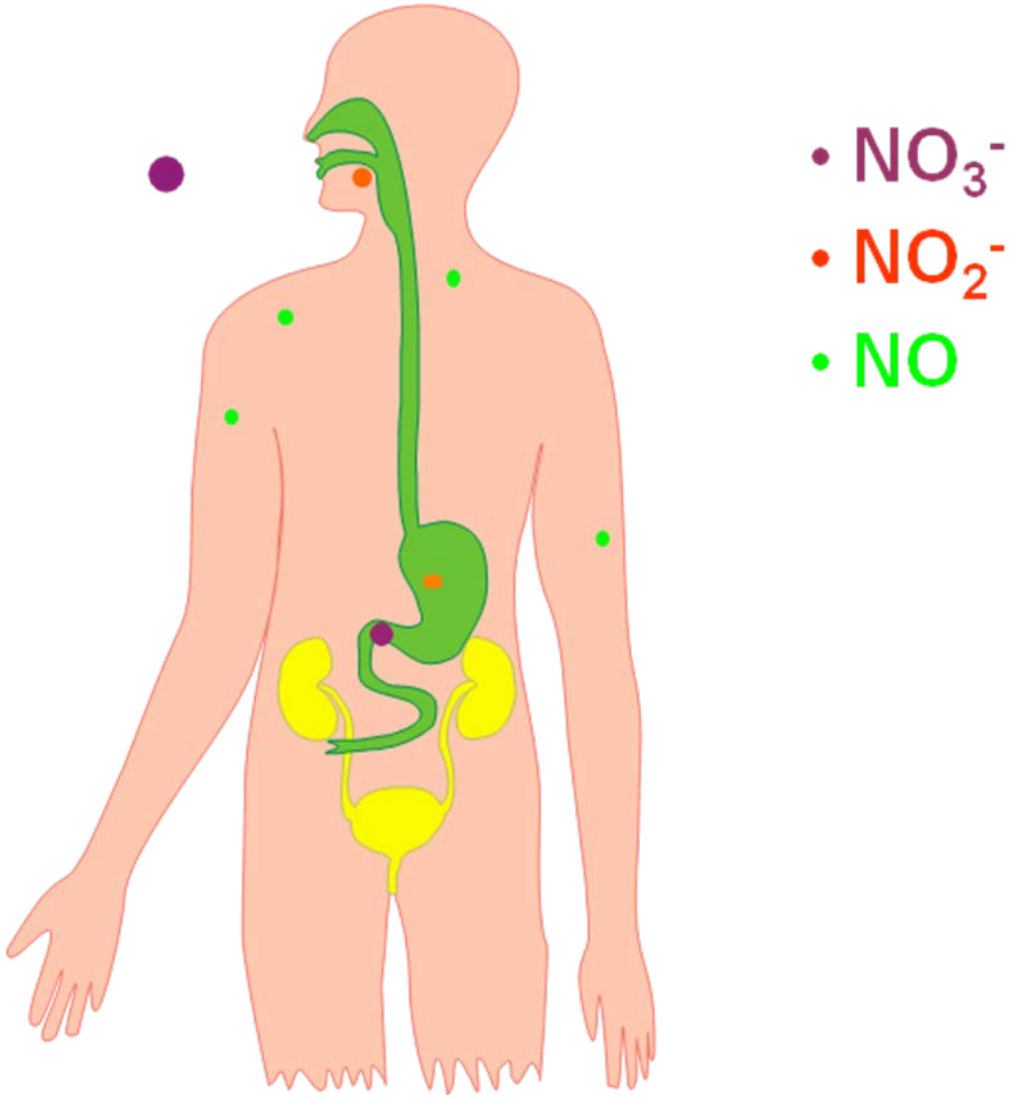


Figure 1.10: ***Enterosalivary circulation of nitrate in humans.*** *A proportion of the absorbed nitrate (purple circle) is taken up by the salivary gland, secreted into the saliva and reduced to nitrite (orange circle) by reductases present on the dorsum of the tongue. This is swallowed and in the acidic environment of the stomach is further reduced to NO (green circle). This enterosalivary circulation of nitrate is thought to provide an alternative source of NO in conditions of hypoxia and acidosis such as during IR injury.*

1.10.3 Nitrites and protection against IR injury

Johnson and colleagues discovered that administration of acidified sodium nitrite, during ischaemia, resulted in a significant reduction of myocardial injury in cats (208). This was indicated by lower creatine kinase (CK) levels and a reduced

necrotic area (expressed as percentage of myocardial area at risk) on nitroblue tetrazolium staining compared to controls. Using the isolated rat Langendorff heart model, Webb et al demonstrated that provision of nitrite during the ischaemic period or at reperfusion significantly reduced myocardial infarct size; an effect associated with comparable improvements in recovery of LV function (209). In this study, the importance of reduction of nitrite to NO was highlighted by the demonstration that protection was lost in the presence of the $NO^{\bullet}$ scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (carboxy-PTIO). This apparent loss of nitrite-induced ischaemic protection with carboxy-PTIO has since been demonstrated in a number of other animal studies (210-212). Using animal models of IR injury, it is now evident that nitrite is protective in the heart (209, 213-217), liver (211, 213, 215), kidney (212) and brain (218, 219). The role of nitrite-dependent NO in ischaemic protection is also highlighted in a study by Bryan et al in which dietary nitrite restored NO and nitrite bioavailability in eNOS knockout mice to steady state levels which were sufficient to protect against myocardial IR injury (214). This study provides evidence that dietary nitrites can act as alternative sources of NO in conditions associated with a dysfunctional NOS pathway.

The exact mechanism of the beneficial effects of nitrite-derived NO is debatable. Nitrite-dependent NO production during hypoxia is thought to regulate mitochondrial respiration by inhibiting respiratory chain complexes, thereby regulating the oxygen gradient and mediating cytoprotection during IR injury (213, 220). Nitrite-derived NO might also activate sGC and promote cGMP-dependent mechanisms of ischaemic protection.

1.10.4 Clinical application of nitrates and nitrites in ischaemic protection

In clinical practice, organic nitrates, mainly in the form of nitroglycerin (GTN), have been used for the symptomatic treatment of coronary artery disease for over a century (221). GTN is thought to exert its biological effects via the release of NO. However, ISIS 4 showed that isosorbide mononitrate had no effect on mortality in acute myocardial infarction, and this has limited enthusiasm for further clinical study of the therapeutic effect of NO-supplementation (222). As for other failed interventions in clinical IR injury, it remains uncertain if the organic nitrate was administered at the optimal time-point.

Another clinically relevant approach to NO delivery to tissues is administration in the inhaled form. Previously, the biological effects of inhaled NO (iNO) were thought to be limited to the pulmonary vasculature without any extrapulmonary bioactivity. Fox-Robichaud and colleagues disproved this by demonstrating, in feline mesentery treated with the NOS inhibitor – L-NAME, that the local vasoconstriction and leukocyte recruitment was abolished by iNO (223). Subsequent studies using animal and human models of IR injury have shown that iNO may exert protective effects in the liver (224), heart (225, 226) lung (227) and lower limb (228). Large scale clinical trials and potential widespread clinical use of iNO in the clinical setting have been hindered by lack of convincing preliminary human data, complex dose titration and storage issues, possibility of rebound hypertension or hypoxia with acute use, the risk of developing methemoglobulinemia and the accumulation of toxic oxidants such as NO_2 and peroxynitrite (229-231). Currently, iNO is licensed only for persistent pulmonary hypertension in neonates (230).

There is now compelling experimental evidence, from several studies, to support nitrite therapy in protection against IR injury in animals. The reduction in infarct size by a sodium nitrite infusion in the last 5 minutes of ischaemia during acute myocardial infarction in dogs sets the stage for the potential clinical use of nitrites as adjuvant cardioprotective therapy (232). Inorganic nitrate and nitrite have potential to deliver NO specifically to ischaemic tissues, in a manner that might optimise the local therapeutic effect and minimise systemic effects.

The inorganic nitrates and nitrites thus provide a substrate for the endogenous NO production in time of need, necessitating further assessment of their role in protection against IR.

1.11 Translation of protective therapies into clinical use

The ultimate aim of strategies to reduce IR injury lies in the translation of research findings into clinical use in order to derive the full benefit of improving outcomes in the management of cardiovascular ischaemia.

Previous therapies aimed at reducing the lethal reperfusion injury in patients with myocardial infarction have not been successfully translated into clinical use. These include antioxidants, calcium overload and Na^+-H^+ exchange inhibitors, anti-inflammatory agents, magnesium, therapeutic hypothermia, and glucose, insulin and potassium (21). Perhaps the pathogenesis of human IR injury is more complex than in the animal models that are used to validate drug targets. Some of the differences that have been highlighted include age and health of subjects, ischaemia/reperfusion times, timing of intervention and end points for cardioprotection (21). Future studies

should be aimed at using more clinically relevant animal models with robust study designs that will correlate with human studies. The utility of human mechanistic studies to bridge the gap between animal data and clinical trials in patients cannot be overemphasised.

1.12 The human forearm model of IR injury

The human forearm has served as a valid *in vivo* model to investigate the mechanisms of ischaemic protection in humans (53, 124, 169, 171, 187, 233). The ability to measure IR induced endothelial dysfunction in the human forearm has enabled the mechanistic assessment of different protective strategies. IPC, PostC and remote conditioning have all previously been shown to protect against endothelial IR injury in the human forearm.

1.13 Aims of thesis

Using the human forearm model of IR injury I sought to determine whether RIPC, RPostC and PostC protect against endothelial IR injury in conduit and resistance vessels.

In chapter 3, I investigated the role of K_{ATP} channels and the mPTP in mechanism of PostC in humans. These targets can be manipulated using pharmacological tools. PostC has previously been shown to protect against endothelial IR injury in the brachial artery (124). I assessed the effects of PostC on endothelial IR injury in the brachial artery and resistance vessels. Using this *in vivo* model of endothelial IR

injury, K_{ATP} channels have previously been implicated in IPC and RIPC (171, 233). I investigated whether the non selective K_{ATP} channel blocker, glibenclamide abrogates the protective effect of PostC and also determined the effects of the more pancreatic selective K_{ATP} channel blocker, glimepiride on PostC. This was to establish a role for K_{ATP} channels in PostC and investigate the effects of agents with different selectivity on PostC. Opening of the mPTP has been implicated as an effector mechanism in PostC (156, 159). I sought to determine if the administration of ciclosporin, a known inhibitor of the mPTP opening, administered around the onset of reperfusion protects against endothelial IR injury. This might be evidence that ciclosporin mimics PostC.

In chapter 4, I investigated the role of the opioid receptor pathway in remote conditioning. Naloxone is a known non-selective inhibitor of the opioid receptors. I sought determine whether naloxone abolishes the protective effects of RIPC or RPostC in an attempt to identify a role for circulating opioids in the transfer of protection by remote conditioning.

In chapter 5, I investigated the role of components of the autonomic nervous system in RIPC and RPostC. Using a human *in vivo* model of endothelial IR, a previous study in this laboratory showed that complete autonomic blockade with trimetaphan abolished the protective effects in the early and late phases of RIPC (187). I sought to determine the effect of alpha adrenergic and cholinergic nervous blockade on RIPC and RPostC using phentolamine and atropine respectively.

Chapter 6 entailed investigations into the role of oral inorganic nitrate, as an endogenous source of NO during IR injury in humans. This is in recognition of the

role of an alternative pathway of NO generation during IR injury (196). I sought to determine whether oral inorganic nitrates in the form of beetroot juice and KNO_3 tablets protected against endothelial IR injury.

The above aims are a component of a larger investigation of the mechanistic aspects of ischaemic protection in humans. This will build on the data in animal models and identify potential pharmacological targets in humans, as a prelude to clinical trials in patients.

Chapter 2

General methods

2.1 The human forearm model of IR injury

By inflating a blood pressure cuff to a suprasystolic pressure it is possible to achieve ischaemia in the forearm. The ability to measure IR-induced endothelial dysfunction in the human forearm has enabled the mechanistic assessment of different protective strategies. Ischaemic protection by IPC, PostC and remote conditioning has previously been investigated using the human forearm endothelial IR injury model (124, 169, 233).

2.1.1 Induction of IR injury

Ischaemia of the non-dominant arm (plethysmography studies)/right arm (vascular ultrasound studies) was achieved by inflating a 12cm or 9cm wide blood pressure cuff respectively, placed around the upper arm to a pressure of 200 mm Hg for 20 min. Thereafter, 20 minutes of reperfusion allowed restoration of baseline diameter and blood flow as described previously (53, 124, 171, 187, 233).

2.1.2 Induction of ischaemic preconditioning (IPC)

IPC was induced by inflating a 9cm-wide blood pressure cuff placed around the upper part of the right (index) arm. The cuff was inflated to 200 mm Hg for 5 minutes (ischaemia of the arm), followed by a 5-minute deflation (reperfusion). This constituted a conditioning cycle and 3 cycles were used in advance of IR, as described previously (233).

2.1.3 Induction of ischaemic postconditioning (PostC)

PostC was induced by short periods of intermittent reperfusion to the ischaemic arm in the first 60 seconds of reperfusion (124). At the end of the 20 minute period of index ischaemia, the upper arm cuff was deflated for 10 seconds (allowing reperfusion), after which the cuff was again inflated to 200 mmHg for 10 seconds (restoring ischaemia). The alternating deflation/inflation cycle was repeated 3 times (1 minute total duration), after which continuous reperfusion of the arm occurred.

2.1.4 Induction of remote preconditioning (RIPC)

RIPC was induced by inflating a 9cm-wide blood pressure cuff placed around the upper part of the contralateral arm (ArmRIPC) or 12cm blood pressure cuff around the upper part of the thigh (LegRIPC). The cuff was inflated to 200 mm Hg for 5 minutes (ischemia of the arm), followed by a 5-minute deflation (reperfusion). This constituted a conditioning cycle and 3 cycles in the arm or 2 cycles in the leg were used in advance of IR, as described previously (171, 187).

2.1.5 Induction of remote postconditioning (RPostC)

RPostC was induced by inflating a 12cm-wide blood pressure cuff placed around the upper part of the thigh. The cuff was inflated to 200 mm Hg for 5 minutes (ischemia of the arm), followed by a 5-minute deflation (reperfusion). This constituted a conditioning cycle and 2 cycles were used during the 20 min index ischemia, as described previously (171).

2.2 Measurement of endothelial function

It is now well established that the single layer of cells lining blood vessels, termed endothelium, subserves several important functions that maintain the integrity of the cardiovascular system. In view of its varied function it is perhaps not surprising that no single measure describes all aspects of endothelial function. In addition, endothelial function may vary depending on the vascular bed, disease condition or even the type of flow (eg laminar versus turbulent) to which the endothelium is exposed (234, 235). However the vasomotor function has been easiest to measure in humans. In response to certain physical and chemical stimuli the endothelium releases NO which causes blood vessels to dilate (236-238). The degree of vasodilatation and/or blood flow can be measured and is an assessment of endothelial function. This can be done by invasive (venous occlusion plethysmography; intravascular coronary ultrasound) and non-invasive (brachial/radial artery FMD; pulse wave analysis, pulse amplitude tonometry) techniques. In this thesis, endothelial function assessment has been performed in two vascular beds – conduit vessel (brachial artery) and resistance vessels.

2.2.1 Assessment of conduit vessel endothelial function

By using vascular ultrasound, the endothelial response to shear stress (increased blood flow) can be measured. This phenomenon is known as flow mediated dilatation (FMD) and is used to assess endothelial function in conduit arteries such as the brachial or radial artery. The mechanisms that underlie an increase in NO bioactivity in response to shear stress include increased levels of calcium which occurs as a result of the opening of calcium-activated potassium channels (236). This leads to increased eNOS activity; subsequent increased NO generation and

vasodilatation. The increase in vessel diameter is measured using vascular ultrasound. FMD in the forearm is largely NO- dependent because administration of a NOS inhibitor abolishes FMD (238, 239). Factors which affect the magnitude of FMD include temperature, food, vasoactive drugs, physical exercise, sympathetic stimuli such as acute exercise and mental stress, phase of the menstrual cycle and the magnitude of the blood flow stimulus (236). These factors should be taken into account when performing FMD studies.

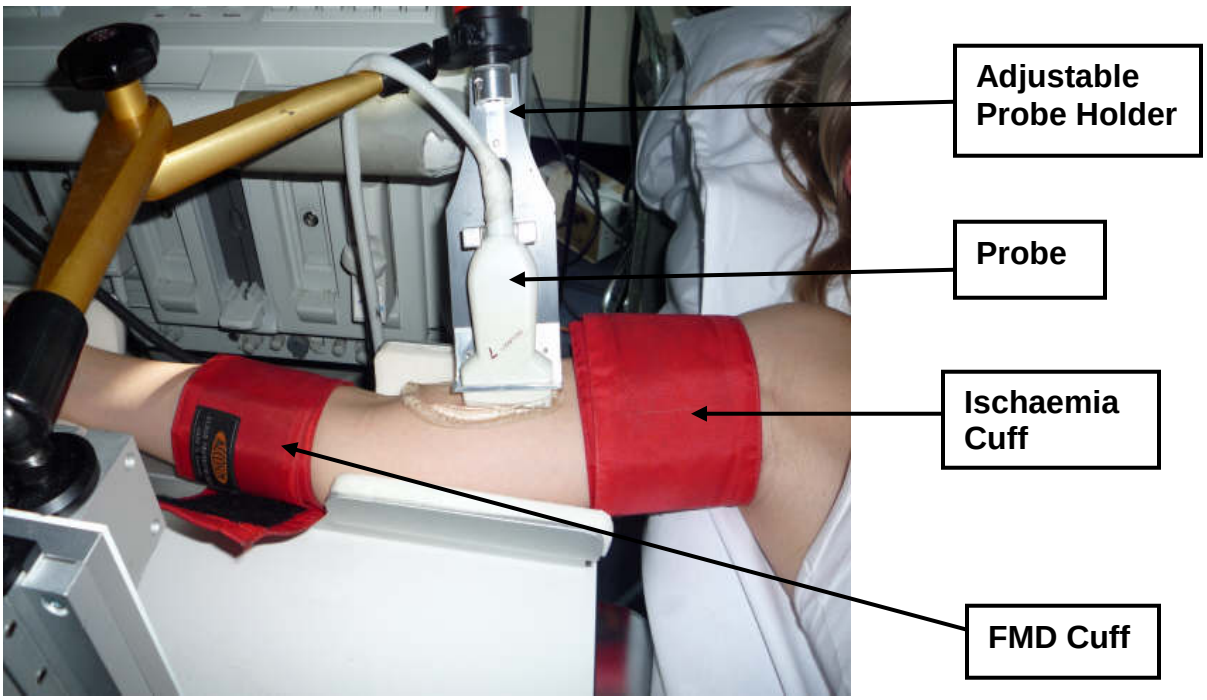
2.2.1.1 Subject preparation

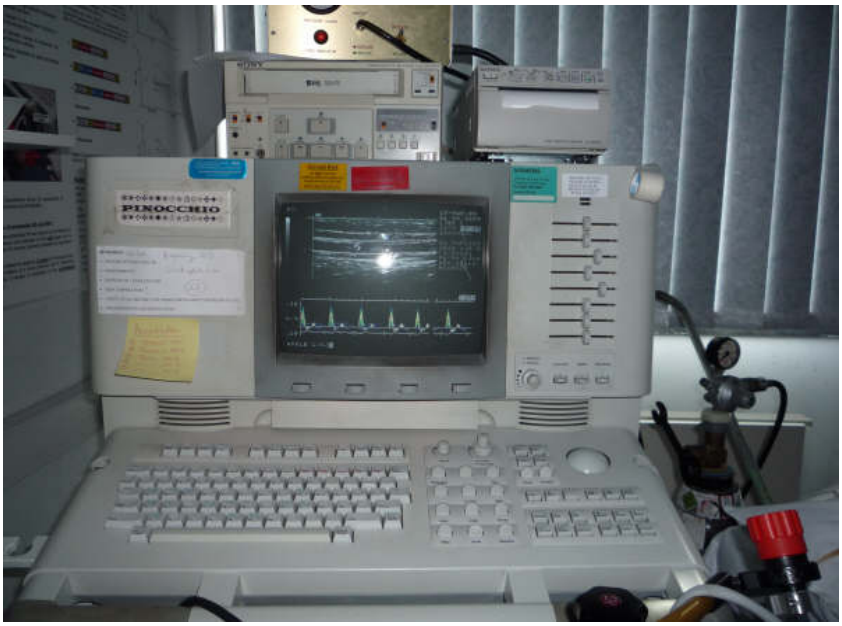
FMD studies in this thesis were performed at the Vascular Physiology Unit, Institute of Child Health, UCL and the Clinical Research Facility UCL Hospital. Studies were performed on healthy, non-smoking volunteers (18-45 years of age) in a quiet, temperature-controlled laboratory (24^{o}C to 26^{o}C). Volunteers were asked to refrain from caffeine-containing drinks and fatty meals for 4 hours prior to each study and refrain from excessive exercise for 24 hours prior to each study. The exclusion criteria were a history of any illness, volunteers taking systemic medication, pregnancy or age <18 years or >45 years. Studies repeated in same volunteers were at least 7 days apart and in a random order sequence. All protocols were undertaken after review by an NHS research ethics committee.

2.2.1.2 Experimental technique

Subjects were positioned comfortably (Fig 2.1) and brachial artery flow mediated dilatation (FMD) of the right arm was assessed. Reactive hyperaemia of the forearm (achieved by means of the FMD cuff) was used as a stimulus to increase blood flow in the brachial artery resulting in brachial artery dilatation (239). A B-mode

ultrasound scan of the brachial artery was obtained in longitudinal section between 5 and 10 cm above the antecubital fossa with 7.0-MHz linear-array transducer [spatial resolution of 0.1 mm] (240) and a standard Acuson XP10 system. Longitudinal, ECG-gated, end-diastolic images were acquired every 3 seconds for offline analysis (Fig 2.2). Arterial diameter over a 1- to 2- cm segment was determined for each image using automatic B-mode edge-detection software (Brachial Tools, Medical Imaging Applications).





Vascular Ultrasound machine

Fig. 2.1: *These photographs show the subject positioning and experimental set up during an FMD study.*

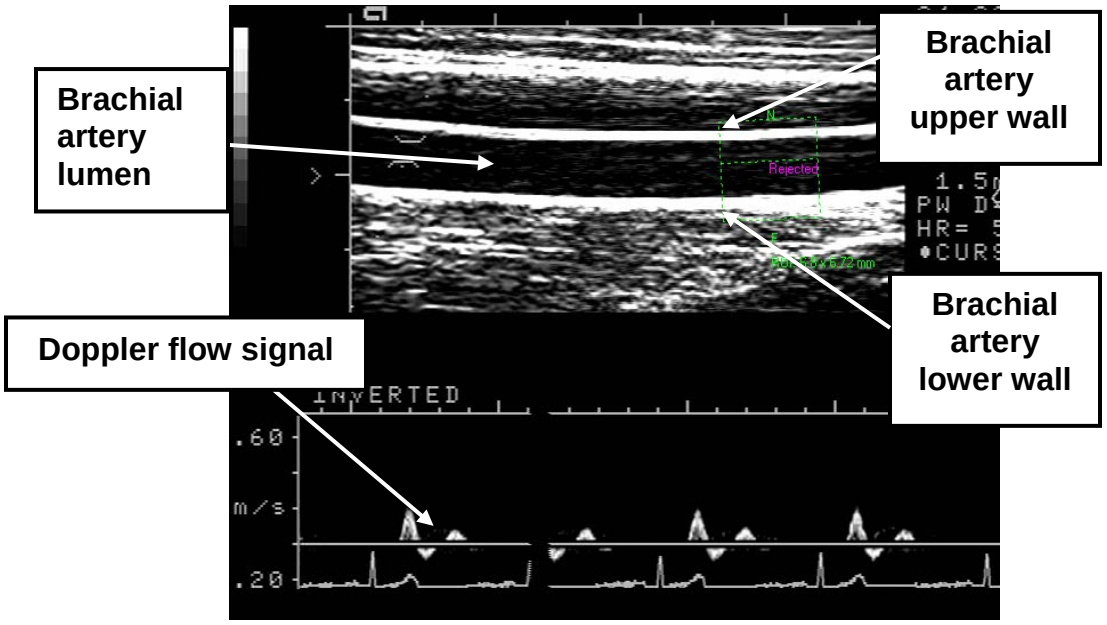


Fig 2.2: *Image of longitudinal section of the brachial artery on ultrasound machine.*

Blood flow was manipulated in the brachial artery by means of a 9-cm-wide pneumatic cuff (Scanmed, Moreton-in-Marsh, Gloucestershire, UK) placed around the forearm immediately below the antecubital fossa (FMD cuff). After 1 min of baseline flow, the cuff was inflated to 300 mm Hg for 5 minutes and then released, resulting in a brief episode of reactive hyperaemia. Brachial artery diameter changes in response to blood flow were assessed for a further 5 min. Blood flow velocity was continuously monitored by pulsed-wave Doppler.

2.2.1.3 FMD calculation

FMD in this thesis is expressed as peak percentage change in arterial diameter from baseline (Fig. 2.3). FMD may also be expressed as absolute change in arterial diameter.

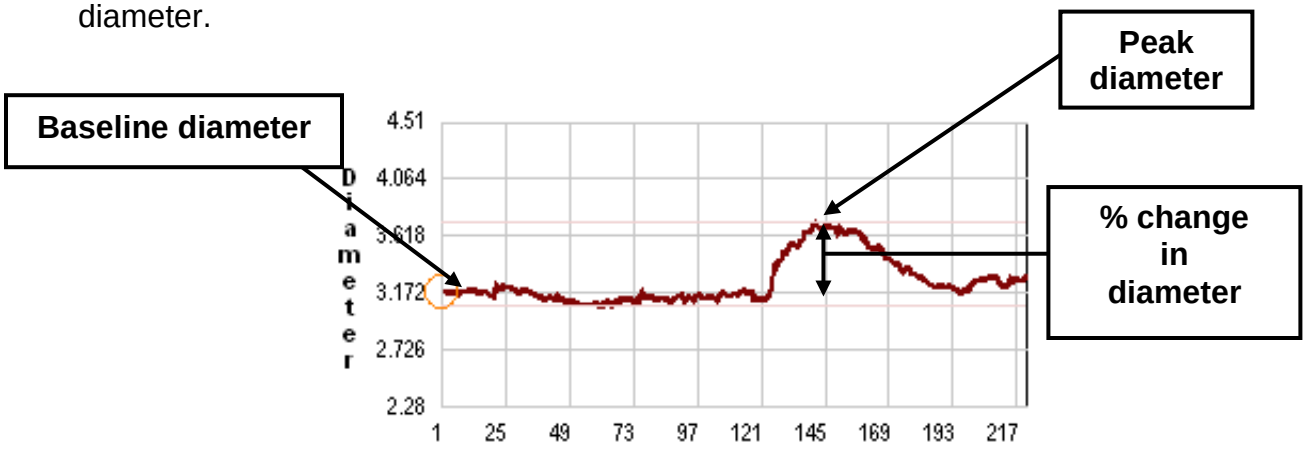


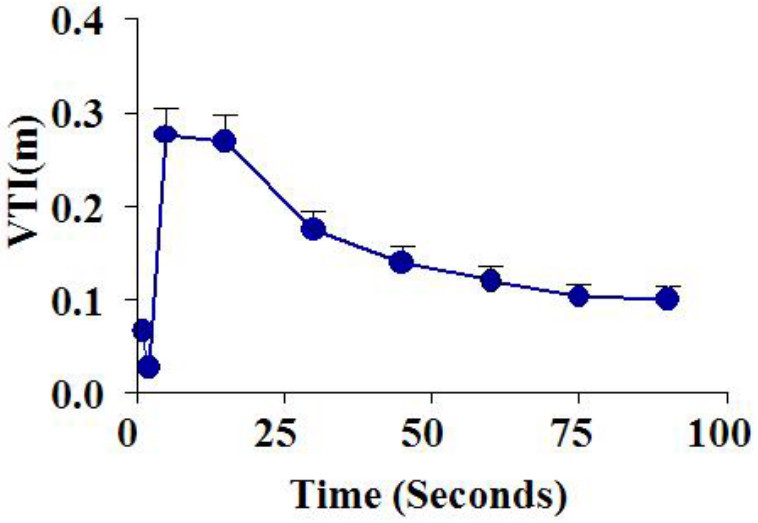
Fig. 2.3: *FMD expressed as percentage change in brachial artery diameter from baseline at peak dilatation.*

2.2.1.4 Measurement of blood flow

Blood flow velocity was measured using the pulsed wave doppler flow signal [Figure. 2.2]. This is the velocity-time profile for a single cardiac cycle and is displayed as a spectral doppler curve. The area under the curve of the velocity-time profile is the

velocity-time integral [VTI = velocity (m/s) x time (s)] and approximates to the average distance, measured in metres, travelled by a pulse of blood during one cardiac cycle, typically at rest 0.01 – 0.05 m [Figure 2.4].

a.



b.

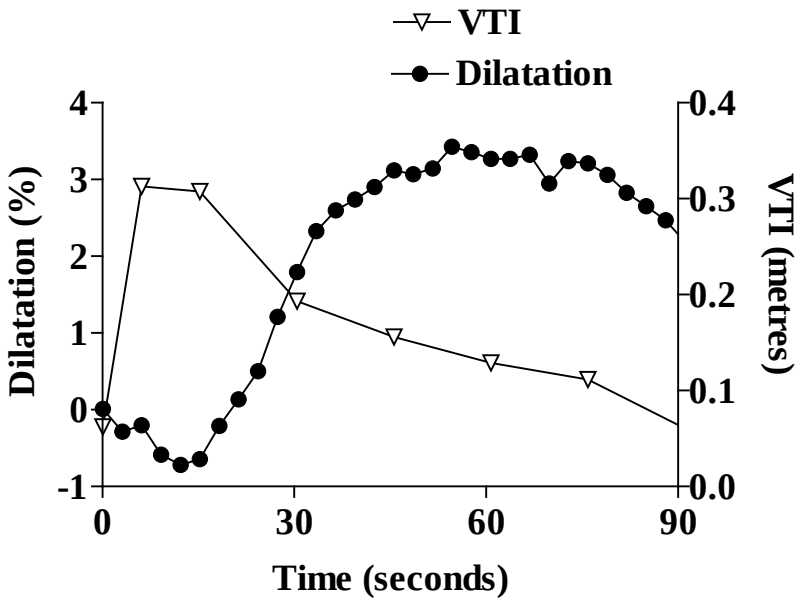


Fig. 2.4: (a) Blood flow / time profile; velocity time integral (VTI). (b) VTI and dilatation as a function of time

Blood flow is that volume of blood that passes a point in a specified period of time. Assuming that we can approximate a section of artery to a cylinder, the calculation of the blood flow volume will be as follows:

Volume / cardiac cycle = artery cross-sectional area x average distance travelled by pulse = πr^2 (t) $\int$ v(t) dt,

(r(t) = the measured instantaneous vessel radius and v(t) = the instantaneous blood velocity)

The Doppler signal is measured at an angle of approximately 70º to the axis of the blood vessel, giving $\int$ v(t) dt = cos 70º x measured VTI. Therefore,

Volume / cardiac cycle = cos 70º.πr^2 (t).VTI

Volume per minute is calculated by multiplying this value by heart rate (HR).

Volume / min= HR cos 70º.πr^2 (t).VTI.

If the heart rate, the doppler angle of incidence and arterial diameter (2 x arterial radius) are assumed to remain constant during the period of the study, VTI can be considered proportional to volume flow per minute. Measurement of the radius for the period of peak flow (up to 30 seconds after cuff release) confirms that there is minimal change in arterial diameter at this time. Furthermore, during the same time period heart rate and doppler angle are also constant. Thus a valid conclusion is that peak volume following cuff release coincides with peak VTI. A small change in arterial diameter will introduce an error into the assessment of flow based on VTI. This error may be calculated as follows:

Volume / min = HR (t=0) cos 70º.πr^2 (t=0).VTI [1 + 2∆r/ r (t=0)]

r (t=0) is the arterial radius at the time when the blood pressure cuff is released and it is equal to the radius of the artery prior to forearm ischaemia. Therefore fractional error is typically (2∆r / r (t=0)) and is maximal at about t=60s (approximately 6%). However, for the 30 seconds following cuff release this is negligible, as the arterial radius remains almost constant and approximately equal to r (t=0).

Taking the above into account, the following formula can be used to calculate peak volume flow per minute during reactive hyperaemia:

Volume / min (peak) = HR(at time of peak VTI) cos 70º.πr(t=0)^2.VTI(peak)

2.2.1.5 Data presentation of blood flow

For studies in this thesis, baseline and peak VTI were calculated and the ratio of peak to baseline absolute volume flow per minute was determined using the following formula:

$$\frac{\text{Volume / min (peak)}}{\text{Volume / min(baseline)}} = \frac{\text{HR (at time of peak VTI).VTI (peak)}}{\text{HR(baseline).VTI(baseline)}}$$

This formula is based on the assumption that r(t=0) is approximately equal to r(baseline). In all of the studies in this thesis there were no significant changes in heart rate. However to avoid the risk of confounding effects of changes in heart rate affecting the measurement of the flow stimulus, heart rate values were recorded at

the same time as the VTI measurements at baseline and peak hyperaemia and incorporated into the calculation of the volume flow per minute ratio.

2.2.1.6 Accuracy and reproducibility of the technique

The validity of any measuring technique depends on its degree of accuracy and reproducibility. Error arises when the same observer makes repeated measures (intraobserver error) and when different observers make the same measures (interobserver error). To enable the detection of a reliable effect of an intervention, knowledge of the reproducibility is essential. FMD has a high degree of reproducibility as evidenced by studies which show a low coefficient of variation (CV) (241, 242). A pre-requisite to reproducibility of FMD is standardisation of the technique and operator training both of which are part of the standard operating procedures within the laboratories where these studies were performed. Comparison of FMD responses in a single subject measured on multiple occasions or between groups of subjects is possible provided that there are no major differences in basal blood flow, or arterial diameter. Image analysis is another factor that influences the reproducibility of FMD. B mode edge detection software was used for analysis in this thesis and compared with an alternative method of analysis (A-mode wall tracking (A-WT) (Vadirec, Medical Systems Arnhem, Oosterbeek, the Netherlands), has a lower coefficient of variation (242).

Having performed detailed assessments of brachial artery FMD, Donald et al have expressed reproducibility of FMD as the percentage of coefficient of variation (CV) = [(standard deviation of the paired differences/the overall mean)/$\sqrt{2}$] x 100 (242). Reproducibility of FMD in this thesis has been presented in a similar manner. I

assessed intraobserver variability by performing two sequential scans on 8 subjects at least 72 hours apart. Interobserver variability was assessed by performing repeated FMDs in 8 subjects on two separate days at least 72 hours apart. One of the scans was performed by an experienced vascular sonographer (Ann Donald) and the other by me.

My intraobserver CV was 6.24%; n=8 and interobserver CV was 7.6%; n=8. These are comparable to the published data for FMD CV of 7.1% for adults and 6.3% for children (241).

2.2.2 Assessment of resistance vessel endothelial function

Forearm venous occlusion plethysmography measures endothelial function in resistance vessels in response to chemical stimuli. Local administration of endothelium dependent vasodilator drugs such as acetylcholine (ACh) and bradykinin increases forearm blood flow which is an index effect of these drugs on the endothelium (237). By so doing, the endothelial function in forearm resistance vessels can be indirectly assessed. Forearm plethysmography is based on the principle that if venous return from the arm is obstructed and arterial inflow continues, the result is forearm swelling at a rate proportional to the arterial inflow (237, 243). The rate of swelling is measured as a change in the forearm circumference which reflects a change in forearm blood flow (FBF). FBF is predominantly in skeletal muscle, so the hands are excluded during measurements because blood flow in the hand is predominantly through the skin. Another reason for excluding the hand is a high proportion of arteriovenous shunts (243).

Absolute values of basal blood flow in the forearm may vary with the time of day or sympathetic tone but should not alter the ratio of flow in the two arms or the

percentage response to drugs calculated from the ratio (237, 243). The presence of a control arm (contralateral arm) for each study is an advantage of the technique. To minimise variability, studies should be undertaken in a quiet, temperature-controlled environment and results expressed as a ratio of flow in the two arms.

2.2.2.1 Subject preparation

Venous occlusion plethysmography studies in this thesis were performed in the Clinical Pharmacology Clinical Laboratory, and subsequently in the Clinical Research Facility, University College Hospital London. Other aspects of subject preparation were as for FMD studies highlighted in section 2.2.1.1.

2.2.2.2 Experimental technique

Subjects were positioned comfortably (Fig 2.5) and bilateral forearm blood flow was measured by means of mercury-in-silastic strain gauge plethysmography, as described previously (233, 237, 243). The brachial artery of the non-dominant arm was cannulated with a 27-gauge needle (Cooper's Needle Works, Birmingham, UK) under aseptic technique using 2ml of 2% lidocaine (subcutaneous) for anaesthesia (Fig. 2.6). Drugs were administered in saline (0.9% [wt/vol] sodium chloride) and infused at a rate of 0.5ml/min. During recording periods, the hands were excluded from the circulation by inflation of the wrist cuffs to 200 mm Hg. Forearm blood flow in response to the endothelium-dependent dilator acetylcholine (ACh; 25, 50 and 100nmol/min; each dose for 3 minutes) was measured 15 minutes after cannulation to establish baseline endothelial function. For some studies the response to the endothelium-independent dilator, glyceryl trinitrate (GTN; 4, 8 and 16 nmol/min; each dose for 3 minutes) was determined.

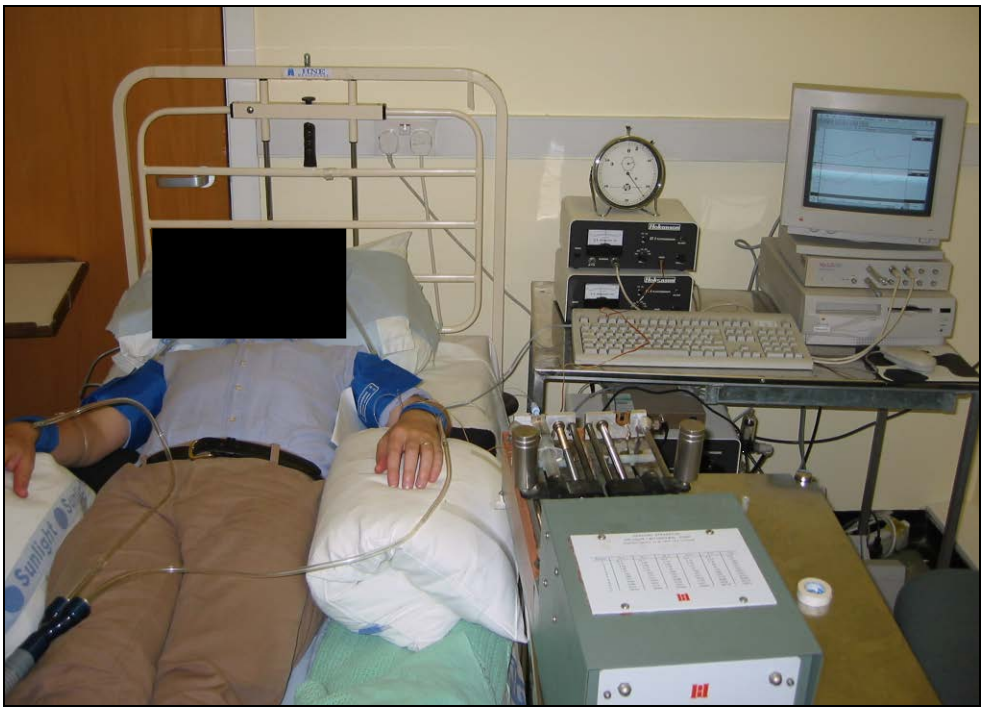


Fig 2.5: *Experimental set up during venous occlusion plethysmography*

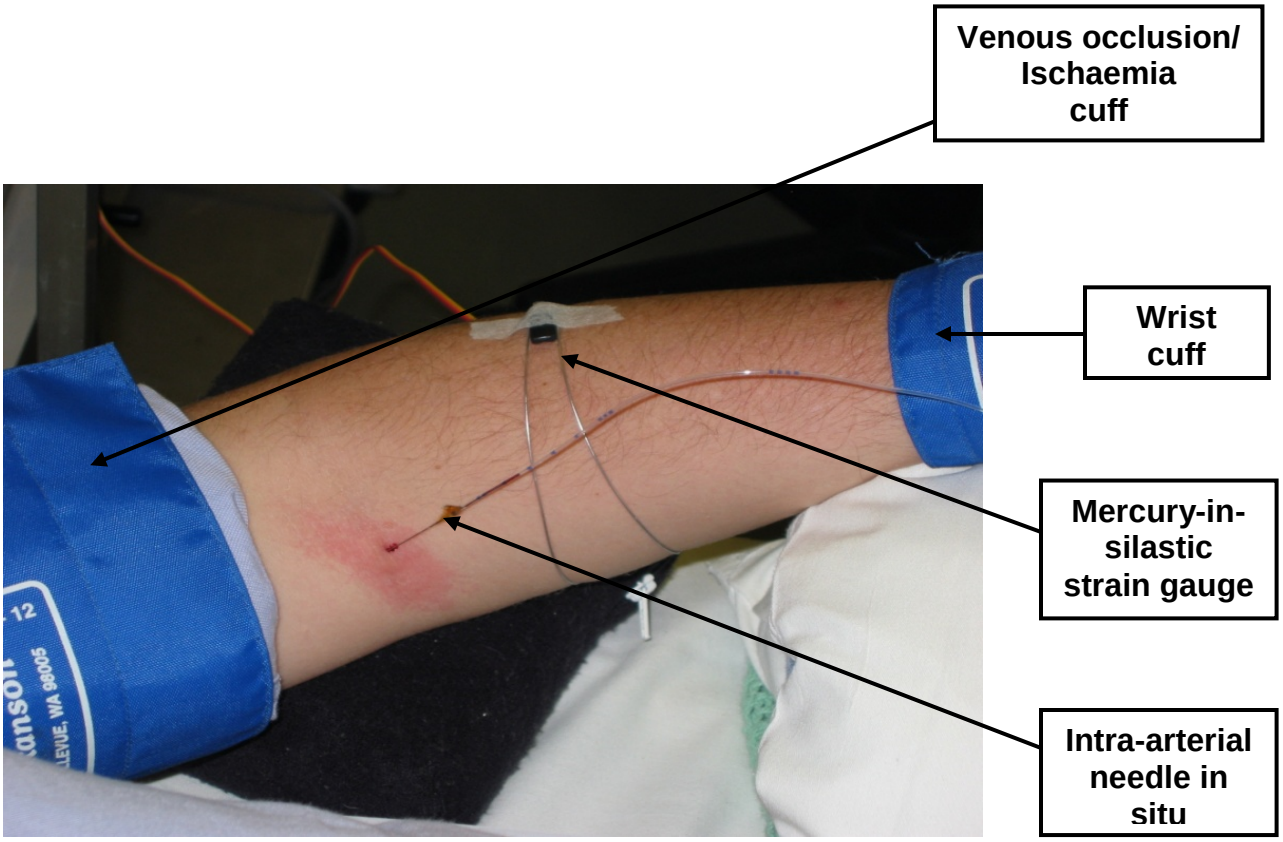


Fig 2.6: *Needle positioning during venous occlusion plethysmography*

2.2.2.3 Data analysis

Calibration of the strain gauges occurred at the start of each experiment for each subject. The mean slope of the last four recordings (over a minute duration) during the infusion of normal saline and each subsequent drug infusion (the last minute of recording of each dose of the infused drug) was used for analysis. Forearm blood flow was expressed as ml (flow)/100 ml (forearm volume)/minute. In this thesis, data were expressed as the percentage change in ratio of blood flow (infused/non-infused arm). This method enabled adjustment for non-specific changes that may change resting blood flow (243). Dose-response curves of percentage change in ratio of blood flow (infused/non-infused arm) against dose of vasodilator (ACh or GTN) were obtained. Other possible methods of expression of this data are as absolute blood flow, or as percentage change in absolute blood flow.

2.2.2.4 Accuracy and reproducibility of technique

Blood flow measured by strain gauge plethsymography correlates with that measured using doppler ultrasound (244). However factors such as mental arousal, sympathetic activity and ambient temperature can contribute to significant difficulty in interpretation of forearm blood flow (FBF) measurements. As a result of considerable intra-individual variation in a single limb (coefficient of variation – CV >30%), FBF measurements are performed in both forearms and expressed as changes in relation to the other (FBF ratio of intervention to control forearms) (245). This eliminates the confounding effects of background changes in FBF and reduces the variability of the technique (CV 20%).

In this thesis, intra-individual variation in baseline FBF ratio was assessed in 13 subjects who had repeated studies between three and seven times on separate

occasions. The mean CV for baseline FBF was 24.4%; n=13. After vasodilatation with acetylcholine (25, 50, 100nmol/min) the mean CV in dose response increased to 31.2%; n=13. These CVs compare with those obtained in other studies (245, 246). There is evidence that endothelial function measurements by plethysmography do not relate to arm length or circumference, but results may be affected by distal misplacement of the strain gauge (246, 247).

2.3 Biochemical assays

Biochemical assays in this thesis were performed at the department of clinical biochemistry, King's College Hospital (KCH) under the supervision of Dr Roy Sherwood and Ms Tracy Dew for plasma beta endorphins and the Wolfson Institute for biomedical research, UCL in collaboration with Dr Adrian Hobbs for plasma nitrates and nitrites.

2.3.1 Measurement of plasma beta endorphins

Endogenous opioids are found in the brain, heart, sympathetic nerves and adrenal medulla. They have been identified as important peptides in the triggering and/or mediation of ischaemic protection, particularly in the myocardium (248). There are three well characterised endogenous opioids (enkephalins, dynorphins, and endorphins) and they interact with G-protein coupled receptors (GPCRs) opioid receptors (δ, μ, κ) to exert their biological effects (249-251).

Beta endorphin is a 31 amino acid peptide first isolated from camel pituitary (252). It is derived from the C-terminal fragment of a 31kDa precursor molecule known as proopiomelanocortin (POMC) and has been found in a variety of tissues including brain, heart, adrenal gland and peripheral nerves (253). Historically, measurement

of plasma levels of beta endorphin was performed by immunologic reactions which involved radioisotope antigen markers (254, 255). Although still used today, this laboratory technique [radioimmunoassay (RIA)] is fraught with problems of safety, complicated set up procedures and inconvenient storage, handling and disposal techniques. More recently, enzyme linked immunosorbent assay (ELISA), which has the advantage of simplicity and ease of use, has been used as a sensitive assay for beta endorphins (254, 255). Importantly, a good correlation (r=0.95) exists between ELISA and RIA for quantitative determination of beta endorphins in plasma (256)

2.3.1.1 Beta endorphin ELISA

This assay is based on the principle of competitive inhibition enzyme immunoassay which was first developed as a two step ELISA technique and had a sensitivity of 10 picograms (3 femtomoles) per well (254). Based on this principle, other researchers have subsequently developed more sensitive ELISA assays for beta endorphin detection in humans (255).

Beta endorphin is a relatively small peptide and competitive inhibition assays are often used for small analytes because of the risk of steric hindrance which may occur with other techniques that entail the binding of two antibodies to the molecule at the same time, as in the monoclonal – polyclonal sandwich immunoassay.

The non-specific binding sites of a secondary antibody, on a pre-coated microtitre immunoplate, are blocked. This enables the secondary antibody to bind to the Fc fragment of the primary antibody (beta endorphin antibody) whose Fab fragment will be competitively bound by both biotinylated beta endorphin and beta endorphin standard or targeted beta endorphin in the sample. The biotinylated beta endorphin is able to interact with streptavidin-horseradish peroxidise (HRP) which catalyzes the

substrate solution composed of 3, 3', 5, 5'- tetramethylbenzidine (TMB) and hydrogen peroxide to produce a blue coloured solution. This enzyme substrate reaction is stopped by hydrogen chloride and the solution turns to yellow. The intensity of the yellow is directly proportional to the amount of biotinylated beta endorphin – HRP complex but inversely proportional to the amount of beta endorphin in standard solutions or samples. This is due to the competitive binding of the biotinylated beta endorphin and the beta endorphin in standard solutions or samples to the beta endorphin antibody (primary antibody). A standard curve of a beta endorphin with known concentration can then be established accordingly. The beta endorphin with unknown concentration in samples can be determined by extrapolation to this standard curve (ref: MD biosciences® beta endorphin ELISA protocol; www.mdbiosciences.com).

2.3.1.2 Preparation of plasma

Venous blood was collected in EDTA tubes containing 312µl aprotinin (0.6 TIU/ml of blood) and shaken gently. This was to prevent clotting and degradation of proteins by proteinases. 4-mls of blood at specified time points during RIPC were collected (details of experimental protocol in chapter 4). Venous blood samples were centrifuged (3000rpm for 15 minutes at 4°C), to obtain plasma. The plasma samples were aliquoted and stored at -80°C until laboratory analysis was performed within one month. Plasma samples were transported to KCH on dry ice.

2.3.1.3 Assay procedure

All reagents were prepared as recommended by the assay manufacturers. Microtitre well plate (Fig 2.8) A-1 was left empty as a blank and 50 µL assay buffer was added into B-1 as total binding.

50 µL standard (1.0 µg of human beta endorphin; Lyophilized) + positive control (0.4-0.6 ng/ml of human beta endorphin lyophilized) were added to the remaining wells in duplicate. 25 µL of primary antiserum (rabbit anti beta endorphin IgG) was added into each well except the blank. 25 µL of biotinylated beta endorphin was added into each well. The well plates were then covered and incubated for 2 hours at room temperature. Thereafter each well (except blank) was washed 6 times with 300 µL assay buffer and blotted dry. 100 µL HRP solution was added to each well (except blank). The wells were then covered and incubated at room temperature. A repeat wash with assay buffer (as above) was performed and then 100 µL of substrate solution (TMB) was added to each well (including blank). The wells were covered and incubated for 1 hour at room temperature. Finally, 100 µL 2N HCl was added to each well (including blank) and absorbance at 450nm was read by a microplate reader.

2.3.1.4 Data analysis

The concentration of beta endorphin in a sample was determined by reference to the standard curve and expressed in ng/ml.

2.3.1.5 Accuracy and reproducibility of the assay

The sensitivity of the assay is 0.15 ng/ml (range 0.15 –1.96ng/ml) and is comparable to other beta endorphin ELISA assays used by other researchers (254,

255). Intra-assay variation of <5-10% and inter assay variation of <15%. Cross reactivity specificity for human beta endorphin was 100%.

2.3.2 Measurement of plasma nitrates and nitrites

The reduction of nitrite and nitrate to NO at room temperature is facilitated by an acidic milieu containing vanadium III. However at room temperature nitrates are only very slowly reduced. Heating to temperatures of 80 – 90 ºC enables the rapid reduction of both nitrite and nitrate (257). This forms the basis of the chemiluminescence detector-based method for trace nitrites and nitrates in aqueous samples. Earlier chemiluminescence methods employed glacial acetic acid and potassium Iodide to reduce nitrite to nitric oxide. However this only resulted in reduction of nitrite but not nitrate, thus providing a method to determine nitrite concentration in the large excess of nitrate in plasma (258).The chemiluminescence analysis method is used in environmental analyses and human fluids where trace nitrite and nitrate data are needed. Other methods to measure nitrite levels in plasma include flow injection analysis (FIA) and high pressure liquid chromatography (HPLC). However ozone chemiluminescence is more sensitive to trace quantities of NO, making it the method of choice when extremely low concentration of nitrites must be quantified in a complex matrix and sample volumes are limited (258, 259).

2.3.2.1 Ozone chemiluminescence analysis of NOx

Acidic vanadium III at 98 ºC quantitatively reduces both nitric acid and nitrate to NO. The released NO is carried by inert gas to a detector where it reacts with ozone to

produce a chemiluminescence signal proportional to the concentration and this is quantified (258).

2.3.2.2 Preparation of samples

Venous blood samples were collected from a large superficial arm vein, at specified time points (see chapter 6) and transferred into heparin tubes containing 5 IU/ml of blood). The samples were centrifuged at 1300G at 4^0C for 15 minutes. The plasma supernatant was separated from the red blood cells and both samples were stored at - 80 ºC until analysis. The blood sampling and centrifugation was performed without delay to prevent the nitrite from rapidly reacting with haemoglobin to form nitrate under oxygenated conditions or iron-nitrosylhaemoglobin under de-oxygenated conditions (259).

2.3.2.3 Experimental procedure

Prior to ozone chemiluminescence, plasma samples were filtered using Microcon® Ultracel YM (3 kDa) filters (Millipore Corporation, Billerica, USA) and then [nitrate] and [nitrite] in the filtrate determined (260). Samples and standards containing nitrite and nitrate were first reduced to NO, which was then quantified using a NO analyzer (NOA 280, Sievers, Boulder, USA). To determine total [nitrite] and [nitrate] (NOx), samples were added to 0.1 mol/L vanadium (III) chloride in 1M hydrochloric acid refluxing at 90°C under nitrogen. Nitrite concentrations were determined by addition of samples to 1.5% potassium iodide in glacial acetic acid under nitrogen at room temperature. Concentrations of nitrate were calculated by subtraction of [nitrite] from NOx values.

2.3.2.4 Data analysis and accuracy

The sensitivity of the ozone chemiluminescence is 1±1nmol/L with a coefficient of variation of 5%. Plasma concentrations (µmol/L) of nitrate and nitrite were determined after construction of standard curves.

2.4 Drugs and reagents

Acetylcholine (ACh) was obtained from Merck Biosciences, Nottingham, UK; Lidocaine Hydrochloride from Antigen Pharmaceuticals, Roscrea, Ireland; 0.9% sodium chloride from Baxter Healthcare, Norfolk, UK; Glibenclamide (Glib) from APS, Eastbourne, UK; Glimepiride (Glim) from Alpharma, Barnstable, UK; Ciclosporin (Sandimmun®) from Novartis Pharma, UK; Naloxone from CP Pharmaceuticals Ltd, Wrexham, UK; Phentolamine from Alliance Pharmaceuticals Chippenham, Wiltshire, UK; Atropine from Antigen Pharmaceuticals, Roscrea, Ireland; Potassium nitrate from Martindale Pharmaceuticals, UK; Potassium Chloride from Martindale Pharmaceuticals, UK; Beetroot juice from Planet Organic; Aprotinin was obtained from Nordic Pharma, UK.

2.5 Calculations and statistical analysis

All data were analysed with GraphPad prism version 4.0 (GraphPad Software, USA) and expressed as mean±SEM. For conduit vessel studies, data were compared using a paired *t* test or ANCOVA as appropriate. For resistance vessel studies, ACh dose-response curves were constructed and the area under the dose-response

curve (AUC) calculated. Comparisons before and after an intervention were made using paired 2-way ANOVA or ANCOVA as appropriate.

A repeated measure ANCOVA was used to compare post-IR values between interventions. Adjustment for baseline FMD and AUC of the baseline dilator response to ACh values was made by including the pre-IR values as a baseline co-variate. Post hoc comparisons between pairs of interventions were performed and adjustment for multiplicity was made using the Scheffe's test. In all cases, a value of $P<0.05$ was considered statistically significant. The D'Agostino-Pearson normality test was used to check for ANCOVA normality assumption.

Sample size calculations used prior estimates of FMD (mean 8.6%, within individual SD of 2.3) and resistance vessel dilatation to ACh (mean area under the dose-response curve to ACh 13,000 units, within subject SD of 6000). To detect a 50% reduction in dilatation, at an α value of 0.05 and a β level of 0.8, required n=7 (FMD) and n=16 (plethysmography).

Chapter 3

The role of the K_{ATP} channel and the mitochondrial permeability transition pore in the mechanism of postconditioning in humans

3.1 Introduction

Ischaemic postconditioning (PostC) elicited by intermittent restoration of blood flow *at the onset of* reperfusion and has been proven to be effective in reducing experimental and clinical IR injury (see section 1.8). PostC is mechanistically similar to ischaemic preconditioning (IPC); however IPC is activated by brief periods of non-lethal ischaemia *in advance of* an injurious ischaemic insult. IPC and PostC cause similar degrees of tissue protection in experimental settings of IR injury, and these observations suggest that much of reversible tissue damage caused by IR injury occurs early in the reperfusion phase. Cardiovascular events are unpredictable and this makes PostC an attractive intervention, especially in acute ischaemic syndromes where there is a degree of mechanical control over the schedule of reperfusion. This has been successfully demonstrated in clinical studies of PostC in ST-segment elevation myocardial infarction (see section 1.8.5).

The control of reperfusion in the majority of acute ischaemic syndromes has remained technically challenging. Pharmacological activation of PostC mechanisms could be a useful adjunct to reperfusion therapy in other domains. Much is known of the mechanism of PostC in animal models, with an important role identified for mitochondria, through the opening of the mitochondrial K_{ATP} channel associated with closure of the mitochondrial permeability transition pore (mPTP) (145, 155, 156). In this chapter, I sought to determine a role for K_{ATP} channels in PostC and a role for inhibition of the mitochondrial permeability transition pore at reperfusion in ischaemic protection. I have used an inhibitor of the mPTP (ciclcosporin) and K_{ATP} channel blockers with different tissue specificity (glibenclamide and glimepride) to achieve this.

3.2 Methods

3.2.1 Subjects

One hundred and thirty nine studies were performed on 24 healthy, non-smoking volunteers aged 18-45 years. Of these, 9 were recruited to the FMD studies and 17 recruited to the venous occlusion plethysmography studies receiving interventions based on K_{ATP} channel blockade. 11 volunteers were recruited to the venous occlusion plethysmography studies receiving interventions based on mPTP blockade.

3.2.2 Assessment of Conduit Vessel Endothelial Function

Brachial artery flow mediated dilatation (FMD) of the right arm was assessed, as described in section 2.2.1.

3.2.3 Assessment of Resistance Vessel Endothelial Function

Bilateral forearm blood flow was measured using mercury-in-silastic strain gauge plethysmography, as described in section 2.3.1

3.2.4 Induction of IR injury

Ischaemia of the non-dominant arm (plethysmography studies)/right arm (vascular ultrasound studies) was achieved as described in section 2.1.1. Uninterrupted reperfusion occurred on cuff deflation.

3.2.5 Induction of Postconditioning

PostC was induced by short periods of intermittent reperfusion to the ischaemic arm in the first 60 seconds of reperfusion as described in section 2.1.3. The alternating deflation/inflation cycle was repeated 3 times (1 minute total duration), after which continuous reperfusion of the arm occurred.

3.3 Experimental Protocols

3.3.1 Effect of IR on Endothelial Function

To determine the effect of IR on endothelial function (EF), brachial artery FMD and forearm blood flow in response to ACh (Figure 3.1a) was assessed before ischaemia (baseline) and at 20 minutes after reperfusion. It has previously been demonstrated that this protocol results in endothelial dysfunction in the brachial artery and forearm resistance vessels (53, 124, 169, 171, 187, 233).

3.3.2 Effect of IR on vascular smooth muscle function

To determine the effect of IR on smooth muscle function in resistance vessels forearm blood flow in response to GTN was assessed before ischaemia and at 20 minutes after reperfusion (Figure 3.1b). Previous studies in this laboratory have demonstrated that this protocol did not result in vascular smooth muscle dysfunction in conduit vessels (187, 261).

3.3.3 Effect of postconditioning on endothelial IR Injury

To establish that protection against endothelial IR can be achieved by modifying reperfusion, PostC was induced in the same group of volunteers. The PostC schedule was applied at the onset of reperfusion, immediately after index ischaemia (Figure 3.1c)

3.3.4 Mechanism of protection by PostC in humans: Role of K_{ATP} channels

The effect of K_{ATP} channel blockade on PostC was investigated using the 5mg oral glibenclamide [non-selective K_{ATP} channel blocker] and 1mg oral glimepiride [pancreas-selective K_{ATP} channel blocker] (262-264). Drugs were administered 45 minutes before assessment of baseline endothelial function of conduit and resistance vessels (Figure 3.2a) (171, 233). This was followed by 20 minutes of arm ischaemia, PostC and a repeat assessment of endothelial function. To exclude a direct vascular effect of either K_{ATP} channel blocker on the endothelial response to IR injury, endothelial function was determined before and after IR injury in the presence of glibenclamide (n=4) or glimepiride (n=5), (Figure 3.2b). In all studies a high-carbohydrate meal (59g carbohydrates; 15g fat; 2.4g protein; 386 Kcal) was given immediately and 3 hours (115g carbohydrates; 50g total sugars; 20g fat; 27g protein; 750 Kcal) after the administration of glibenclamide and glimepiride (171, 233). Blood glucose was monitored throughout the study.

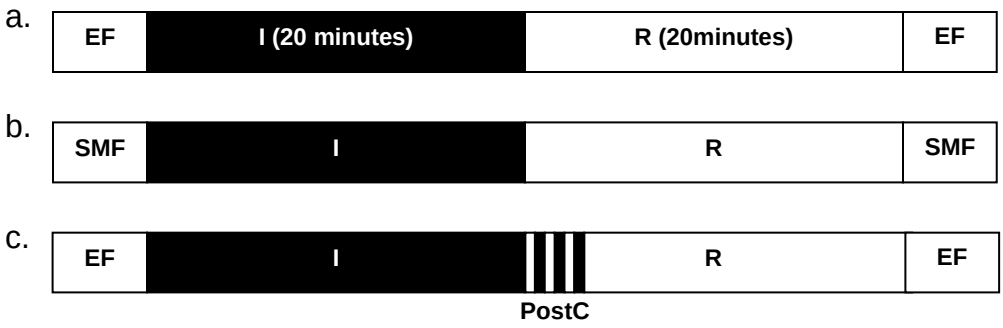


Figure 3.1: ***Protocol of studies to determine the effect of PostC on endothelial IR-injury*** *(a) Endothelial function (EF) in the brachial artery and forearm resistance vessels was assessed before 20 minutes of arm ischaemia (I) and at 20 minutes of reperfusion (R). (b)The vascular smooth muscle function (SMF) in the forearm resistance vessels was assessed before and after IR injury. The effect of PostC was determined (c) by applying 3 cycles of 10 seconds of arm reperfusion and 10 seconds of arm ischaemia.*

Figure 3.2: ***Protocol of studies to determine the role of K_{ATP} Channels in PostC*** *(a) To determine the effect of Glibenclamide on PostC; Baseline EF was assessed in the brachial artery (FMD) and resistance vessels (dose response to ACh). 3 cycles of PostC were applied after 20 minutes of ischaemia of the arm (at the onset of reperfusion) in the presence of oral glibenclamide 5mg which was administered 45 minutes before IR. EF was reassessed at 20 minutes of reperfusion. To determine the effect of glimepiride on PostC; Baseline EF was assessed in the brachial artery and resistance vessels. 3 cycles of PostC were applied after 20 minutes of ischaemia of the arm in the presence of oral glimepiride 1mg which was administered 45 minutes before IR. EF was reassessed at 20 minutes of reperfusion. (b) Experiments to exclude any direct effects of glibenclamide or glimepiride on endothelial response to IR injury.*

3.3.5 Effect of inhibition of the mPTP on endothelial IR Injury

In cohort 2 (n=11), the role of the mPTP in PostC was assessed using ciclosporin (non-selective mPTP blocker) administered intra-arterially at reperfusion. Baseline response to ACh was assessed before and after IR injury as above (Figure 3.3a).

On a separate study day, subjects underwent IR injury, during which ciclosporin (Sandimmun-Novartis; 0.6µmol/min), was administered during the last 2 minutes of ischaemia and the first minute of reperfusion (Figure 3.3b). After 20 minutes of reperfusion the response to ACh was repeated. To assess for a direct effect of ciclosporin on endothelial function, the protocol was repeated without IR (Figure 3.3c).

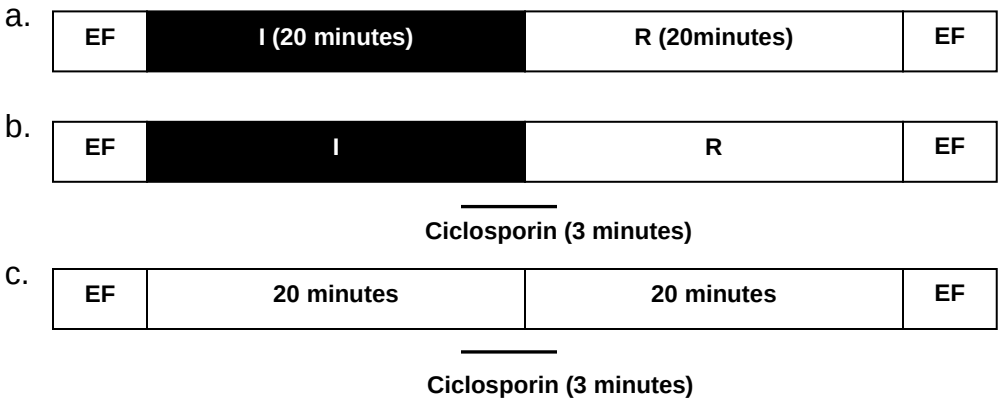


Figure 3.3: ***Protocol of studies to determine the role of the mPTP in PostC.*** *(a) Endothelial function (EF) in the forearm resistance vessels was assessed before 20 minutes of arm ischaemia (I) and at 20 minutes of reperfusion (R) (b) Baseline EF was assessed in the resistance vessels (dose response to ACh). To determine if mPTP inhibition mimics, the mPTP inhibitor ciclosporin (0.6µmol/min) was infused into the brachial artery during the last 2 minutes of ischaemia and the first minute of reperfusion (3 minutes in total). EF function was reassessed at 20 minutes of reperfusion.*

3.4 Calculations and Statistical Analysis

See section 2.5 of chapter 2.

3.5 Results

All subjects tolerated the procedure. There was no significant difference in blood glucose between the glibenclamide- and glimepiride- treated subjects (Figure 3.4). Symptomatic hypoglycaemia was treated in two subjects in the glibenclamide group and one subject in the glimepiride group. There were no effects on blood pressure,

heart rate, baseline brachial artery diameter or FMD flow stimulus during reactive hyperaemia (Table 3.1).

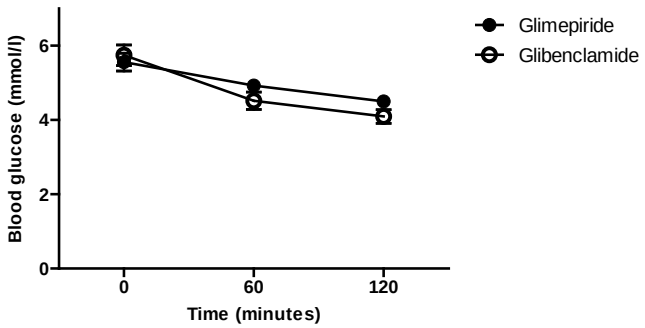


Figure 3.4: *Comparison of blood glucose concentration (mmol/l) in glimepiride and glibenclamide studies (p=0.2; Paired t test).*

3.5.1 Effect of IR on endothelial function

IR reduced brachial artery FMD (7.1±0.9% before IR vs 2.8±0.4% after IR, P<0.001; Paired t test; n=9) [Figure 3.5a] and resistance vessel forearm blood flow (P=0.0014; ANOVA; n=17) [Figure 3.5c]. In the KATP studies, a significant correlation existed between baseline and post-IR values for FMD (Pearson's r=0.6, P< 0.001) and vasodilator response to ACh AUC (Pearson's r=0.7, P<0.0001). In the mPTP studies, a significant correlation existed between baseline and post-IR values for the vasodilator response to ACh AUC (Pearson's r=0.7, P<0.001). These correlations justified using ANCOVA to adjust post-IR values for differences in baseline endothelial function (EF) between the different protocols, so that post-IR EF could be directly compared with greater statistical power (Tables 3.2, 3.4 and 3.5)

	IR Alone *(n=9)*		***IR+PostC*** *(n=9)*		***IR+PostC+Glib*** *(n=9)*		***IR+PostC+Glim*** *(n=9)*	
	Pre	*Post*	*Pre*	*Post*	*Pre*	*Post*	*Pre*	*Post*
SBP (mmHg)	105±3	110±2	114±5	116±4	113±4	117±5	111±3	114±4
DBP (mmHg)	63±2	65±2	61±2	65±2	61±2	64±2	63±3	66±2
HR (bpm)	67±5	66±5	65±3	63±3	64±3	64±3	66±3	65±2
Baseline arterial diameter (mm)	3.6±0.1	3.5±0.1	3.6±0.1	3.6±0.1	3.6±0.1	3.6±0.1	3.5±0.1	3.6±0.1
Flow Stimulus (no units)	10.1±1.1	10.4±1.3	11.6±0.9	10.6±1.3	7.5±0.4	10.5±1.2	8.1±0.4	9.3±0.4

Table 3.1: *Summary of blood pressure, heart rate, baseline brachial artery diameter and FMD flow stimulus (no units) during reactive hyperaemia.*

3.5.2 Effect of PostC on endothelial IR injury

PostC prevented the IR induced endothelial dysfunction in the brachial artery FMD (6.8%±0.9% before IR+PostC vs 6.1±0.7% after IR+PostC, p>0.05; n=9) [Figure 3.5b] and resistance vessels (P=0.38; n=16) [Figure 3.5d]

3.5.3 Effect of IR on vascular smooth muscle function

IR did not affect vascular smooth muscle function in the resistance vessel (P=0.92; n=9) [Figure 3.5e].

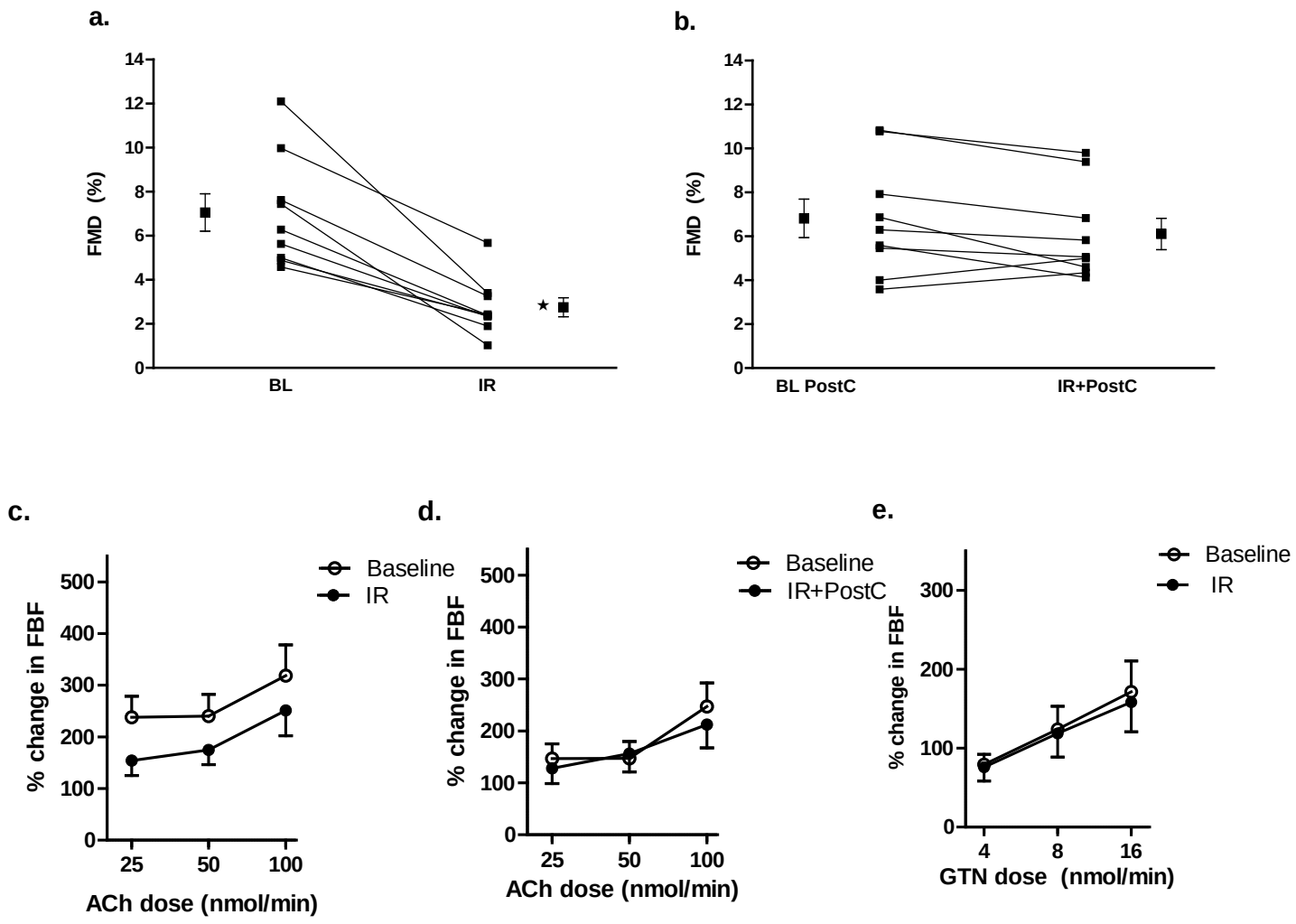
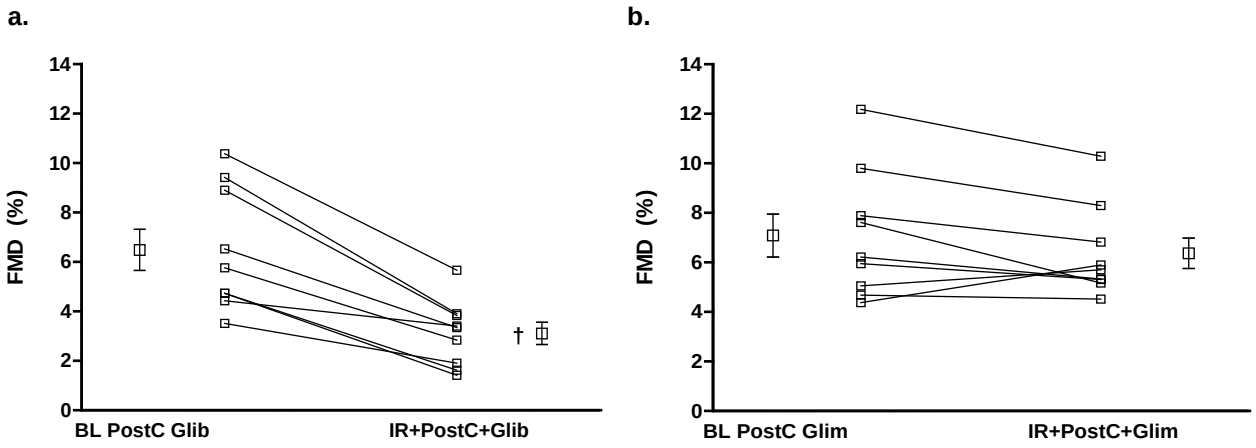


Figure 3.5: *IR reduced endothelial function in the (a) brachial artery (FMD 7.1±0.9% pre-IR versus 2.8±0.4% post-IR; *P<0.001; n=9). The IR induced endothelial dysfunction was prevented by PostC, in the (b) brachial artery (FMD 6.8%±0.9% pre- versus 6.1±0.7% post-IR+PostC; P>0.05; n=9). Similarly, IR induced endothelial dysfunction and in the (c) resistance vessels (*P=0.0014; n=18)* and this was also prevented by PostC *(d) (P=0.38; n=16). (e) IR did not affect vascular smooth muscle function in resistance vessels (P=0.92; n=9). BL=baseline; FBF=forearm blood flow.*

3.5.4 Mechanism of Protection by PostC: Role of K_{ATP} channels

Glibenclamide abolished the protection induced by PostC against IR-induced endothelial dysfunction in the brachial artery (FMD 6.5±0.8% before IR + PostC + glibenclamide vs 3.1±0.4% after IR + PostC + glibenclamide, $p<0.05$; n=9) [Figures 3.6a and 3.8] and resistance vessels (P=0.014; n=9) [Figures 3.6c and 3.8]. In contrast, glimepiride had no effect on PostC in the brachial artery (FMD 7.1±0.9% before IR + PostC + glimepiride vs 6.4±0.6% after IR + PostC + glimepiride, $p>0.05$; n=9) [Figures 3.6b and 3.8] or resistance vessels (P=0.56; n=10) [Figures 3.6d and 3.9]. Neither glibenclamide (FMD 6.3±1.5 pre- versus 2.9±1.0 post-IR+Glib; P=0.03; n=4) [Figure 3.7a] nor glimepiride (FMD 6.9±1.1 pre- versus 3.6±0.7 post-IR+Glim; P=0.001; n=5) [Figure 3.7b] had any effect on the endothelial response to IR injury.



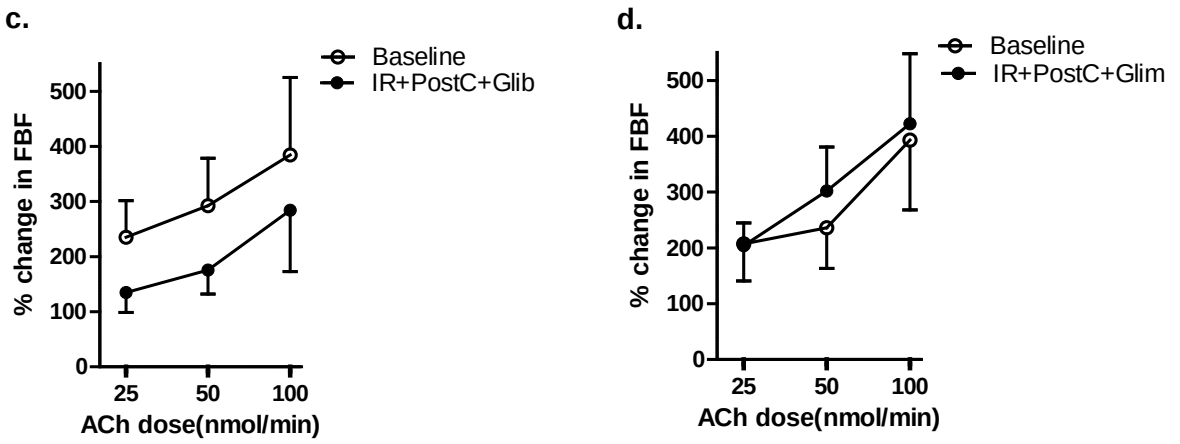


Figure 3.6: *In the presence of glibenclamide, the protection by PostC against IR was lost in the (a) brachial artery (FMD 6.5±0.8% pre- versus 3.1±0.4% post IR+PostC+Glib, †P<0.05; n=9) and (c) in the resistance vessels (P=0.014; n=9). In the presence of glimepiride the protection by PostC was preserved in the (b) brachial artery (FMD 7.1±0.9% pre- versus 6.4±0.6% post-IR+PostC+Glim, P>0.05; n=9) and (d) resistance vessels (P=0.56; n=10).*

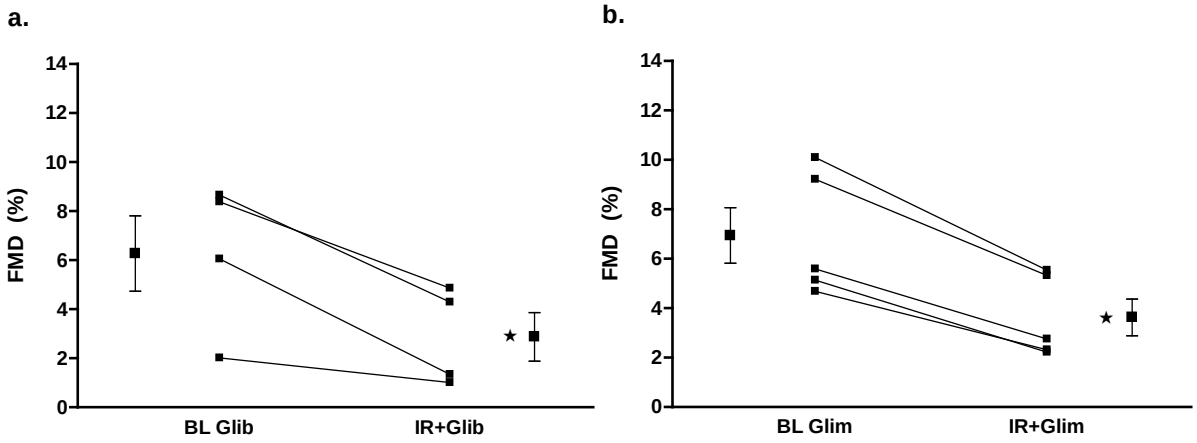


Figure 3.7: *Endothelial response to IR injury was not affected by (a) Glibenclamide (FMD 6.3±1.5 pre- versus 2.9±1.0 post-IR+Glib; P<0.05; n=4) or (b) Glimepiride (FMD 6.9±1.1 pre- versus 3.6±0.7 post-IR+Glim; P<0.01; n=5).*

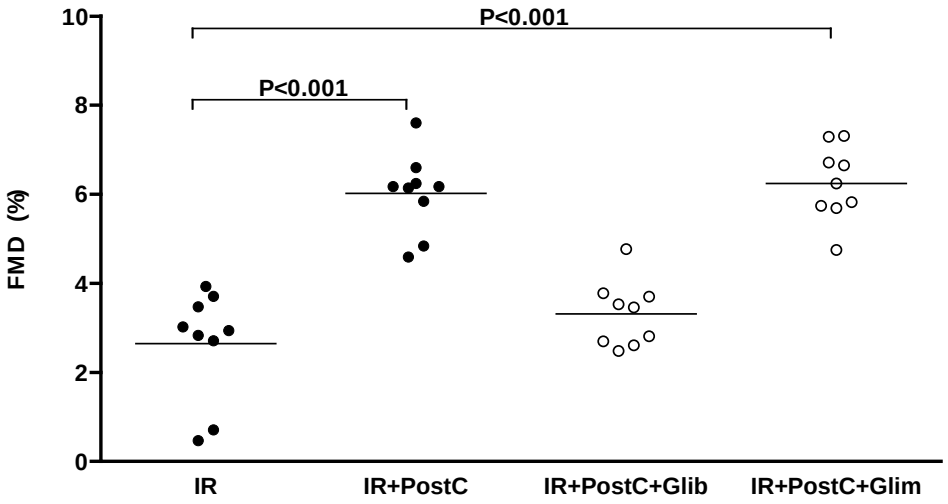


Figure 3.8: *Role of K_{ATP} channels in the mechanism of protection by PostC in the brachial artery. PostC prevented IR-induced reduction in FMD (post-IR FMD, 6.0±0.3%, n=9). The protective effects of PostC were abrogated by systemic glibenclamide (post-IR FMD, 3.3±0.2%, n=9). In the presence of glimepiride PostC prevented IR-induced reduction in FMD (post-IR FMD, 6.2±0.3%, n=9). Post-IR values were adjusted for baseline FMD (ANCOVA).*

.

Study	n	Baseline FMD	Post-IR FMD	Post-IR FMD (ANCOVA Adjusted)
IR	9	7.1±0.9	2.8±0.4	2.6±0.4*
IR+PostC	9	6.8±0.9	6.1±0.7	6.0±0.3†
IR+PostC+Glibenclamide	9	6.5±0.8	3.1±0.4	3.3±0.2‡
IR+PostC+Glimepiride	9	7.1±0.9	6.4±0.6	6.2±0.3

Data are expressed as mean±SEM. Post-IR FMD values were adjusted for baseline FMD by ANCOVA (Regression coefficient 0.56±0.13). For comparisons between the 4 groups, P values by ANCOVA were Scheffé adjusted.

**P*<0.001, IR vs IR+PostC and IR+PostC+Glimepiride
†*P*<0.001, IR+PostC vs IR+PostC+Glibenclamide
‡*P*<0.001, IR+PostC+Glibenclamide vs IR+PostC+Glimepiride

Table 3.2: *Summary of Baseline and Post-IR FMD data*

Study	n	Baseline AUC	Post-IR AUC	Post-IR AUC (ANCOVA Adjusted)
IR	17	2.1±0.4	1.5±0.2	1.0±0.1*
IR+PostC	16	1.3±0.3	1.3±0.3	1.9±0.2†
IR+PostC+Glibenclamide	9	2.4±0.7	1.5±0.3	1.1±0.2‡
IR+PostC+Glimepiride	10	2.1±0.6	2.4±0.6	2.0±0.2

Data are expressed as mean±SEM. Post-IR dilator response to ACh AUC values ($x10^4$) were adjusted for baseline AUC by ANCOVA (Regression coefficient 0.55±0.07). For comparisons between the 4 groups, P values by ANCOVA were Scheffé adjusted.

*P<0.001, IR vs IR+PostC and IR+PostC+Glimepiride
†P<0.001, IR+PostC vs IR+PostC+Glibenclamide
‡P<0.01, IR+PostC+Glibenclamide vs IR+PostC+Glimepiride

Table 3.3: *Summary of Baseline and Post-IR dilator response to ACh AUC data*

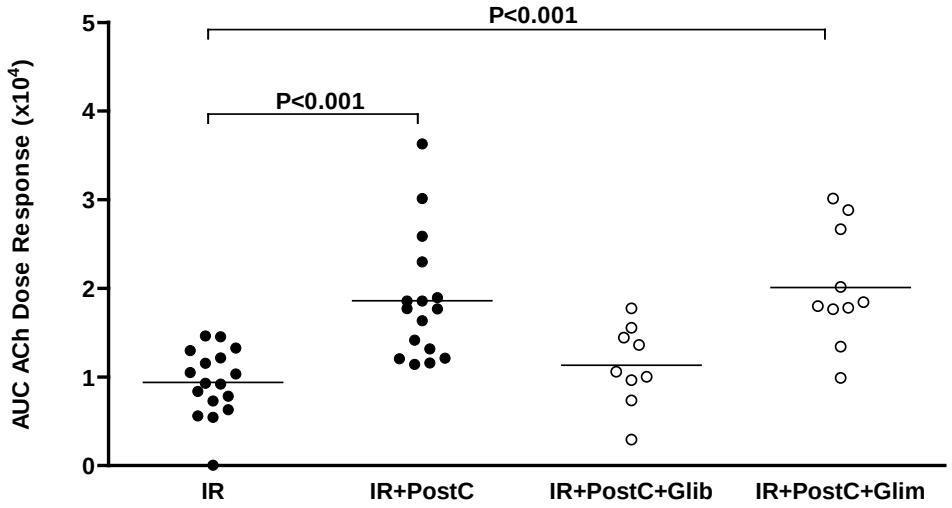


Figure 3.9: *Role of K_{ATP} channels in the mechanism of protection by PostC in the resistance vessels. PostC prevented IR-induced reduction in the dilator response to ACh (Post-IR AUC 1.9±0.2 x 10^4; n=16). The protective effects of PostC were abrogated by systemic glibenclamide (Post-IR AUC 1.1±0.2 x 10^4; n=9). In the presence of glimepiride, PostC prevented the IR induced reduction in dilator response to ACh (Post-IR AUC 2.0±0.2 x 10^4; n=10). Post IR values were adjusted for baseline dilator response to ACh AUC (ANCOVA). AUC=area under curve; FBF=forearm blood flow.*

3.5.5 Effect of inhibition of the mPTP on endothelial IR Injury

IR reduced the vasodilator response to ACh (P=0.006; n=11) [Figures 3.8a and 3.11]. Infusion of ciclosporin around the onset of reperfusion mimicked the protective effect of PostC (p=0.44; n=11) [Figure 3.8b and 3.11]. Ciclosporin had no direct effect on endothelial function (p=0.76; n=11) [Figure 3.8c].

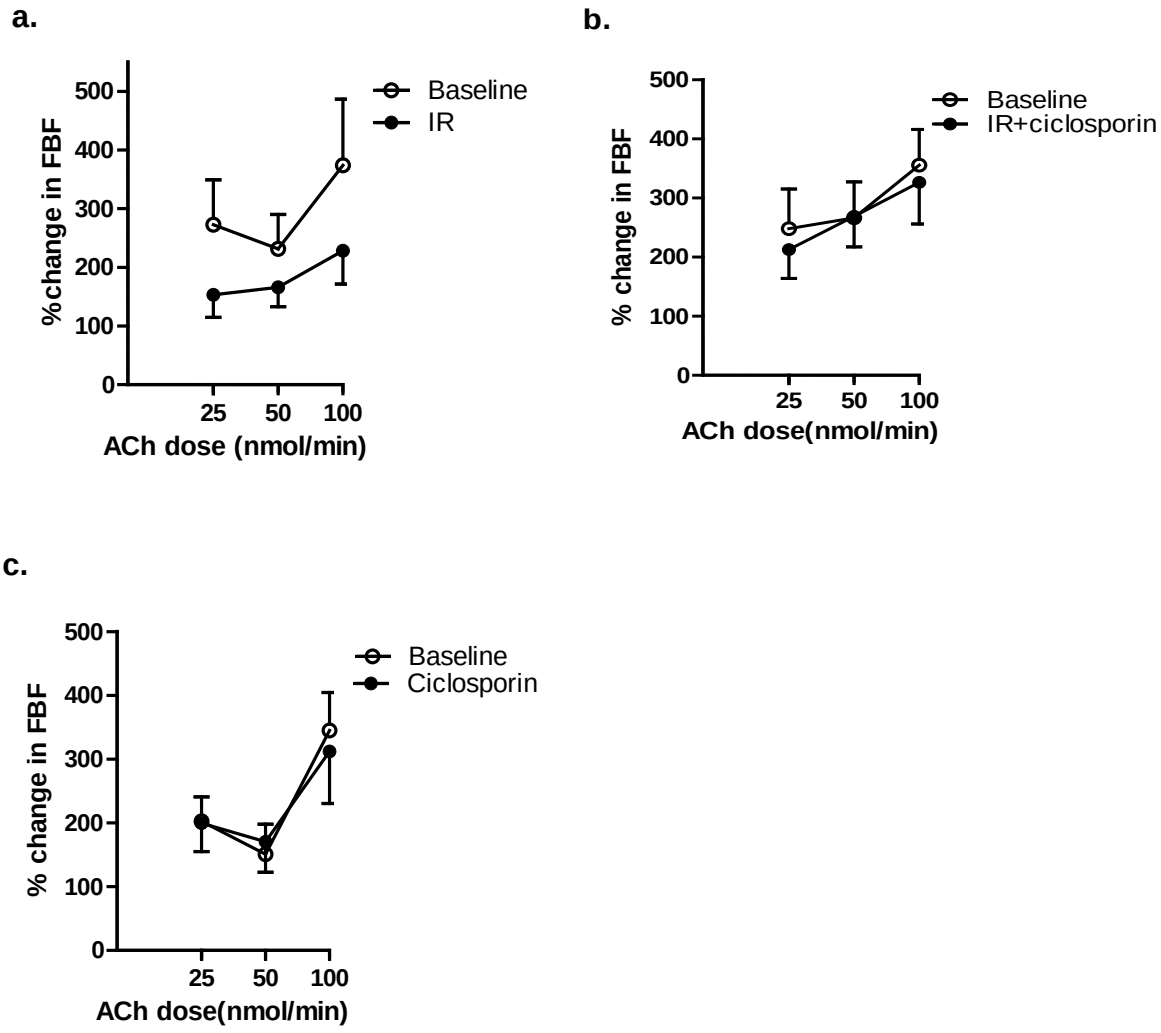


Figure 3.10: *(a) IR caused a reduction in endothelial function* (P=0.006; n=11) *(b) Administration of Ciclosporin around the onset of reperfusion protected against endothelial IR injury in the resistance vessels (P=0.44; n=11). (c) Ciclosporin had no effect on endothelial function (P=0.76; n=11).*

Study	n	Baseline AUC	Post-IR AUC	Post-IR AUC (ANCOVA Adjusted)
IR	11	2.3±0.5	1.4±0.3	1.4±0.2*
IR+Ciclosporin	11	2.2±0.4	2.1±0.4	2.2±0.3

Data are expressed as mean±SEM. Post-IR dilator response to ACh AUC values ($x10^4$) were adjusted for baseline AUC by ANCOVA (Regression coefficient 0.53±0.12).

**P*<0.05, IR vs IR+Ciclosporin

Table 3.4: Summary of Baseline and Post-IR dilator response to ACh AUC data (Cohort 2)

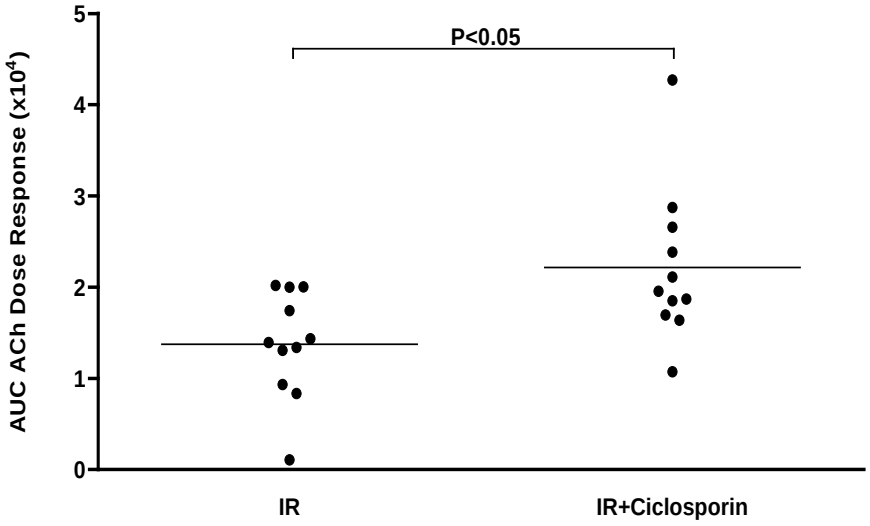


Figure 3.11: *Role of the inhibition of the mPTP in protection against IR-induced endothelial dysfunction in resistance vessels. The IR-induced reduction in dilator response to ACh (Post-IR AUC 1.4±0.2 x 10^4; n=11) was prevented by ciclosporin administered around the onset of reperfusion (Post-IR AUC 2.2±0.3 x 10^4; n=11). Post IR values were adjusted for baseline dilator response to ACh AUC (ANCOVA).*

3.6 Discussion

This chapter demonstrates that PostC is dependent on K_{ATP} channel activity. In conduit and resistance vessels, the non-selective K_{ATP} channel blocker, glibenclamide abolished protection. In contrast, glimepiride, a K_{ATP} blocker that is selective for non-vascular tissues, had no effect on PostC. Furthermore, this study demonstrates that protection by PostC is mimicked by inhibition of the mPTP, as administration of the non-specific inhibitor of mPTP – ciclosporin, around the onset of reperfusion, protected against endothelial IR injury.

3.6.1 PostC protects against endothelial IR injury

IR injury to any organ causes damage directly to the tissue parenchyma, but a vascular component of IR injury can interfere with reperfusion and so contribute to tissue damage. This vascular injury, mainly in the form of endothelial dysfunction, has been well described in both animal and human models (see sections 1.3 & 1.6 of chapter 1). In the present study I used IR-induced endothelial dysfunction as a convenient proxy to explore the mechanism of PostC in humans. As demonstrated previously, IR had no effect on vascular smooth muscle in resistance vessels (187, 261). Also consistent with previous work, results of this chapter demonstrate that PostC preserves endothelial function in conduit vessels exposed to IR, and extends these findings to the resistance vasculature (124). It is possible that these vasculoprotective effects in conduit and resistance vessels contribute to the clinical effect of PostC to reduce tissue injury in patients.

3.6.2 Mechanisms of protection by PostC

Studies of the mechanisms of PostC in the mammalian heart have revealed a number of themes. There is involvement of endogenous ligands which include adenosine, bradykinin and opioids to trigger protection which is then dependent on activation of a number of intermediate pathways, including the NO/cGMP pathway, reperfusion injury salvage kinases (RISK pathway) and K_{ATP} channels. Ultimately, modulation of mitochondrial energetics (critical for many types of ischaemic protection) seems to be a key aspect of PostC, because inhibition of the mitochondrial permeability transition pore (mPTP) at reperfusion mimics PostC (156, 265). These pathways, identified in animal models, provide opportunities to pharmacologically probe the mechanism of PostC in humans.

3.6.3 A role for K_{ATP} channels in PostC in humans

It has previously been shown in humans that K_{ATP} channel activation mimics and blockade of K_{ATP} channels inhibits ischaemic preconditioning in humans (233). The results of this chapter demonstrate that glibenclamide (non-selective K_{ATP} channel blocker) largely abolishes PostC-induced protection against endothelial IR injury, in conduit and resistance vessels. These data implicate the K_{ATP} channel in the mechanism of PostC, and are consistent with animal data (145, 154-156, 266, 267). The molecular structure of K_{ATP} channels offers an explanation for the differential effect of glibenclamide and glimepiride (see section 1.4.3 of chapter 1). K_{ATP} channels in cardiac and vascular tissue comprise mainly $K_{IR}6.2$/SUR2A and $K_{IR}6.1$/SUR2B channels respectively, and are more sensitive to non-selective blockade by glibenclamide (262, 263). In contrast, glimepiride preferentially blocks $K_{IR}6.2$/SUR1 channels, which predominate in the pancreas. Animal and human

studies have demonstrated that glimepiride has fewer cardiac actions compared to glibenclamide (262-264, 268, 269). I observed a clear effect of glibenclamide but not glimepiride to abolish the protective effects of PostC in both vascular beds studied. The results are not easily explained by differences in dose, as the hypoglycaemic effect of both drugs was similar (Figure 3.3). Epidemiological studies have not consistently identified whether glibenclamide increases the risk of ischaemic tissue damage when used to treat diabetes, but the differential effects of K_{ATP} channel blockers identified in this study provide a theoretical basis for choosing between them. Although some studies suggest that glimepiride may induce ischaemic protection by other mechanisms, my results do not show this in humans *in vivo* (270, 271).

3.6.4 Mimicking PostC by mPTP inhibition at reperfusion

Although the exact molecular structure of the mPTP is yet to be determined its role in cell death after IR injury is well recognised (272). During ischaemia the mPTP remains closed but at reperfusion it opens (32). Opening of the mPTP leads to inability of the cells to generate ATP for the repair of damage caused by the Ca^{2+} dependent proteases, nucleases and phospholipases activated during IR injury, a process that contributes to cell death (24).

This chapter demonstrates that the non-specific mPTP inhibitor – ciclosporin administered intra-arterially at a dose of 0.6µmol/min around the time of reperfusion protects against endothelial IR injury in human forearm resistance vessels. A key aspect of the study design was to ensure that ciclosporin was administered close to the moment of reperfusion. The cumulative dose of ciclosporin used was comparable to that which achieved a protective effect in similar studies in isolated rat

hearts and human atrial tissue (273, 274). This was in an attempt to limit the intra-arterial dose of ciclosporin to subsystemic levels and also to avoid higher doses that may lead to a reversal of the protective effect (273). However, experimental evidence suggests that mPTP opening can be transient and may not necessarily lead to cell death (275, 276). Therefore, a plausible explanation for my results is that ciclosporin afforded protection in this model of IR injury by inhibition of long lasting mPTP opening.

Available evidence suggests that PostC induces protection against IR injury by modifying early stages of reperfusion; such that a delay in the application of the PostC stimulus for as short as 1 minute results in loss of the protection (124). Protection against IR injury by pharmacologically inhibiting the mPTP at reperfusion suggests this as a possible mechanism of PostC. These data are in agreement with animal data in which mPTP inhibitors such as ciclosporin, NIM811, debio-025 and sanghliferin A, given at the time of reperfusion after prolonged ischaemia, limited IR injury (156, 277, 278). The possibility of a direct effect of ciclosporin on endothelial function was considered, but my data show that ciclosporin had no direct effect on endothelial function. In a study in patients presenting with acute myocardial infarction, ciclosporin administered around the time of reperfusion caused a significant reduction of creatine kinase a biochemical marker of myocardial infarct size (157). My results provide further evidence that ciclosporin might be exploited as an adjuvant during reperfusion therapy in humans.

3.6.5 Conclusion

In summary, these data provide further support for ischaemic postconditioning as an effective protective strategy against IR injury in humans. In addition, an immediate

therapeutic implication of my results is that preserving vascular K_{ATP} channel activity will be necessary to facilitate the development of new treatments, irrespective of whether they are based on old or new drugs. Evidence from my study and the study in patients presenting with myocardial infarction suggests that inhibition of the mPTP to limit IR injury in humans might be clinically useful.

Chapter 4

The role of endogenous opioids in the transfer of ischaemic protection by remote conditioning stimuli

4.1 Introduction

Ischaemic preconditioning (IPC) protects against damage from ischaemia-reperfusion (IR) injury. Though first noted to cause localized protection, it is now evident that there is a cotemporaneous systemic mechanism that is activated by IPC and which causes ischaemic protection in tissues remote from those undergoing IPC. Two variants of remote protection have been identified; remote ischaemic preconditioning (RIPC), where the preconditioning stimulus is applied before IR injury and remote ischaemic postconditioning (RPostC) where the preconditioning stimulus is applied during IR injury. The discovery that limb ischaemia activates RIPC and RPostC has led to their validation in humans, and a number of clinical trials have reported promising biological effects of RIPC in patients (189).

Understanding the mechanism whereby protection spreads to distant tissues has scientific and therapeutic implications. In animals and humans, a neuronal mechanism has been proposed, because RIPC-induced protection is abolished in the presence of ganglionic blockade (178, 179,187). There is also evidence for haematogenous spread of RIPC; ischaemic protection transfers in blood to preconditioning-naïve animals (177, 279). In humans, plasma extract obtained after activation of RIPC pathways contains a factor that reduces IR injury in vitro (174). The identity of the transferable factor(s) remains unknown, though it is opioid-dependent and crosses species (174, 177, 279). It has not yet been possible to demonstrate in humans that there is inter-individual haematogenous spread of remote ischaemic protection. This reflects the logistical problems and safety concerns of using blood or blood products to transfer protection between individuals. However differences between IPC, RIPC and RPostC might enable the question of intra-individual haematogenous spread to be addressed. Local protection of IPC is

independent of any circulating factor, the key difference in its mechanism compared to RIPC (280). In addition, the model of RPostC that I used in this thesis uses a remote postconditioning stimulus simultaneous with the ischaemic phase of IR injury. The consequence of this is that the injured tissue is isolated from the circulation during induction of remote ischaemic protection. This scheduling of protective stimulus and ischaemic injury minimises the influence of any circulating factor. Therefore, in this chapter I undertook experiments to test the following hypotheses in healthy volunteers;

a. RIPC requires the activation of opioid pathways in vivo and is blocked by the opioid antagonist naloxone

b. IPC and RPostC do not require a circulating factor and are not blocked by opioid antagonism

4.2 Methods

97 studies were performed on 19 healthy non-smoking male volunteers aged between 18 and 45 years who were recruited from the University College London staff and student community. 9 volunteers participated in the RIPC and RPostC studies (cohort 1) while 10 volunteers participated in the IPC studies (cohort 2). FMD was used to assess brachial artery endothelial function (section 2.2.1) and measurement of plasma beta endorphins was performed by ELISA (section 2.3.1).

4.2.1 Induction of IR injury

IR injury was induced as described in section 2.1.1.

4.2.2 Induction of remote preconditioning

RIPC was performed as described in section 2.1.4.

4.2.3 Induction of remote postconditioning

RPostC was performed as described in section 2.1.5.

4.2.4 Induction of ischaemic preconditioning (IPC)

This was performed as described in section 2.1.2.

4.2.5 Assessment of plasma beta endorphins

Volunteers were seated comfortably and a 12-cm blood pressure cuff was placed around the upper part of the thigh. A 19G butterfly needle was placed into a vein in the upper arm and a second was positioned into a vein in the leg. Blood samples, at specific time points (see below) were collected in EDTA tubes that contained 312µl aprotinin (0.6 TIU/ml of blood) and centrifuged to obtain plasma. The plasma samples were aliquoted and stored at -80°C until laboratory analysis was performed (see section 2.3.1).

4.3 Experimental protocols

4.3.1 Effect of IR on endothelial function

To determine the effect of IR on endothelial function (EF), brachial artery FMD was assessed before ischaemia (baseline) and at 20 minutes after reperfusion [Figure 4.1a].

4.3.2 Effect of remote preconditioning on endothelial IR Injury

FMD was assessed before and after IR but immediately preceded by ArmRIPC and LegRIPC to establish that protection against endothelial IR can be achieved by two different RIPC stimuli [Figure 4.1b & c].

4.3.3 Effect of remote postconditioning on endothelial IR Injury

FMD was assessed before and after IR but during the index ischaemia the RPostC stimulus was applied. This was to establish that protection against endothelial IR can be achieved by RPostC [Figure 4.1d].

4.3.4 Effect of ischaemic preconditioning on endothelial IR Injury

FMD was assessed before and after IR but immediately preceded by local IPC to establish that protection against endothelial IR can be achieved by IPC [Figure 4.1e].

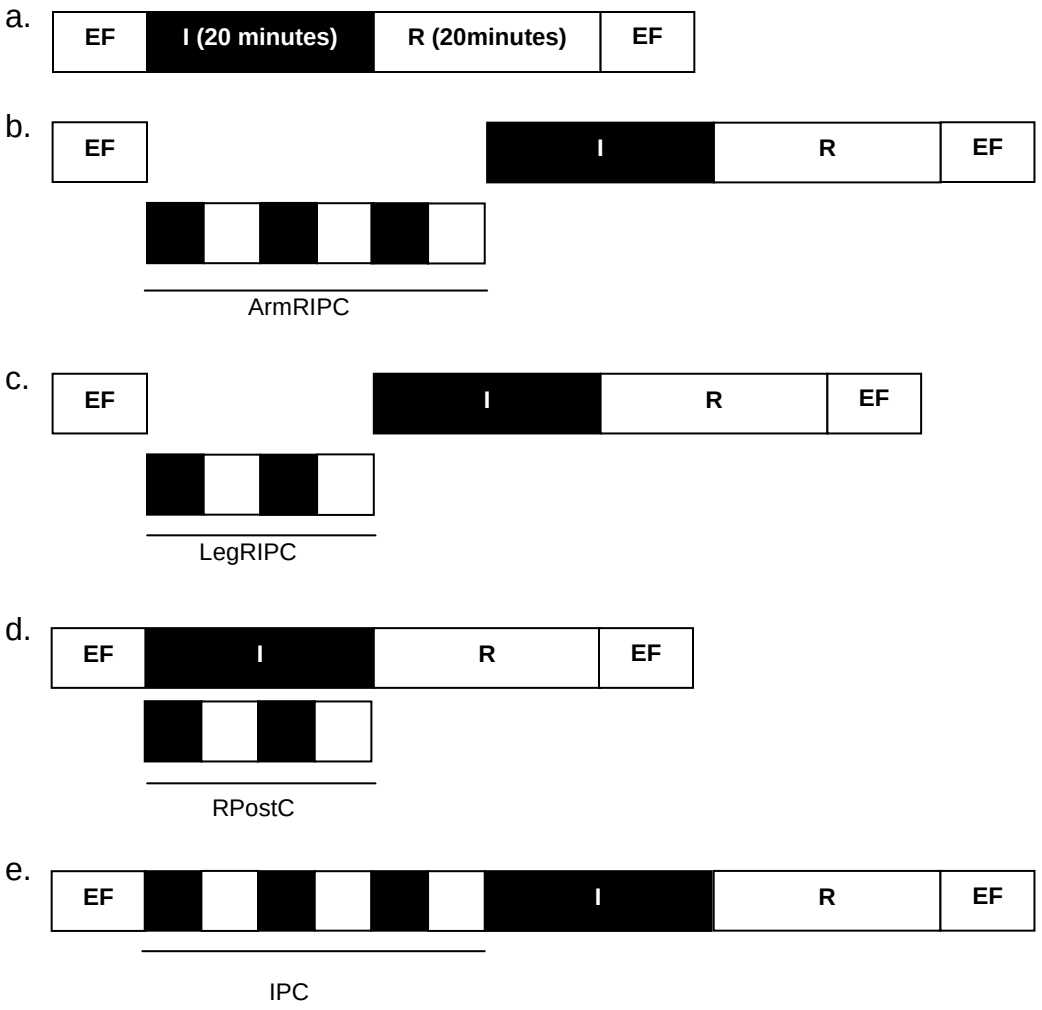


Figure 4.1: *Protocols to determine the effect of (a) IR on endothelial function (EF), (b) ArmRIPC on endothelial IR injury; (c) LegRIPC on endothelial IR injury; (d) RPostC on endothelial IR injury and (e) IPC on endothelial IR injury.*

4.3.5 Effect of opioid receptor blockade on RIPC, RPostC and IPC

The non-selective opioid receptor blocker, naloxone was administered intravenously at a bolus dose of 6mg, then a continuous infusion at a dose of 0.1 mg/min throughout the duration of the protective stimulus [Figures 4.2 a - d]. This dose has been used in a previous study in patients and was determined by the increase of β-endorphin in the plasma to levels that indicate effective opioid receptor blockade (281). This protocol was to establish the role of opioid receptors in the different modalities of ischaemic protection.

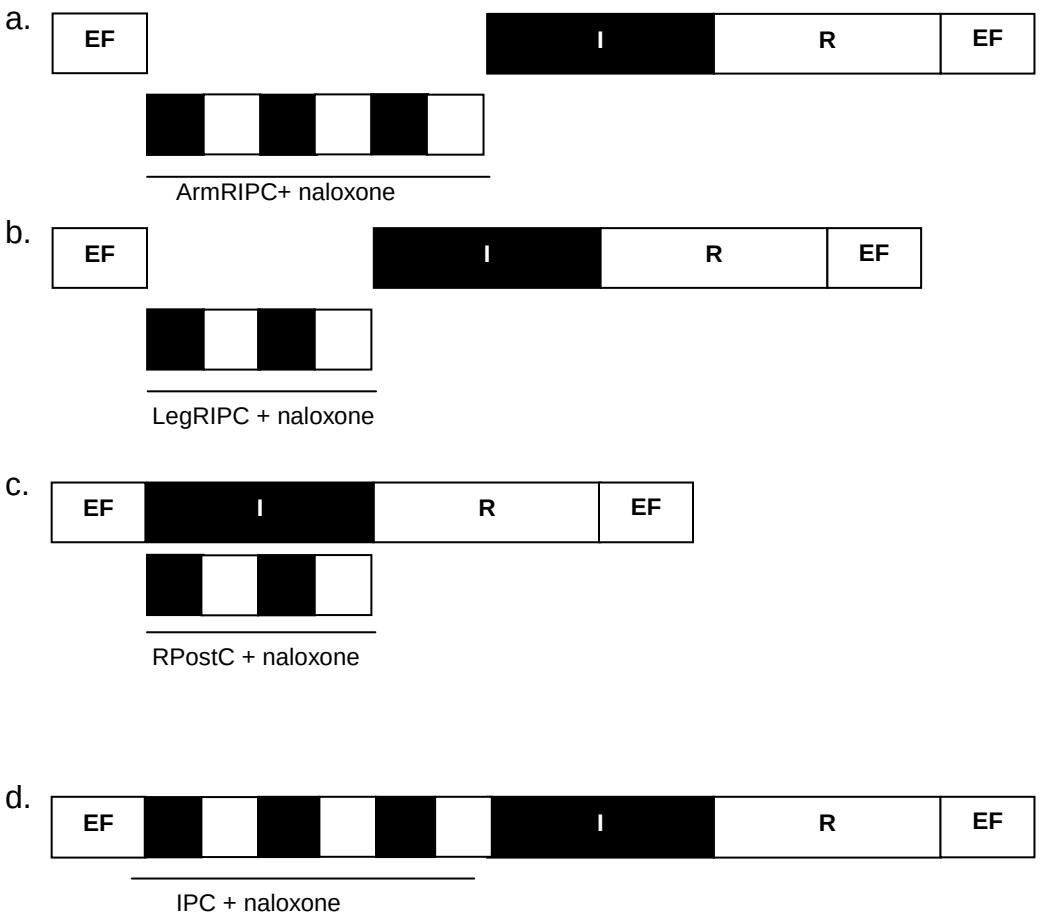


Figure 4.2: *Protocols to determine the effect of naloxone on (a) Arm RIPC (b) Leg RIPC (c) RPostC and (d) IPC.*

4.3.6 Effect of naloxone on endothelial IR injury

Experiments to assess the direct effect of naloxone on endothelial IR injury were also performed [Figures 4.3a & b].

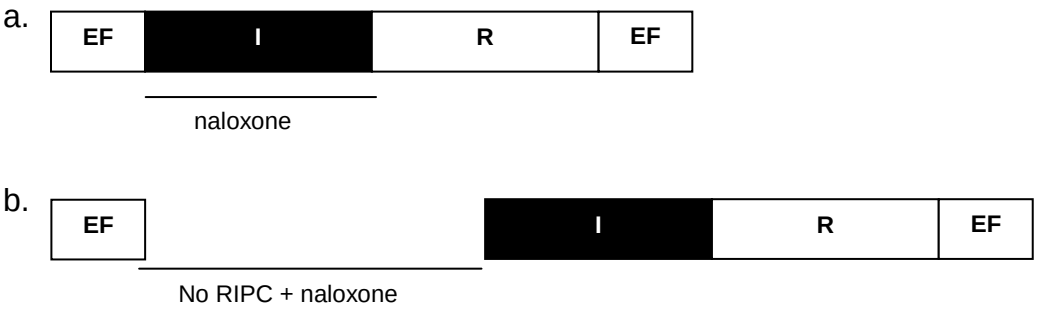


Figure 4.3: *Protocols to determine the effect of naloxone on endothelial IR injury with (a) IR alone and with (b) IR + No RIPC.*

4.3.7 Effect of remote conditioning stimulus on local production of beta endorphins

Baseline blood samples were obtained from the arm and the leg as described in figure 4.4.

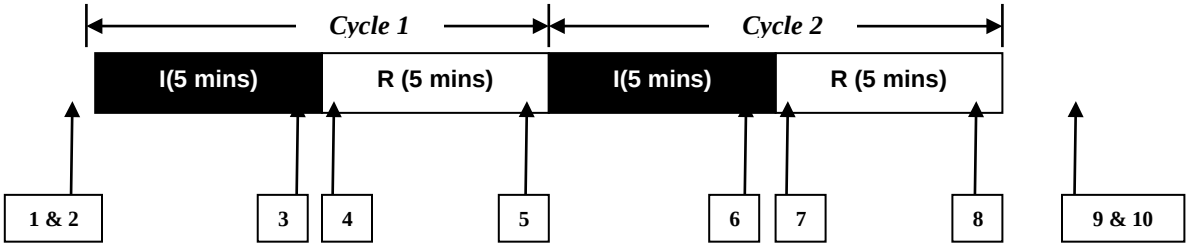


Figure 4.4: ***Protocol to determine the effect of remote conditioning stimulus on plasma beta endorphins.*** *Baseline blood samples were obtained from the arm [1] and the leg [2]. After the cuff was inflated for 4.5 minutes a blood sample [3] was collected from the butterfly needle in the leg vein. Immediately after cuff deflation (approximately at 5 minutes) a 2nd blood sample [4] was collected from the leg vein. A 3rd blood sample [5] was collected just before the end of cycle 1 (before cuff inflation for cycle 2) and this occurred at approximately 9.5 minutes. During cycle 2, blood samples were collected after the cuff had been inflated [6], immediately after the cuff deflation [7] and before the end of cycle 2 [8]. Blood samples were collected 5 minutes after the remote conditioning stimulus, from the arm [9] and from the leg [10].*

4.4 Calculations and Statistical Analysis

For FMD these are as described in section 2.4. Plasma beta endorphin concentrations were measured in ng/ml. In all cases, a value of $P<0.05$ was considered statistically significant.

4.5 Results

All subjects tolerated the procedure. There were no significant effects on blood pressure, heart rate, baseline brachial artery diameter or FMD flow stimulus during reactive hyperaemia (Table 4.1).

	IR Alone (n=8)		***IR+ArmRIPC (n=8)***		***IR+RPostC (n=9)***		***IR+ArmRIPC + naloxone (n=9)***		***IR+RPostC + naloxone (n=8)***	
	Pre	***Post***	***Pre***	***Post***	***Pre***	***Post***	***Pre***	***Post***	***Pre***	***Post***
SBP (mmHg)	108±8	109±8	111±4	112±3	105±3	108±2	111±3	114±4	114±5	110±2
DBP (mmHg)	66±5	67±5	60±3	66±1	58±2	60±4	63±3	66±2	66±4	64±3
HR (bpm)	63±4	63±4	60±4	58±4	59±3	64±3	66±3	65±2	63±3	61±2
Baseline arterial diameter (mm)	3.9±0.2	3.8±0.2	4.0±0.2	4.0±0.2	3.7±0.1	3.7±0.1	3.9±0.1	3.8±0.1	3.7±0.1	3.8±0.1
Flow Stimulus	9.1±1.1	10.2±1.3	10.3±1.1	10.9±1.4	9.4±0.6	10.6±1.3	10.1±0.4	11.5±0.4	9.3±1.6	10.2 ±1.1

Table 4.1: *Summary of blood pressure, heart rate, baseline brachial artery diameter and FMD flow stimulus (no units) during reactive hyperaemia.*

4.5.1 Effect of IR on endothelial function

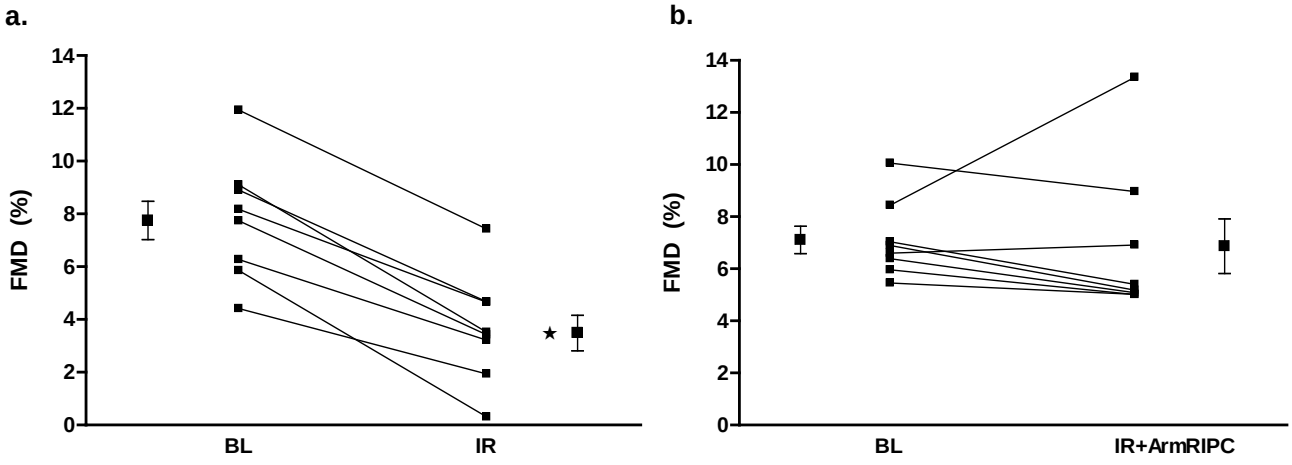
IR reduced brachial artery FMD (FMD 7.8±0.8% before IR vs 3.6±0.7% post-IR; *p<0.0001; paired t test; n=8) [Figures 4.5a & 4.7]. A significant correlation existed between baseline and post-IR values for FMD (Pearson's r=0.5, P<0.001). These correlations justified using ANCOVA to adjust post-IR values for differences in baseline endothelial function (EF) between the different protocols, so that post-IR EF could be directly compared with greater statistical power (Figure 4.7)

4.5.2 Effect of RIPC on endothelial IR injury

RIPC prevented the IR induced reduction in brachial artery FMD (FMD 7.1±0.5% before IR+Arm RIPC vs 6.9±1.0% after IR+Arm RIPC, p=NS; n=8 and FMD 7.2±0.8% before IR+Leg RIPC vs 7.3±0.7% after IR+LegRIPC, p=NS; n=7) [Figures 4.5 b & c & 4.7].

4.5.3 Effect of RPostC on endothelial IR injury

RPostC prevented the IR induced reduction in brachial artery FMD (FMD 6.5±0.8% before IR+RPostC vs 6.6±1.0% after IR+RPostC, p=NS; n=7) [Figures 4.5d & 4.7].



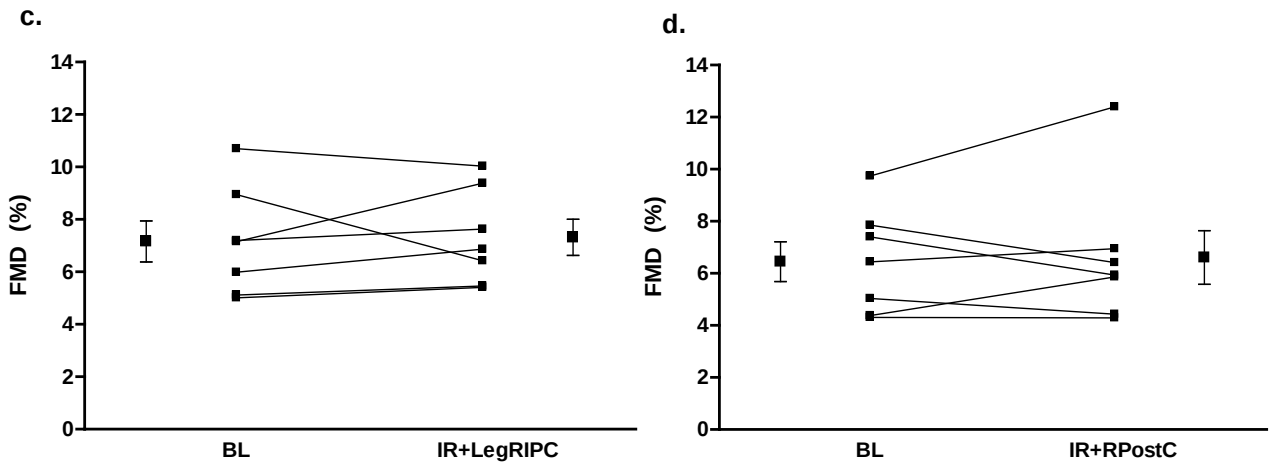
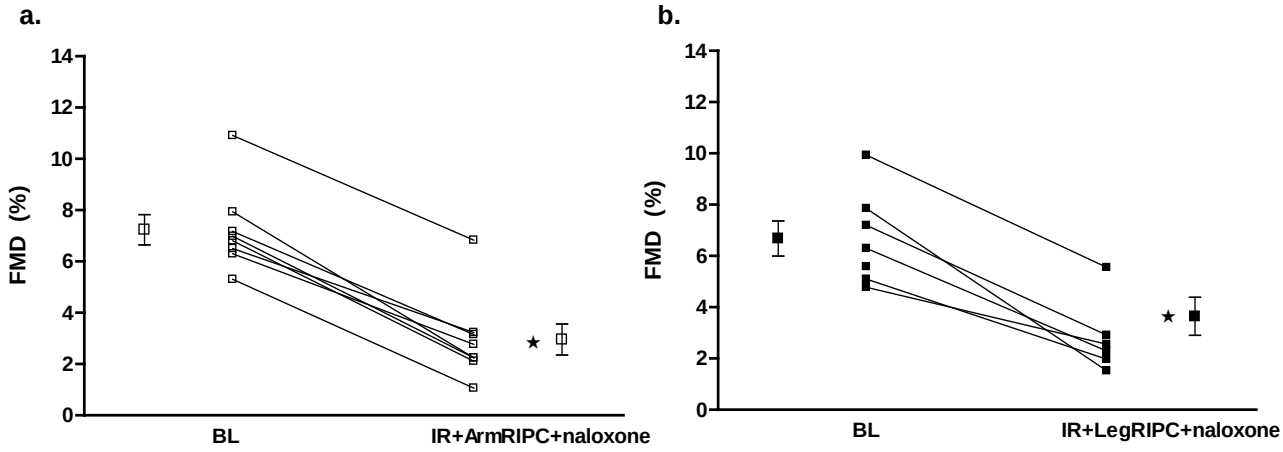


Figure 4.5: *IR reduced endothelial function in the (a) brachial artery (FMD* 7.8±0.8% *pre-IR versus* 3.6±0.7% *post-IR; *P<0.0001; n=8).The IR induced endothelial dysfunction was prevented by (b) ArmRIPC (FMD* 7.1±0.5% *pre- versus* 6.9±1.0% *post-IR+ArmRIPC; P=NS; n=8), (c) LegRIPC (FMD* 7.2±0.8% *pre-versus* 7.3±0.7% *post-IR+LegRIPC; P=NS; n=7) and (d) RPostC (FMD* 6.5±0.8% *pre- versus* 6.6±1.0% *post-IR+RPostC; P=NS; n=7). BL=baseline.*

4.5.4 Role of opioid receptors in protection by RIPC and RPostC

Naloxone abolished the protection by RIPC (arm or leg) against IR-induced endothelial dysfunction in the brachial artery FMD (7.0±0.6% before IR+Arm RIPC+naloxone vs 2.8 ± 0.6% after IR+ArmRIPC+naloxone, p<0.001; n=8) and 6.7±0.7% before IR+Leg RIPC+naloxone vs 3.0 ± 0.5% after IR+ LegRIPC+naloxone, p<0.001; n=6) [Figures 4.6a & b & 4.7]. In contrast, naloxone did not affect the protection afforded by RPostC (7.1±0.6% before IR+RPostC+naloxone vs. 7.0 ± 0.7% after IR+RPostC+naloxone, p =NS; n=8) [Figures 4.6c & 4.7].



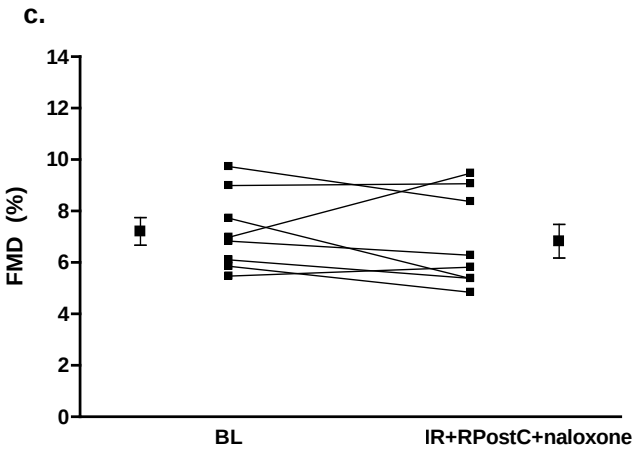


Figure 4.6: *Naloxone abolished the protective effects of (a) ArmRIPC (FMD* 7.0±0.6% *pre-IR versus* 2.8±0.8% *post-IR; *P<0.001; n=8) and (b) LegRIPC (FMD* 6.7±0.7% *pre- versus* 3.0±0.5% *post-IR+LegRIPC+naloxone; *P<0.001; n=8). Naloxone did not affect the protection afforded by (c) RPostC (FMD* 7.1±0.6% *pre- versus* 7.0±0.7% *post-IR+LegRIPC+naloxone; P=NS; n=8) BL=baseline*

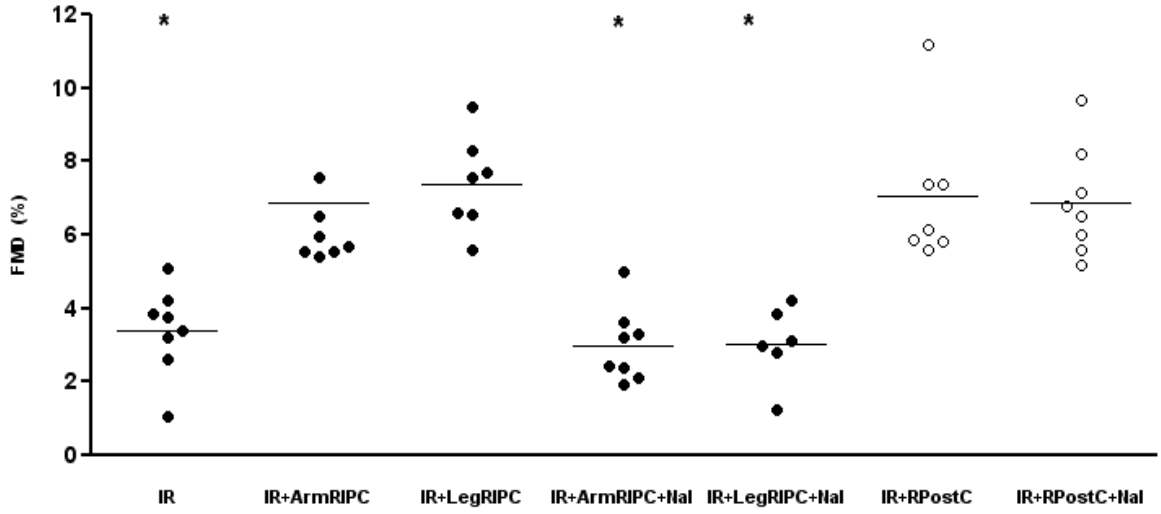


Figure 4.7: *Role of opioid receptors in protection by RIPC and RPostC in the brachial artery. ArmRIPC (post-IR FMD, 6.9±0.9%, n=8), LegRIPC (post-IR FMD, 7.4±0.5%, n=7) and RPostC (post-IR FMD, 7.0±0.7%, n=7) prevented IR-induced reduction in FMD. The protective effects of ArmRIPC (post-IR FMD, 3.0±0.4%, n=8) and LegRIPC (post-IR FMD, 3.0±0.4%, n=6) were abrogated by systemic naloxone. In contrast, the protective effect of RPostC was preserved in the presence of naloxone (post-IR FMD, 6.9±0.5%, n=8). Post-IR values were adjusted for baseline FMD (ANCOVA).*

4.5.5 Role of opioid receptors in protection by IPC

In the cohort of volunteers that took part in the IPC studies (n=10), IR reduced brachial artery FMD (FMD 6.7±0.7% before IR vs 2.5±0.4% after IR, *p<0.0001; n=10) (Figure 4.8a). IPC prevented the IR induced reduction in brachial artery FMD (FMD 6.3±0.5% before IR+IPC vs 6.5±0.6% after IR+IPC, p=NS; n=10) [Figure 4.8b]. Naloxone had no effect on protection against IR-induced endothelial dysfunction by IPC (6.1±0.4% before IPC + vs. 6.5±0.6% after IPC, p =NS; n =10) [Figure 4.8c]

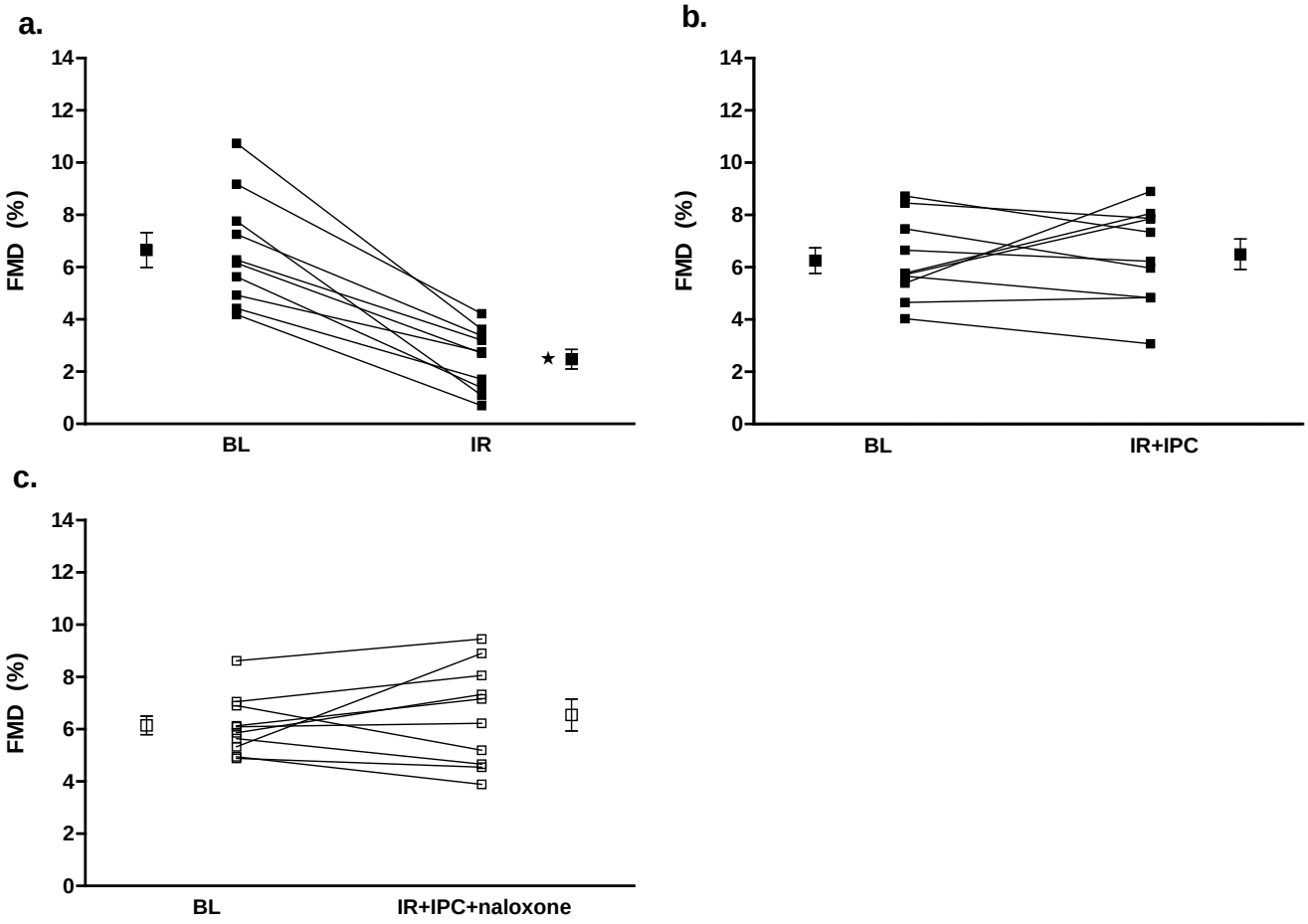


Figure 4.8: *IR reduced endothelial function in the (a) brachial artery (FMD* 6.7±0.7% *pre-IR versus* 2.5±0.4% *post-IR; *P<0.0001; n=10).The IR induced endothelial dysfunction was prevented by (b) IPC (FMD* 6.3±0.5% *pre-versus* 6.5±0.6% *post-IR+IPC; P=NS; n=10). (c) Naloxone did not affect the protection afforded by IPC (FMD* 6.1±0.4% *pre- versus* 6.5±0.6% *post-IR+IPC; P=NS; n=10)*

4.5.6 Effect of remote conditioning on beta endorphins

The concentration of plasma beta endorphins did not change after LegRIPC (p=NS; ANOVA; n=6) [Figure 4.9].

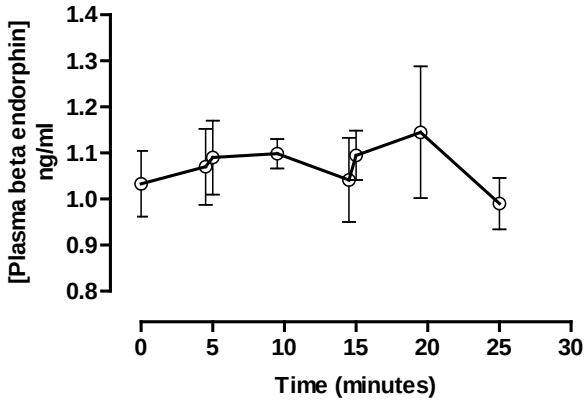


Figure 4.9: *There was no significant change in plasma beta endorphin concentrations during the remote conditioning stimulus comprising 2 leg cycles of 5 minutes ischaemia and 5 minutes reperfusion (p=NS; ANOVA; n=6).*

4.6 Discussion

Results in this chapter demonstrate that naloxone inhibits RIPC-induced endothelial protection from IR injury. Naloxone had no effect on other ischaemic conditioning stimuli that have little or no requirement for circulating mechanisms to effect protection. The most plausible explanation is that opioid antagonism blocks a humoral mediator that contributes to the systemic spread of ischaemic protection in RIPC, but which is not required in IPC or RPostC. Control studies confirmed that naloxone had no direct effect on the endothelial response to IR injury.

4.6.1 Effect of opioid antagonism on IPC

Ischaemic preconditioning represents the best characterised example of hormesis, whereby controlled doses of a potentially injurious stimulus (brief periods of ischaemia) induce transient protection against a larger toxic dose of the same stimulus. IPC induces release of multiple triggers that activate membrane bound receptors (94). These in turn stimulate a complex kinase-based mechanism which leads to changes in mitochondrial energetics and subsequent tissue protection. The human forearm model of IR injury and IPC mimics many of the facets of the mechanisms of IR and IPC identified in pre-clinical models. IR injury is dependent on endogenous oxidant stress, is reduced by exogenous anti-oxidants, and NO-based interventions (41, 282, 283). Activation of IPC requires a threshold stimulus; protection requires activation of mitochondrial ATP-sensitive potassium channels and is mimicked by inhibition of the mitochondrial permeability transition pore (94). Though endogenous opioids have been shown to trigger IPC in some animal models, at the systemic dose that was administered in this thesis, there was no effect of opioid antagonism to block IPC-induced protection. My observations compare with the results of a previous study in isolated rabbit hearts; in which naloxone did not inhibit local preconditioning but abolished the protection afforded by transfer of an IPC concentrate (280). This research group postulates that ischaemic protection by IPC is mediated by multiple, independent or ‘in-parallel’ protective mechanisms such that blocking the opioid pathway alone does not affect protection. The dose of naloxone I have chosen has been previously demonstrated to inhibit the effects of endogenous opioids in humans and increase plasma concentration of endorphins via receptor displacement (281). Whether larger doses or co-

administration with other agents (to target multiple triggers) would be needed to block IPC remains to be determined. Therefore, although the present study does not rule out a role for endogenous opioids in the mechanism of IPC, it strongly suggests that opioids are not necessary to effect IPC in humans in vivo.

4.6.2 Systemic spread of protection in RIPC

Tissue mechanisms of ischaemic protection by RIPC resemble those of IPC with respect to the involvement of triggers, mediators and effectors (see section 1.7.1). However the systemic spread of protection from a localised stimulus implies the involvement of humoral and/or neuronal mechanisms and evidence for both has emerged. Autonomic ganglionic blockade inhibits RIPC in animals and humans (168, 172,187). However, there is strong evidence that a humoral mechanism can also effect systemic protection. Organ denervation does not block RIPC, and there is inter-individual transfer of ischaemic protection by blood or blood products (174, 177, 279, 284, 285). The nature of the active principle in the blood remains uncertain, with circumstantial evidence supporting each of adenosine, bradykinin, opioids and cannabinoids (286). The relative contribution of the neural and humoral components and the degree to which they interact might vary between species and the nature of the IR injury and the preconditioning stimulus.

In a previous study, ganglionic blockade with trimetaphan inhibited the effect of RIPC to prevent endothelial IR injury, a finding that supported a neuronal mechanism in humans. However a dialysable plasma extract from volunteers in whom preconditioning pathways have been activated has been identified that

reduces myocardial IR injury in the rat isolated heart (174). The activity of this unidentified substance was blocked by naloxone, strongly suggesting that it activates opiate receptors. Results in this thesis provide corroborative in vivo evidence that RIPC is opioid-dependent. As previously shown, the contrast with IPC is best explained by naloxone inhibiting the spread of protection rather than interfering with local activation or effector mechanisms, given the similarities of these aspects of the mechanism between IPC and RIPC (280). However, these studies do not themselves differentiate between an opioid based humoral or neuronal mechanism.

4.6.3 Role of opioids in RPostC

Differences in the scheduling of the conditioning ischaemia between RIPC and RPostC provide an opportunity to examine the role of a circulating factor in humans. In the RPostC protocol that was used in this thesis, the index ischaemia and conditioning ischaemia are co-temporaneous. The corollary of this is that the limb undergoing index ischaemia is completely isolated from the circulation during the application of the ischaemic preconditioning stimulus on the contra-lateral limb. This would be expected to minimise any influence of a blood-borne protective substance, but leave neuronal mechanisms intact. RPostC had a similar protective effect to RIPC but this was unaffected by naloxone administration. This is strong evidence that the opioid component of remote protection requires an intact circulation and is humoral rather than neuronal.

4.6.4 Relative contribution of neuronal and humoral pathways

RIPC is sensitive to neuronal or opioid blockade, with inhibition of either pathway blocking protection. This could be because the neuronal and humoral pathways are arranged in series. An alternative mechanism is that they are parallel pathways and each is required to breach a threshold stimulus above which ischaemic protection is triggered. The present study provides evidence that the pathways are not simply arranged in series, because protection from RPostC (activated principally neuronally) persisted after administration of naloxone. It remains to be determined how activation of one pathway during RPostC is sufficient to cross a threshold to effect remote ischaemic protection, whereas for RIPC both pathways need to be activated. One explanation is that the conditioning stimulus of RPostC is closer in time to the index injury, and it is possible that the closer in time the protective stimulus is to the index ischaemia, the greater is the degree of protection. The hypothesis would be that at this earlier timepoint, RPostC crosses a threshold for protection.

4.6.5 Beta endorphins in remote conditioning

The endogenous opioids (endorphins, dynorphins and enkephalins), mediate their effects via the activation of the mu-, kappa- and delta – opioid receptors respectively (251). In this thesis plasma beta endorphins were assayed as a sensitive marker of activation of the endogenous opioid pathway during the remote conditioning stimulus. The lack of a significant change in the concentration of plasma beta endorphin in response to the conditioning stimulus might possibly be due to absence

of a plasma extraction process by acidification to purify the samples. This was based on protocols of the KCH laboratory, which does not recommend extraction when dealing with relatively small polypeptides such as beta endorphin, because of the risk of denaturation. Another possibility is that other endogenous opioids such as the enkephalins or dynorphins might have played a more prominent role during the remote conditioning stimulus. Further studies will be needed to explore this in more detail.

4.6.6 Conclusion

Results in this chapter provide evidence in vivo for a circulating factor that contributes to ischaemic protection in humans. It is sensitive to opioid antagonism and may act independently of nervous system control. There is a complex interaction between the circulating factor and the nervous system, which is explored in more detail in the next chapter. It is possible that the identification of this humoral factor will allow supra-physiological activation of ischaemic conditioning pathways, with greater potential to reduce IR injury than has been possible to date.

Chapter 5

The role of the neurogenic pathway in remote ischaemic protection

5.1 Introduction

Chapter 4 of this thesis described evidence for a circulating opioid pathway in the transfer of protection by RIPC. Previous studies in this laboratory have demonstrated that complete autonomic blockade using trimetaphan abolished protection against endothelial IR injury by early and late RIPC in healthy volunteers (187). This builds on data from animal models which have shown that the ganglion blocker hexamethonium can block the protective effects of RIPC (178, 179). However, available data do not give a clear indication of the relative contribution of the neurogenic or humoral pathways in the mediation of protection by a remote stimulus. The plausibility of a complex interaction between humoral factors and the neurogenic system is highlighted in studies which show that an intact nerve supply is essential to facilitate remote protection by circulating factors (175).

Using an *in vivo* model of endothelial IR injury I sought to further investigate the role of the autonomic pathways in protection by RIPC and RPostC. In this chapter phentolamine and atropine were used as pharmacological probes to determine the role of the alpha adrenergic and cholinergic neural pathways respectively.

5.2 Methods

5.2.1 Subjects

83 studies were performed on 17 male, healthy, non-smoking volunteers aged 18-45 years. Cohort 1 (n=10) were recruited for the RIPC protocols and cohort 2 (n=10) were recruited for the RPostC studies. Missing data are attributable to the inability of some of the participants to complete the protocol which entailed several visits.

5.2.2 Assessment of Conduit Vessel Endothelial Function

Brachial artery flow mediated dilatation (FMD) of the right arm was assessed as described in section 2.2.1.

5.2.3 Induction of IR injury

IR injury was induced as described in section 2.1.1.

5.2.4 Induction of remote preconditioning

ArmRIPC was performed as described in section 2.1.4.

5.2.5 Induction of remote postconditioning

RPostC was performed as described in section 2.1.5.

5.3 Experimental Protocols

5.3.1 Effect of IR on Endothelial Function

To determine the effect of IR on endothelial function (EF), brachial artery FMD was assessed before ischaemia (baseline) and at 20 minutes after reperfusion [Figure 5.1a].

5.3.2 Effect of ischaemic conditioning stimuli on Endothelial IR Injury

FMD was assessed before and after IR injury + ArmRIPC or RPostC [Figures 5.1b & c].

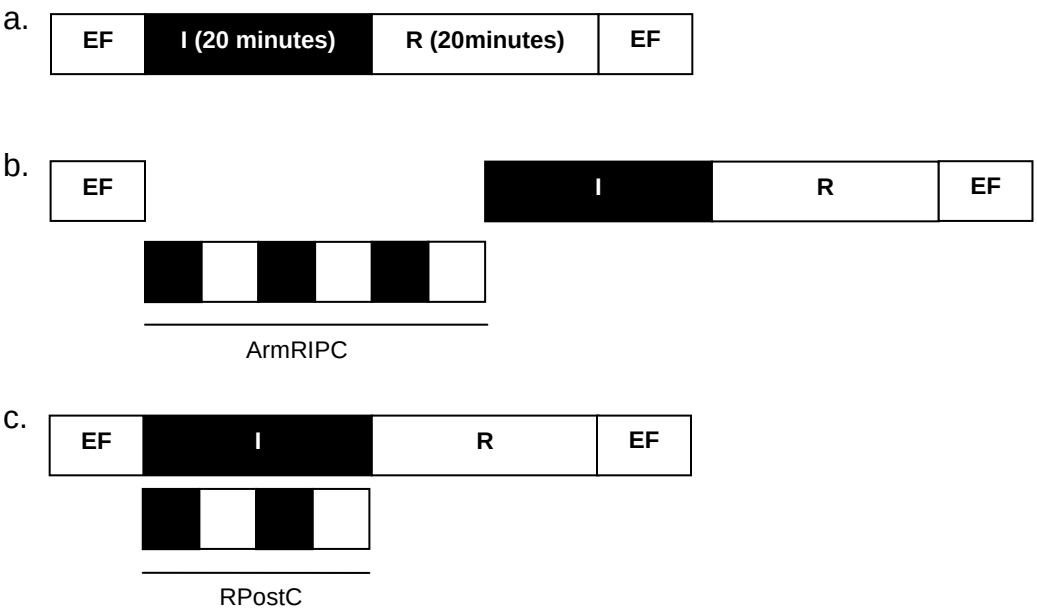


Figure 5.1: *Protocols to determine the effect of RIPC and RPostC on endothelial IR.*

5.3.3 Effect of alpha adrenergic receptor blockade on RIPC and RPostC

The alpha adrenergic receptor blocker, phentolamine was administered intravenously at a dose of 0.2 – 0.7mg/min, as described previously (287). The infusion rate for each volunteer was titrated according to the response observed with dose increments every 5 minutes as appropriate; the drug was considered to be at the effective dose when there was a stable drop of 10mmHg in the systolic arterial pressure or a sustained increase of 10 beats per minute in heart rate (287) [Table

5.1]. The phentolamine infusion, at the effective dose, was commenced at least 5 minutes before RIPC and RPostC and was continued through these interventions. This was to determine the effect of phentolamine on ArmRIPC and RPostC [Figures 5.2a and b].

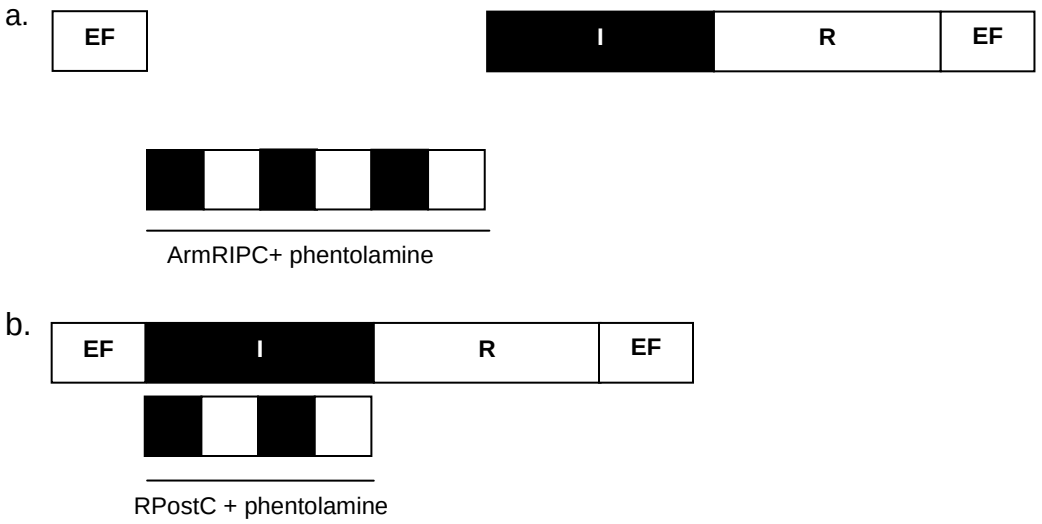


Figure 5.2*: Protocols to determine the effect of alpha adrenergic blockade on RIPC and RPostC*

	Phentolamine effective dose (n=22)	
	Pre	***Post***
SBP (mmHg)	115±4	119±4
DBP (mmHg)	63±3	68±2
HR (bpm)	63±3	81±4

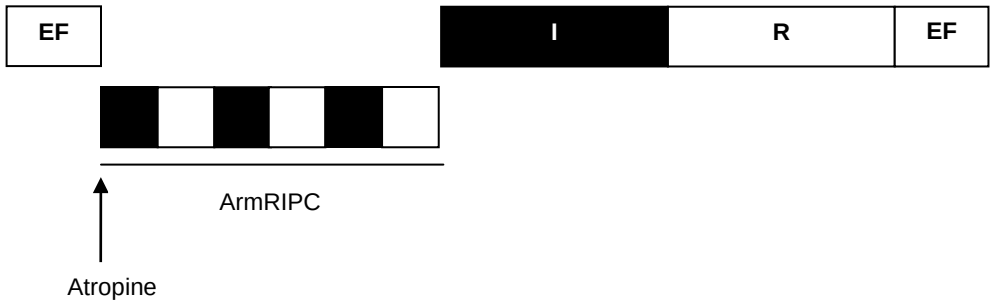
Table 5.1: *Evidence of the effective dose of phentolamine was a sustained increase in heart rate of >10 beats per minute above baseline.*

5.3.4 Effect of cholinergic receptor blockade on RIPC and RPostC

The cholinergic receptor blocker, atropine was administered intravenously at a bolus dose of 10mcg/kg. This is a dose that is similar to that in clinical use and has also been used previously in healthy volunteer studies (288, 289). Atropine was administered 5 minutes before ArmRIPC and RPostC [Figures 5.3a and b].

a.

.



b.

Figure 5.3: *Protocols to determine the effect of the cholinergic receptor blockade on (a) RIPC and (b) RPostC*

5.3.5 Effect of combined alpha adrenergic + cholinergic receptor blockade on RIPC

Both phentolamine and atropine were administered, as described above, simultaneously [Figure 5.4].

.

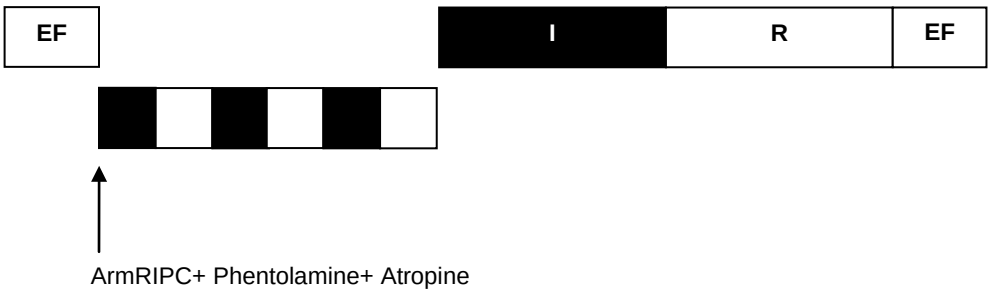
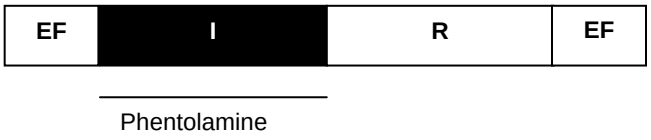


Figure 5.4: *Protocol to determine the effect of combined alpha adrenergic and cholinergic receptor blockade on RIPC.*

5.3.6 Effect of phentolamine and atropine on endothelial IR injury

Phentolamine or atropine was administered, as described above, to determine the effect of administration of phentolamine and atropine on endothelial IR injury (Figures 5.5a and b).

a.



b.

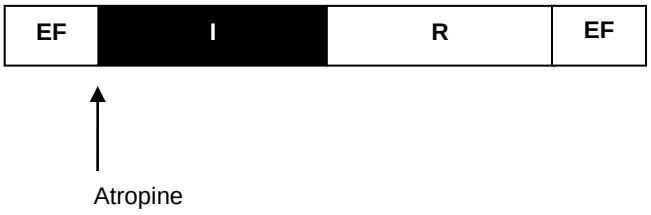


Figure 5.5: *Protocols to determine the effect of atropine or phentolamine on endothelial IR injury.*

5.4 Calculations and Statistical Analysis

As described in section 2.5 of chapter 2.

5.5 Results

The studies were generally well tolerated with most volunteers experiencing nasal congestion during the phentolamine studies and a dry mouth with atropine studies. In two subjects, during phentolamine studies transient episodes of palpitations with tachycardia were managed conservatively and the studies were stopped. There were no significant confounding effects on blood pressure, heart rate, baseline brachial artery diameter or FMD flow stimulus during reactive hyperaemia during FMD measurements [Table 5.2].

	IR Alone (RIPC studies)		***IR+RIPC***		***IR+RIPC+Phent***		***IR+RPostC+Phent***	
	Pre	*Post*	*Pre*	*Post*	*Pre*	*Post*	*Pre*	*Post*
SBP (mmHg)	113±2	117±2	112±3	116±2	115±2	115±3	111±2	116±2
DBP (mmHg)	65±1	67±2	62±2	64±3	65±3	68±2	70±3	67±4
HR (bpm)	64±2	63±4	65±3	63±3	65±3	69±6	65±3	69±4
Baseline arterial diameter (mm)	3.9±0.2	3.9±0.2	3.9±0.2	3.9±0.3	3.9±0.2	4.0±0.2	3.9±0.2	4.0±0.3
Flow Stimulus	7.1±1.0	10.2±1.3	8.3±1.1	9.9±1.4	8.4±0.3	9.6±1.1	8.4±0.3	8.8±0.4

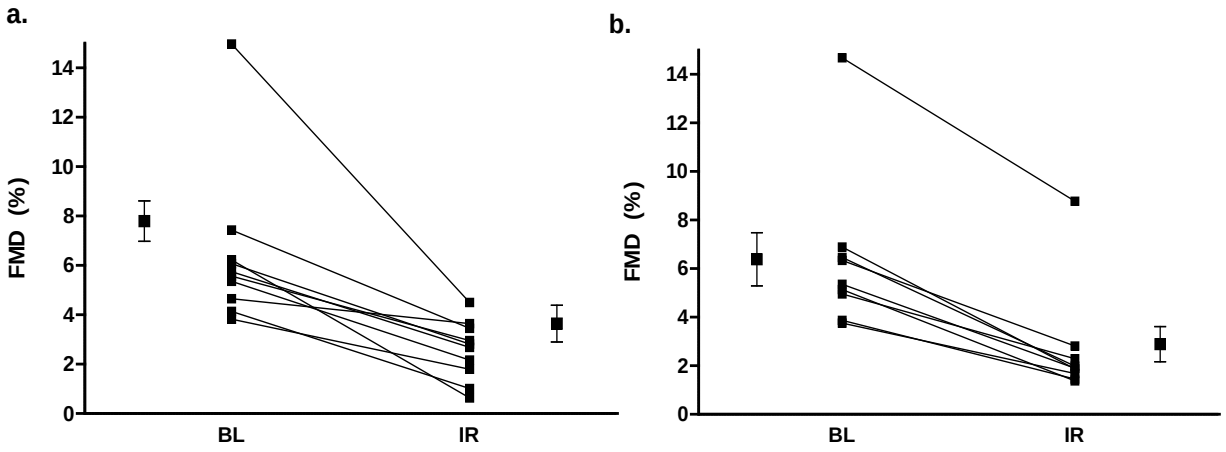
Table 5.2: *Summary of blood pressure, heart rate, baseline brachial artery diameter and FMD flow stimulus (no units) during reactive hyperaemia.*

5.5.1 Effect of IR on vascular dilator function

IR reduced brachial artery endothelial dysfunction in the RIPC study (6.4 ± 1.0% before IR vs 2.6±1.2 after IR, p<0.0001; paired t test; n=10) [Figures 5.6a & 5.8] and RPostC study (6.4±1.1% before IR vs 2.7± 0.8% after IR, p<0.0001; paired t test; n=9) [Figures 5.6b & 5.9] A significant correlation existed between baseline and post-IR values for FMD in the RIPC studies (Pearson's r=0.5, P<0.001) and RPostC studies (Pearson's r=0.5, P<0.01). These correlations justified using ANCOVA to adjust post-IR values for differences in baseline endothelial function (EF) between the different protocols, so that post-IR EF could be directly compared with greater statistical power (Figures 5.8 & 5.9)

5.5.2 Effect of RIPC and RPostC on endothelial IR injury

RIPC (FMD 6.3±0.7% before vs 5.9±0.7% after IR+ RIPC, p = NS; n=8) [Figures 5.6c & 5.8] and RPostC (FMD 5.8±0.3% before vs 5.5 ± 0.2% after IR+ RPostC, p = NS; n=9) [Figures 5.6d & 5.9] protected from endothelial IR injury.



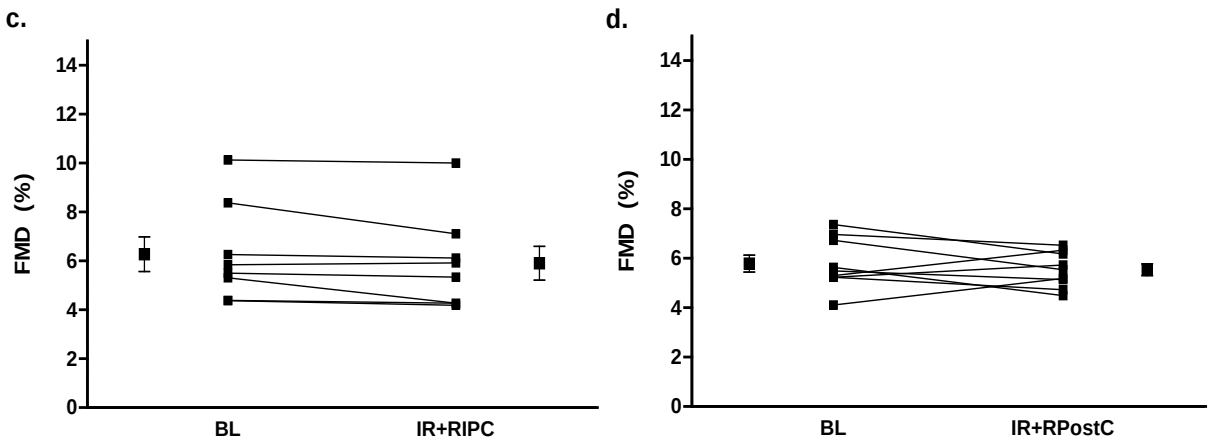


Figure 5.6: *IR reduced endothelial function in the brachial artery in the RIPC study (FMD 6.4±1.0% pre- versus 2.6±1.2% post-IR; *P<0.0001; n=10) and RPostC study (FMD 6.4±1.1% pre- versus 2.7±0.8% post-IR; *P<0.0001; n=9) The IR induced endothelial dysfunction was prevented by (b) RIPC (FMD 6.3±0.7% pre- versus 6.9±1.0% post-IR+RIPC; P=NS; n=8), and (c) RPostC (FMD 5.8±0.3% pre- versus 5.5±0.2% post-IR+RPostC; P=NS; n=9) BL=baseline*

5.5.3 Effect of phentolamine on RIPC and RPostC

Phentolamine had no effect on the RIPC induced protection (FMD 6.8 ±0.7% before vs 5.9±0.7% after IR + RIPC + phentolamine, p=NS; n=9) [Figure 5.7a & 5.8] whereas the administration of phentolamine abolished the protective action of RPostC (FMD 6.0±0.5% before vs 1.8±0.3% after IR + RPostC + phentolamine, p<0.0001;n=8)[Figures5.7b&5.9].

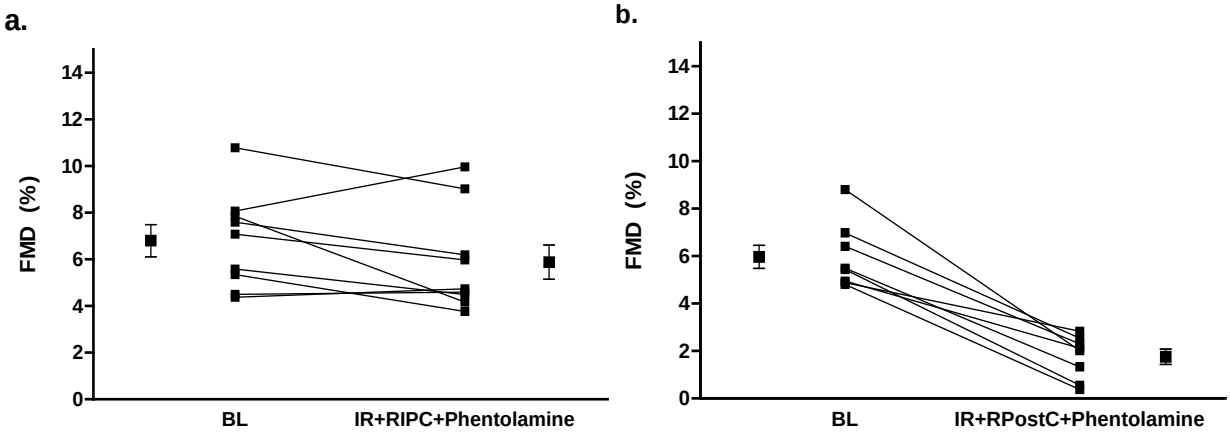


Figure 5.7: *The administration of phentolamine had no effect of protection against endothelial IR by (a) RIPC (FMD* 6.8±0.7% *pre- versus* 5.9±0.7% *post-IR+RIPC+Phentolamine; P=NS; n=9) but abolished the protective effect of (b) RPostC (FMD* 6.0±0.5% *pre- versus* 1.8±0.3% *post-IR+RPostC+Phentolamine; *P<0.0001; n=8). BL=baseline*

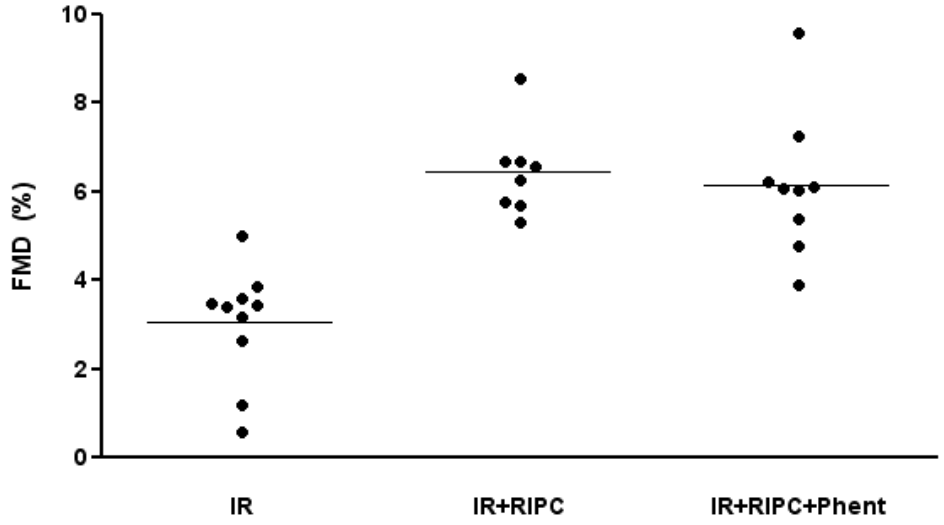


Figure 5.8: *Effect of phentolamine (Phent) on protection by RIPC in the brachial artery. RIPC prevented IR-induced reduction in FMD (post-IR FMD, 6.4±0.4%, n=8). The protective effects of RIPC were preserved in the presence of systemic phentolamine (post-IR FMD, 6.1±0.5%, n=9). Post-IR values were adjusted for baseline FMD (ANCOVA).*

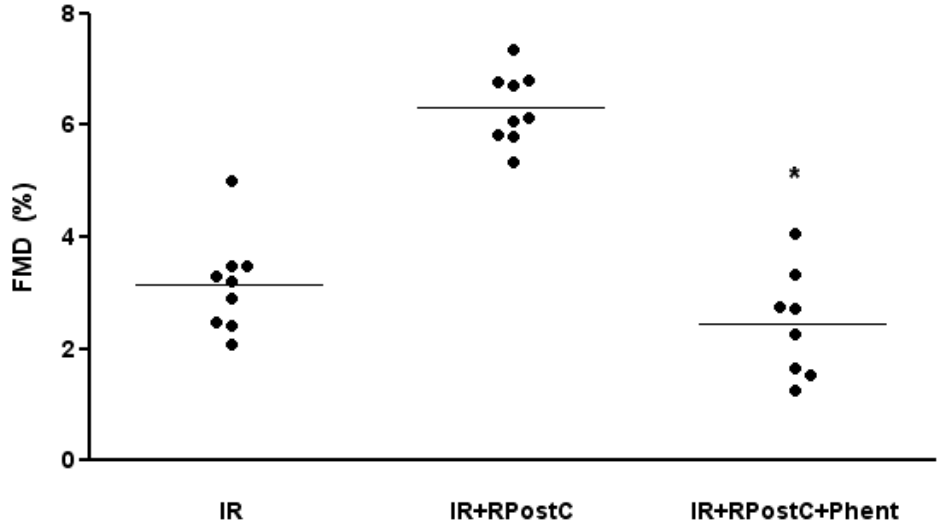


Figure 5.9: *Effect of phentolamine (Phent) on protection by RPostC in the brachial artery. RPostC prevented IR-induced reduction in FMD (post-IR FMD, 6.3±0.2%, n=9). The protective effects of RPostC were abrogated by systemic phentolamine (post-IR FMD, 2.4±0.3%, n=8. Post-IR values were adjusted for baseline FMD (ANCOVA).*

5.5.4 Effect of atropine on RIPC and RPostC

The administration of atropine had no effect on protection against endothelial IR injury induced by RIPC (FMD 4.4±0.4% before vs 4.2±0.4% after IR + RIPC + atropine, p=NS; n=8) [Figure 5.10a] or by RPostC (FMD 4.0±0.7% before vs 3.8±0.8% after IR + RPostC + atropine, p=NS; n=6) [Figure 5.10b].

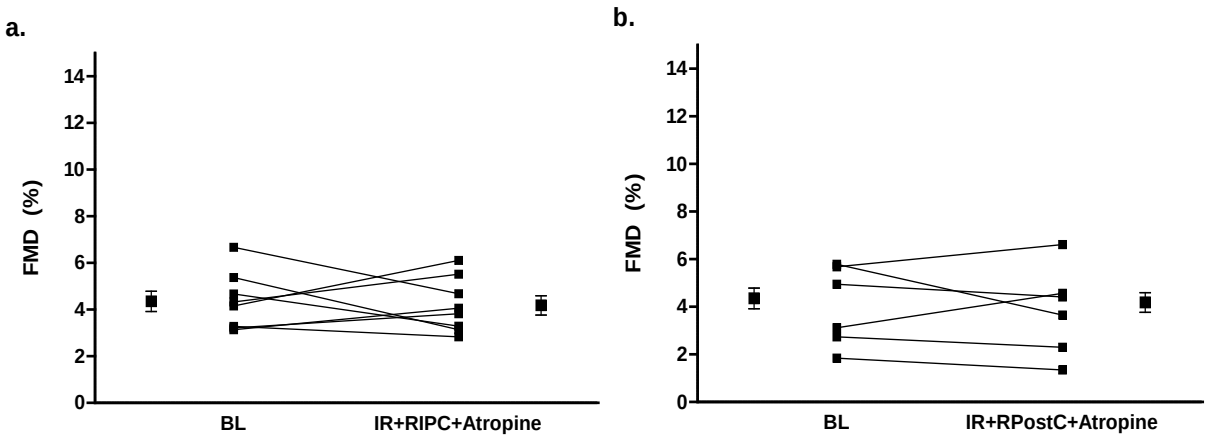


Figure 5.10: *The administration of atropine had no effect of protection against endothelial IR by (a) RIPC (FMD* 4.4±0.4% *pre- versus* 4.2±0.4% *post-IR+RIPC+Atropine; P=NS; n=8) or (b) RPostC RIPC (FMD* 4.0±0.7% *pre- versus* 3.8±0.8% *post-IR+RIPC+Atropine; P=NS; n=6). BL=baseline*

5.5.5 Effect of phentolamine + atropine on RIPC

RIPC induced protection against endothelial IR injury was not significantly affected by the administration of the combination of phentolamine and atropine (FMD 5.0±0.8 % before vs 4.1±0.7% after IR + RIPC + phentolamine + atropine, p=NS; n=7) [Figure 5.11].

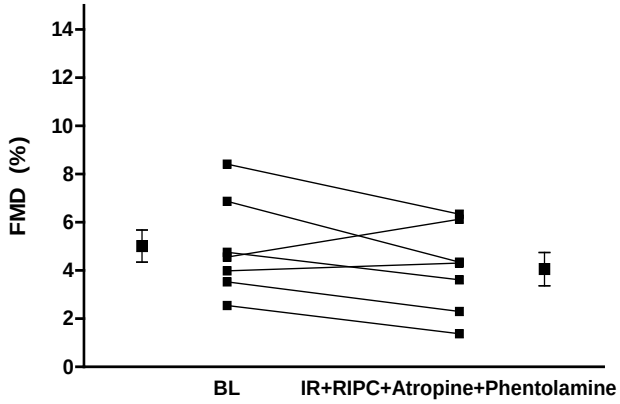


Figure 5.11: *The administration of phentolamine+atropine did not affect protection conferred by RIPC (FMD* 5.0±0.8% *pre- versus* 4.1±0.7% *post-IR+RIPC+Atropine+Phentolamine; P=NS; n=7). BL=baseline*

5.5.6 Effect of phentolamine and atropine on endothelial IR injury

Neither Phentolamine nor atropine, administered during a sham RPostC stimulus, had any effect on the endothelial response to IR injury (FMD 7.0±0.7% before vs 3.2±0.3% after IR + shamRPostC+ phentolamine; p<0.001; n=5) [Figure 5.12a] and (FMD 5.4±1.0% before vs 3.3 ±1.0% after IR + No RPostC+ atropine; p<0.05; n=4) [Figure 5.12b]

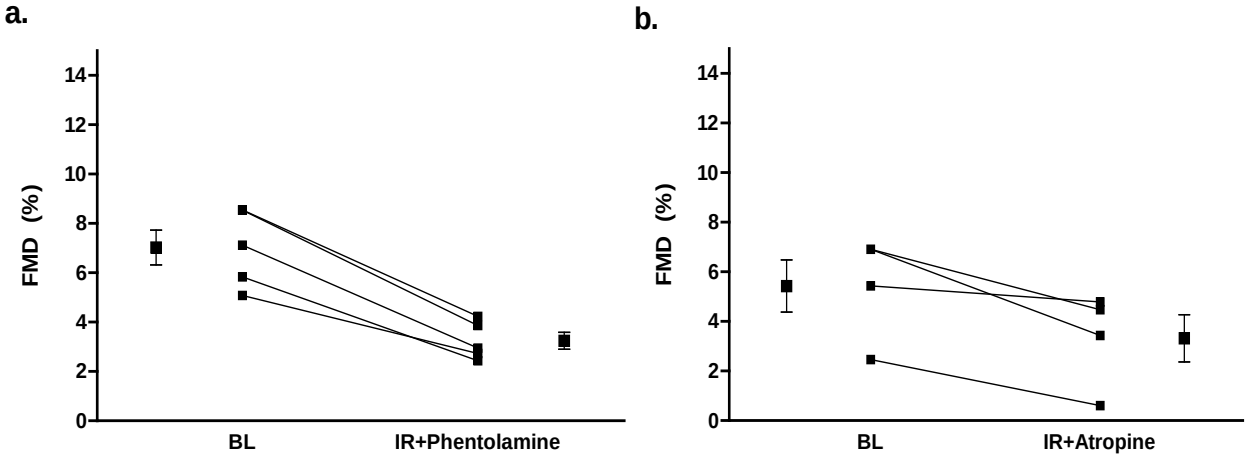


Figure 5.12: *Endothelial response to IR was not affected by (a) Phentolamine (FMD* 7.0±0.7% *pre- versus* 3.2±0.3% *post-IR+Phentolamine; *P<0.001; n=5) or (b) atropine (FMD* 5.4±1.0% *pre- versus* 3.3±1.0% *post-IR+Atropine *P<0.05; n=4). BL=baseline*

5.6 Discussion

This study demonstrates, for the first time in humans, that phentolamine inhibits RPostC-induced protection against IR injury. However, phentolamine had no effect on RIPC. These results suggest a significant contribution of the alpha adrenergic pathway in mediation of protection by RPostC. Furthermore, this study demonstrates that atropine had no effect on the protection by RIPC or RPostC, a suggestion that the cholinergic pathway plays little role in the mediation of a remote protective stimulus.

5.6.1 RPostC is blocked by systemic adrenergic blockade

Systemic phentolamine administered at a dose that caused detectable adrenergic blockade abolished the effect of RPostC to protect remote endothelium from IR injury, whereas atropine had no effect. These data implicate the adrenergic but not the cholinergic component of the autonomic nervous system in the reflex spread of ischaemic protection elicited by RPostC. However it is not possible to be certain that a larger dose of atropine might have had an inhibitory effect. Neither atropine nor phentolamine affected the endothelial response to IR injury, so the effect of phentolamine was unlikely to be explained by a direct effect to exacerbate IR injury (although the sample sizes for these studies were small and limit the robustness of these conclusions).

5.6.2 Relative contribution of neuronal and haematogenous transfer of protection by RPostC

In chapter 4, I presented evidence that a circulating factor contributed to the systemic protection that was triggered by RIPC, and likely activated opioid pathways. I hypothesised that a haematogenous factor would not contribute to IPC (protection was exclusively activated by local pathways) or RPostC (where the ischaemic conditioning stimulus activated systemic pathways in a limb that was isolated from the circulation). The data on the effects of naloxone were consistent with this hypothesis. The corollary was that RPostC could only cause remote protection by neuronal pathways. The adrenergic and cholinergic components of the autonomic nervous system were rational candidates based on the known involvement of the autonomic nervous system in RIPC, and the established role of acetylcholine and noradrenaline as triggers of preconditioning in many tissues. My results suggest a pivotal role for the alpha adrenergic receptors in the transfer of protection by RPostC by the autonomic nervous system. These results also support my conclusion in chapter 4 that the neuronal and humoral pathways probably act in parallel rather than simply in series. Both will be activated by the RPostC stimulus, but it is only by blocking the neuronal component that it is possible to inhibit RPostC. Were the pathways arranged as a single neuro-hormonal mechanism sequential mechanism, blockade of one would necessarily inhibit the entire pathway. Indeed if this were the case, then RPostC would probably not cause any protection, as my data suggest that regardless of the activation of a humoral pathway, it has an insufficient biological effect to contribute to ischaemic protection.

5.6.3 Interaction between the neural and humoral pathways in RIPC

Loukogeorgakis et al have previously demonstrated, using a human *in vivo* model of IR injury, that complete autonomic blockade using trimethaphan abolished the RIPC induced protection against endothelial IR injury (187). Data in this chapter show that the combined effects of alpha adrenergic and cholinergic receptor blockade, by the combined administration of phentolamine and atropine, were insufficient to block the protective effects of RIPC. This may highlight the unrecognised importance of other components of the autonomic nervous system in order to achieve a threshold stimulus for RIPC to occur. It is possible that the dose of atropine was too low, or that there is a requirement for beta adrenergic blockade. These possibilities will require additional study, and it is a weakness of this chapter that these data have not been included. There is also the possibility in the study by Loukogeorgakis and colleagues that the trimetaphan had off target effects which might have affected endothelial function and contributed to protection. However if it is accepted that ganglionic blockade effects a more complete autonomic blockade than I have achieved in this chapter, then it seems likely that a neuro-humoral reflex accounts for the spread of ischaemic protection by RIPC. Given that identical stimuli effect RIPC and RPostC, the conclusions drawn about RPostC imply that the neuro-humoral pathways that underpin the systemic protection of RIPC operate in parallel and both contribute to the crossing of a threshold of protection.

5.6.5 Conclusion

Results in this chapter highlight that the alpha adrenergic autonomic pathway plays a role in facilitating RPostC but not RIPC. The cholinergic pathway does not seem to be implicated in effecting protection by either RPostC or RIPC. More studies are

required to elucidate the role of other components of the autonomic pathway such as the beta adrenoceptor pathway and also to determine the relative contribution of each of the different pathways (neurogenic or humoral) to remote protection.

Chapter 6

The role of inorganic nitrates in protection against endothelial ischaemia reperfusion injury

6.1 Introduction

Vascular endothelial cells are susceptible to the effects of IR injury and this manifest as a measurable endothelial dysfunction (see section 1.5.3). Invariably this affects the endothelial production of NO via endothelial NO synthase (eNOS). NO has been implicated in the protective effect of the various interventions against IR injury that exist to date (290). There is evidence that an increase in nitric oxide (NO) bioavailability, via a complex signalling pathway involving cyclic GMP and various protein kinases, results in a reduction in deleterious intracellular processes such as calcium overload, mitochondrial permeability transition pore opening and increased production of reactive oxygen species (290). This promotes cellular cytoprotection and tissue salvage which is the ultimate goal in protection against IR injury. Emerging data indicate that inorganic nitrates and nitrites, previously considered to be inert end products of NO metabolism, provide a source of NO for cellular processes under hypoxic and acidotic conditions, such as during IR injury, when NOS becomes dysfunctional (see section 1.10 of chapter 1). The implication is that endogenous nitrite and nitrate stores act as a backup source of NO which may be beneficial during IR injury.

Vegetables including beetroot contain a large amount of inorganic nitrate which is thought to play a significant role in their potential health benefits (291-293).Once ingested the inorganic nitrate is rapidly absorbed via the stomach and a proportion of it enters the enterosalivary circulation where it is recycled into NO via reduction to nitrite (see section 1.10.2 of chapter 1). The nitrate-nitrite dependent NO generation might achieve prominence during hypoxic or ischaemic conditions, such that their reduction to NO occurs when NOS–dependent NO synthesis is impaired (196). This process of increasing the bioavailability of NO under ‘stress’ is thought to be the

mechanism underlying protection against IR injury by inorganic nitrates and nitrites as demonstrated in animal models (196, 294).

Oral ingestion of inorganic nitrates produces a dose-dependent increase in nitrite and NO production in the circulation (202, 203, 217). This might enable the therapeutic potential of nitrates to be exploited as a storage pool for nitrite and NO generation given their longer half-lives. Based on this I sought to determine the role of oral inorganic nitrates in the form of beetroot juice and postassium nitrate (KNO3) in protection against endothelial IR injury in the human *in vivo* model.

6.2. Methods

6.2.1 Subjects

Studies were performed on healthy, non-smoking volunteers, aged 18-45 years. In study one (beetroot 500ml), 20 studies were performed on 10 volunteers and in study two (beetroot 250ml & KNO3), 49 studies were performed in 13 volunteers. All studies repeated in same volunteers were at least 7 days apart and were an open-label crossover design for beetroot juice studies and double-blind crossover design for potassium nitrate studies.

6.2.2 Assessment of conduit vessel endothelial function

Brachial artery flow mediated dilatation (FMD) of the right arm was assessed, as described in section 2.2.1.

6.2.3 Induction of IR injury

IR injury was induced as described in section 2.1.1.

6.2.4 Determination of plasma nitrite and nitrate concentration

6.2.4.1 Blood samples

A 19-gauge butterfly needle, with extension set, was inserted prior to capsule or juice ingestion. The blood samples obtained were prepared as described in section 2.3.2.2.

6.2.4.2 Chemiluminescence

Plasma nitrite and nitrate concentrations were determined by chemiluminescence as described in section 2.3.2.1.

6.3 Experimental protocols

6.3.1 Effect of IR on endothelial function

To determine the effect of IR on endothelial function (EF), brachial artery FMD (Figure 6.1a) was assessed before ischaemia (baseline) and at 20 minutes after reperfusion.

Figure 6.1a: *Protocol to determine the effect of IR on endothelial function*

6.3.2 Effect of 500ml beetroot juice on endothelial IR injury

To determine the effect of beetroot juice (Planet Organic®) on endothelial IR injury, healthy volunteers were randomised to 500ml of beetroot juice 2 hours before IR or no treatment before IR. Subjects underwent the IR injury protocol after beetroot juice or no treatment, with both protocols being at least 7 days apart (Figure 6.1b)

Figure 6.1b: *Effect of 500ml beetroot juice on endothelial IR Injury*

6.3.3 Effect of KNO_3 on endothelial IR injury

To determine the effect of KNO_3 (Martindale Pharmaceuticals) on endothelial IR injury, healthy volunteers were randomised in a double-blind crossover design to receive either 24 mmol of KNO_3 or KCl tablets with 500ml of water (Figure 6.1c).

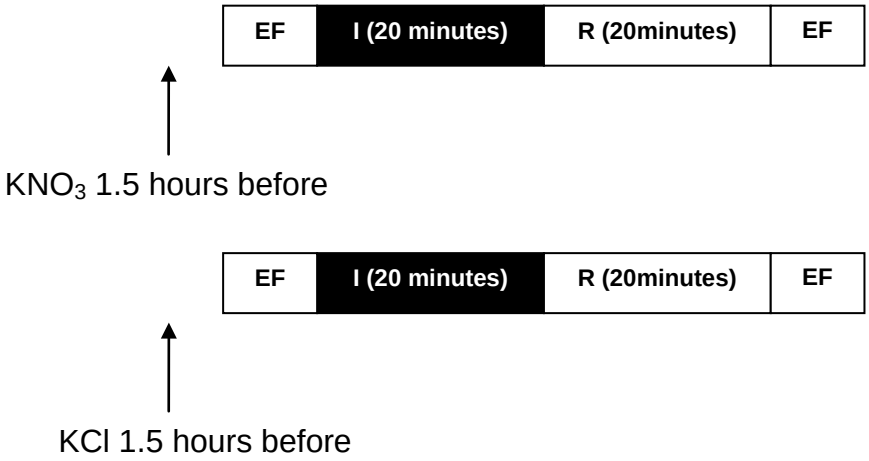


Figure 6.1c: *Effect of KNO_3 on endothelial IR injury*

6.3.4 Dose-dependent effect of beetroot juice on endothelial IR injury

To determine the effect of beetroot juice containing a lower dose of nitrate (5.5mmol) on endothelial IR injury, healthy volunteers received 250ml of beetroot juice (James White Drinks Ltd) 1.5 hours before IR or 250ml of water (Figure 6.1d).

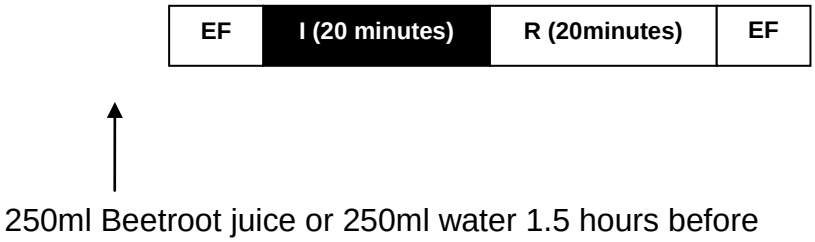


Figure 6.1d: *Effect of 250ml of beetroot juice on endothelial IR injury*

6.3.5 Blood sampling for nitrate and nitrite concentration

To determine the change in nitrate and nitrite concentrations in plasma after KNO_3, blood samples were obtained at baseline; then after KNO_3 every 30 minutes up to 3 hours.

6.4 Calculations and Statistical Analysis

See section 2.5 of chapter 2.

6.5 Results

The studies were well tolerated with beeturia and red stools as expected adverse effects. However one subject developed mild symptoms of gastritis which was associated with the potassium chloride capsules (on unblinding). This participant

was managed conservatively with antacids and withdrawn from the study. The mean nitrate concentration was 45.0±2.6mmol/L in the 500ml beetroot juice and 22.4±3.8mmol/L in the 250ml beetroot juice. The nitrite concentration in both volumes of beetroot juice was <50nmol/L.

6.5.1 Study 1

6.5.1.1 Effect of IR on endothelial function

IR reduced brachial artery FMD (7.5±0.9% before IR vs 3.1±0.4% after IR, p<0.0001; n=10) [Figure 6.2a].

6.5.1.2 Effect of beetroot juice on endothelial IR injury

500ml beetroot juice prevented IR induced endothelial dysfunction (FMD 6.8±1.0 % before IR + BJ 500 vs 5.5±1.0 after IR + BJ 500, p<0.001; n=10) [Figure 6.2a & b].

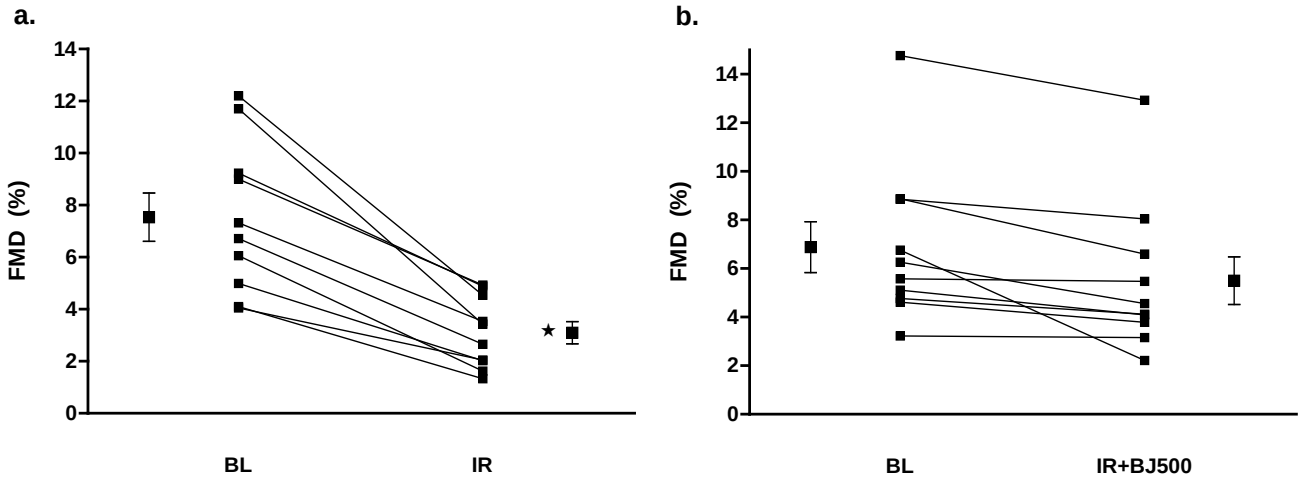


Figure 6.2*: Effect of beetroot juice (500ml) on endothelial IR injury*

6.5.2 Study 2

6.5.2.1 Effect of IR on endothelial function

IR reduced brachial artery FMD (10.3 ±1.0% before IR vs 4.9±0.8% after IR, p<0001; paired t test; n=12) [Figures 6.3a & 6.4]. A significant correlation existed between baseline and post-IR values for FMD (Pearson's r=0.7, P<0.0001).These correlations justified using ANCOVA to adjust post-IR values for differences in baseline endothelial function (EF) between the protocols, so that post-IR EF could be directly compared with greater statistical power [Table 6.1]

6.5.2.2 Effect of KNO_3 on endothelial IR injury

KNO_3 prevented the IR induced endothelial dysfunction (FMD 11.6±1.2% before IR + KNO_3 vs 10.2 ±1.1% after IR + KNO_3, p=NS; n=.12) [Figures 6.3b & 6.4] whereas there was no protection against endothelial IR injury with KCl (FMD 12.1±1.6% before IR + KCl vs 7.7±1.0% after IR + KCl, p<0.001; n=12) [Figures 6.3c & Figure 6.4]

6.5.4 Effect of lower dose beetroot juice derived nitrate

The lower dose of beetroot derived nitrate (250ml) also reduced the IR induced endothelial dysfunction FMD (11.0 ±1.2% before IR + BJ 250 vs 10.7±1.2% after IR + BJ 250, p=NS; n=12) [Figures 6.3d & 6.4].

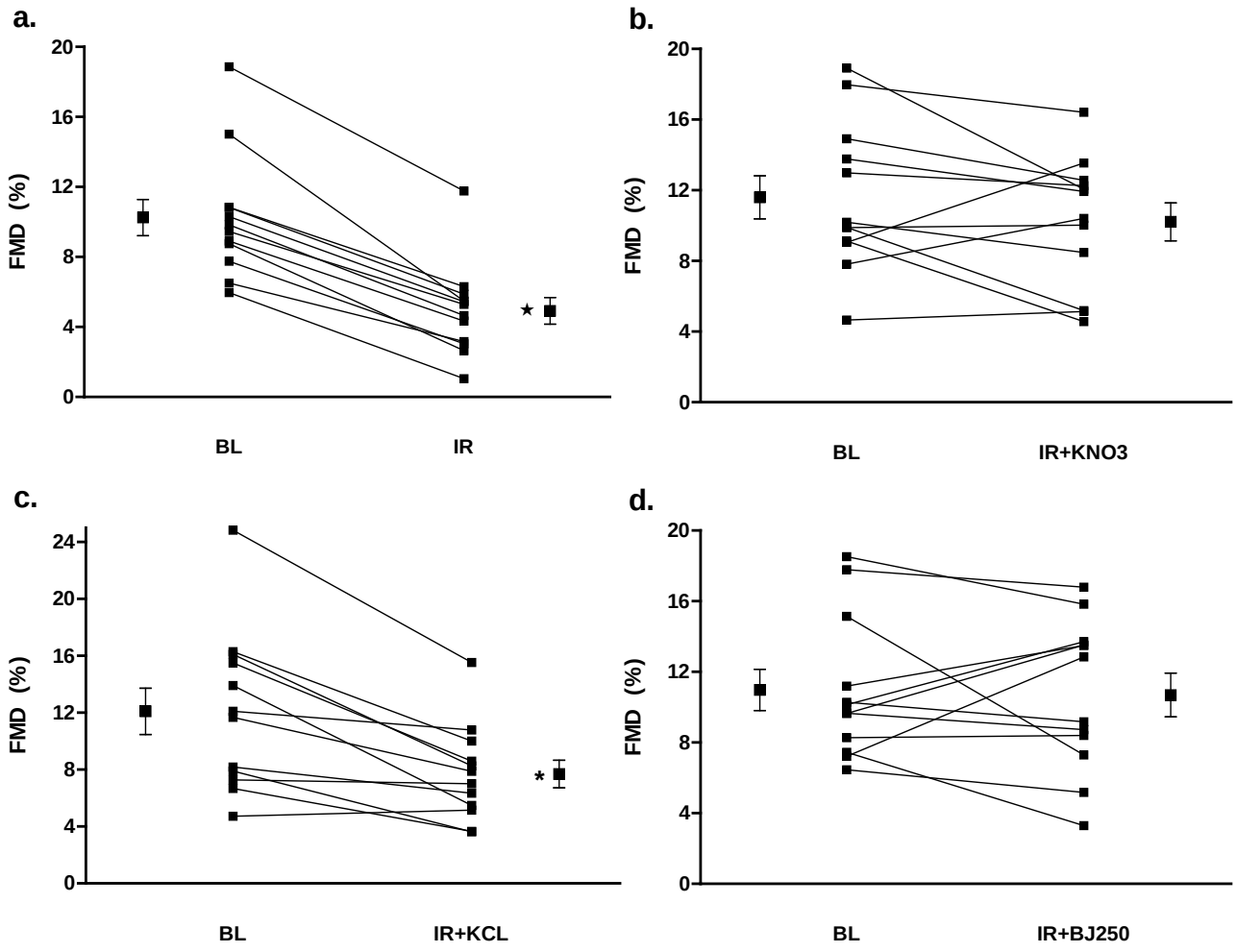


Figure 6.3: *Effect of (b) KNO3, (c) KCl and (d) 250ml of beetroot juice on (a) endothelial IR injury.*

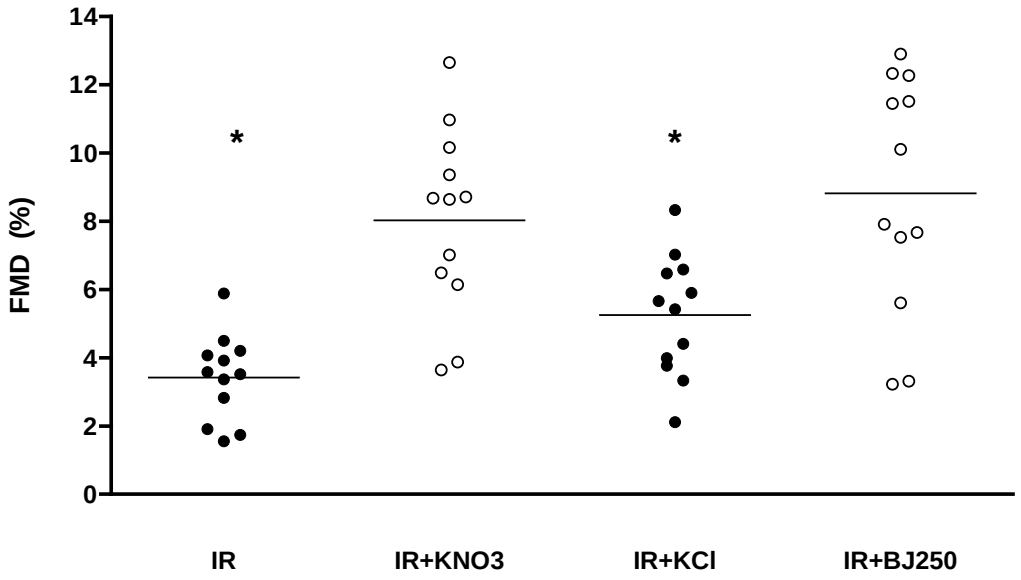


Figure 6.4: *Effect of KNO3 and 250ml of beetroot juice on endothelial IR injury. Both KNO3 (post-IR FMD, 8.0±0.8%, n=12) and 250ml of beetroot juice (post-IR FMD 8.8.±1.0%, n=12) prevented IR-induced reduction in FMD. KCl (post-IR FMD, 5.3±0.5%, n=12) did not protect against IR induced endothelial dysfunction. Post-IR values were adjusted for baseline FMD (ANCOVA).*

Study	n	Baseline FMD	Post-IR FMD	Post-IR FMD (ANCOVA Adjusted)
IR	12	10.3 ±1.0 %	4.9±0.8%	3.4.±0.4*
IR+KNO_3	12	11.6±1.2%	10.2 ±1.1%	8.0±0.8†
IR+KCL	12	12.1±1.6%	7.7±1.0%	5.3±0.5‡
IR+BJ250	12	11.0 ±1.2%	10.7±1.2%	8.8.±1.1

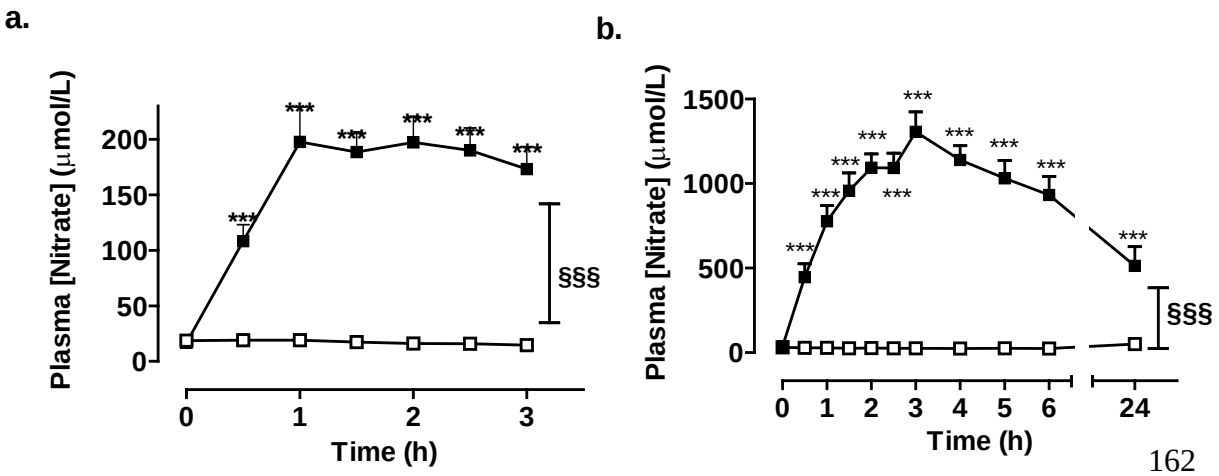
Data are expressed as mean±SEM. Post-IR FMD values were adjusted for baseline FMD by ANCOVA (Regression coefficient 0.56±0.13). For comparisons between the 4 groups, P values by ANCOVA were Scheffé adjusted.

*P<0.001, IR vs IR+KNO_3 and IR+BJ250
†P<0.05, IR+ KNO_3 vs IR+KCL
‡P<0.01, IR+BJ250 vs IR+KCL

Table 6.1: *Summary of baseline and Post-IR FMD data*

6.5.6 Circulating nitrate and nitrite concentration after oral nitrate load

Ingestion of 250ml of beetroot juice (5.5mmol nitrate) or KNO_3 capsules (24mmol nitrate) increased the circulating plasma nitrate concentration within 30 minutes and this peaked at 3 hours [Figure 6.5a & b]. The rise in plasma nitrite concentration was more modest with significantly elevated levels first evident at 1.5 hours and peaking at 2.5 hours [Figure 6.5c and d].



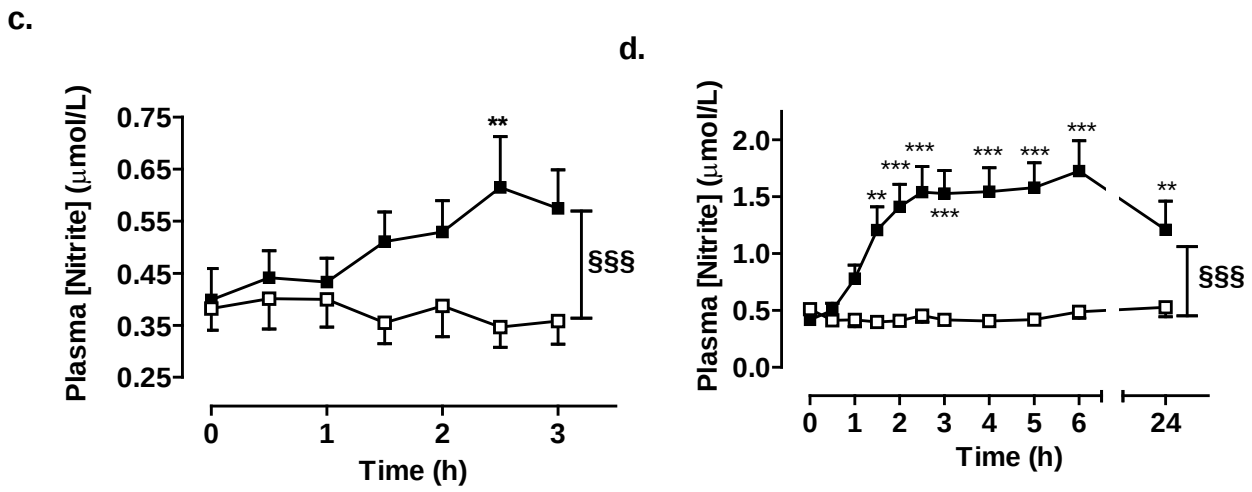


Figure 6.5: *Effect of oral nitrate load on plasma nitrate and nitrite compared with KCl. 250ml beetroot juice (5mmol nitrate) elevated nitrate and nitrite levels [(a) &(c)]. Similarly, KNO_3 (24mmol nitrate) elevated plasma nitrate and nitrite [(b) & (d)]. In contrast, KCl had no significant effect on plasma nitrate or nitrite (unfilled data points). Data are expressed as mean SEM of n=9. Significance shown for comparisons as §§§ P<0.0001 for 2-way ANOVA and *** P<0.001 or **P<0.01 for Bonferroni post hoc tests.*

6.6 Discussion

Results in this chapter demonstrate, for the first time in humans, that oral inorganic nitrate, in the form of beetroot juice or KNO_3 capsules, protects against endothelial IR injury. Notably this protection was not evident after ingestion of KCL capsules which negates potential confounding protective effects of K^+. Both beetroot juice and KNO_3 capsules resulted in increases in plasma nitrate and nitrite indicating that the endogenous handling of oral inorganic nitrate is the same whether the source is a nitrate salt or the diet.

6.6.1 Plasma nitrate and nitrite concentrations after an oral nitrate load

The exact mechanism by which vegetable-rich diets confer protection against cardiovascular disease remains uncertain. However the recent suggestions that their beneficial effects are as a result of nitrate/nitrite derived NO has generated further interest.

My results indicate that an oral nitrate load (beetroot juice or KNO_3) administered before IR produced a rapid (within 30 minutes) rise in circulating plasma nitrate concentration while the plasma nitrite concentration showed a more gradual rise with significant elevation in concentration at 1.5 hours. The important finding with respect to IR injury was that the elevated nitrate and nitrite concentrations were sustained beyond the onset of reperfusion, therefore providing the necessary substrate for NO generation. A potential explanation for this time lag between the plasma nitrate and nitrite peaks is the endogenous production of nitrites which is facilitated by the enterosalivary circuit. This is corroborated by the observation that interruption of this circuit, by avoidance of swallowing, blocks the increase in plasma nitrite but has no effect on plasma nitrate (202). In addition, beetroot juice contained large amounts of nitrate but undetectable levels of nitrite. The reduction of nitrite to NO is thought to be facilitated by enzymes (predominantly xanthine oxidoreductase) or deoxyhaemoglobin, activities of which are enhanced during ischaemic conditions (198, 200, 201).

Bryan et al showed that nitrate supplementation in the drinking water of mice for 7 days, which also protected against the damaging effects of a myocardial IR injury, was associated with higher steady state plasma and heart nitrite levels (217). Conversely, this group observed that feeding mice with a low nitrite/nitrate diet for the same period of time, resulted in reduced levels of plasma and myocardial nitrite

concentration and the protection against IR injury was lost. The acute rise in plasma nitrite concentration I observed is consistent with results from other human studies and is associated with nitrate accumulation in saliva and plasma (202, 203).

6.6.2 Oral inorganic nitrate and ischaemic protection

The early phase of reperfusion is critical in the mediation of IR injury, presenting an immediate therapeutic window of opportunity. My results indicate that oral inorganic nitrate in the form of beetroot juice and potassium nitrate tablets, led to a reduction in endothelial IR injury. Notably the early phase of reperfusion corresponded with elevated plasma concentrations of nitrate and nitrite. I did not observe such an effect with my volume control (water) or potassium control (potassium chloride tablets) interventions, which strongly suggest that the oral nitrate load was responsible for these biological effects. The quantity of nitrite in the diet is limited and the half-life short (see section 1.10.3 of chapter 1); therefore there is a need for further investigation into the benefits which might be derived from oral inorganic nitrate in whatever form.

6.6.3 Clinical perspective

Contributing to an increase in NO bioavailability appears to be the fundamental principle underlying the therapeutic use of oral inorganic nitrates in protection against IR injury. Organic nitrates, such as nitroglycerin (GTN) or isosorbide mononitrate, are widely used in clinical practice for the management of heart failure or relief of angina and cause rapid generation of NO in vascular smooth muscle. This can generate profound vasodilatation which might cause significant hypotension and limit their use. Another significant problem with the use of organic

nitrates is the development of tolerance. Although the exact mechanism of nitrate intolerance is not clear, increased formation of reactive oxygen species have been implicated (221, 295). An additional advantage of inorganic nitrates over the organic nitrates is that their bioactivation to NO is pH- and pO_2- dependent such that therapeutic effects are localised to the ischaemic tissue without an unwanted generalised effect.

6.6.4 Conclusion

Inorganic nitrate and nitrite biology has received a considerable amount of research interest particularly in relation to its contribution to endogenous NO production and protection against IR injury. A parallel study by some of my research collaborators also demonstrates effects of oral inorganic nitrates in BP reduction. Further efforts to harness the full potential of this area of research should be in the form of well designed clinical trials. This will enable us build on the very promising preliminary data in this thesis and might enable the exploitation of oral nitrate supplementation in the acute or chronic ischaemic setting.

Chapter 7

Summary and Conclusions

In this thesis, I investigated the mechanisms of ischaemic protective phenomena (IPC, PostC, RIPC, RPostC) using a human *in vivo* model of endothelial IR injury in the forearm. I utilised pharmacological probes to investigate the role of a) K_{ATP} channels and the mPTP in PostC; b) the opioid pathway in remote conditioning; and c) components of the autonomic nervous system (alpha adrenergic and cholinergic) in remote conditioning. I also determined whether oral inorganic nitrates (beetroot juice and KNO_3) protected against endothelial IR injury in humans.

7.1 Mechanisms of postconditioning

In chapter 3, I demonstrated that PostC protects against endothelial IR injury in humans in two vascular beds (resistance and conduit vessels). This provides more data in support of PostC as a valid therapeutic intervention aimed at reducing IR injury in humans. Furthermore, data in this thesis suggest that the mPTP is involved in the mediation of protection by PostC as ciclosporin, administered around the onset of reperfusion, mimicked the protective effect of PostC. This is consistent with data that showed cardioprotection from ciclosporin administered around the time of percutaneous coronary intervention in patients presenting with acute myocardial infarction (157). An obvious therapeutic implication is pharmacological postconditioning with ciclosporin, a drug with a long term safety profile. This might enable the therapeutic benefits of ischaemic PostC to be derived whilst avoiding the uncertainties of safety and feasibility of the technique.

Results from Chapter 3 also highlight a role for K_{ATP} channels in the mechanism of PostC. This was evident from studies which showed that glibenclamide a non-selective K_{ATP} channel blocker abolished the protective effects of PostC against endothelial IR injury. However with the pancreas-selective K_{ATP} channel blocker,

glimepiride, protection by PostC was preserved. K_{ATP} channel blockers are widely used in clinical practice for the treatment of Type 2 Diabetes. Diabetic patients are at an increased risk of cardiovascular events and are more likely to benefit from protective phenomena such as PostC. The differential effects of subtype specific K_{ATP} channel blocker perhaps need to be considered when using these drugs in this patient cohort.

7.1.1 Future work

Translation of the above findings will require clinical trials to determine the optimal dose of ciclosporin, as preclinical studies indicate that high dose may actually exacerbate IR injury (273). Robust trials with appropriate endpoints should be aimed at validating the protective effect of ciclosporin during IR injury and determining the differential effects of K_{ATP} channel blockers on protective strategies. Such trials will not be trivial because they will need to be large and are unlikely to be sponsored by the pharmaceutical industry. Epidemiological data from various sources might also provide complementary information on cardiovascular outcomes in patients on ciclosporin and K_{ATP} channel blockers.

7.2 Mechanisms of transfer of protection by remote conditioning

Data from chapter 4 of this thesis highlight a role for the opioid receptor pathway in RIPC. The evidence for this is that non selective opioid receptor blocker, naloxone, abolished protection afforded by RIPC. In contrast, protection by IPC was not affected by naloxone.

These results provide a further suggestion that endogenous opioids play a prominent role in the transfer of protection from a remote site during RIPC rather

than for local protection. Furthermore, the data also indicate that RPostC protected against endothelial IR injury and this protection was preserved in the presence of naloxone suggesting a less significant role for the opioid pathway in RPostC.

Experiments in Chapter 5 demonstrate that protection by RPostC is lost when the alpha adrenoceptor blocker, phentolamine, was co-administered while protection by RIPC was unaffected. This suggests that alpha adrenergic component of the autonomic nervous system (ANS) contributes significantly to protection by RPostC. My results also show that blockade of the cholinergic component using atropine did not affect protection by RIPC or RPostC. This suggests that the cholinergic component of the autonomic nervous system is of limited importance during RIPC or RPostC. However, previous studies in this laboratory show that complete autonomic blockade with trimetaphan abolished protection by RIPC (187). A speculation is that in addition to the activation of opioid pathway RIPC depends on a component of the autonomic nervous system to cross a threshold of protection.

The main difference between RIPC and RPostC is in the timing of the application of the stimulus in relation to IR. A plausible mechanism of the mode of transfer of protection is that the two pathways (neurogenic and humoral) act synergistically in parallel, with the autonomic pathway providing an initial predominant stimulus which rises above a threshold for protection for a limited period of time during RPostC. As time elapses the humoral pathway becomes the predominant stimulus and reaches threshold, responsible for protection by RIPC.

7.2.1 Future work

Further studies in order to probe the mechanisms of transfer of protection by remote conditioning are essential. Mechanistic studies should aim to determine the role of

other humoral factors such as bradykinin, hypoxic inducible factor (HIF) and adenosine in the transfer of protection by a remote protective stimulus. Assessing the role of the beta adrenoceptor component of the ANS in facilitating remote protection is also appropriate. Information on the time course of transfer of protection via the neurogenic and humoral pathways will enable an understanding of their interaction and relative contribution to remote ischaemic protection. This might enable full utilisation of this form of ischaemic protection.

7.3 Oral inorganic nitrates in protection against IR injury

Studies in chapter 6 focused on the role of oral inorganic nitrates in protection against endothelial IR injury. Results suggest that oral inorganic nitrates in the form of beetroot juice and potassium nitrate capsules provide protection against endothelial IR injury. These data build on previous data which until now have been based on animal models of IR injury. The L-arginine-NOS system is a major source of NO and is critical in maintaining cardiovascular homeostasis. An important advance, with potential therapeutic application, is in the recognition of alternative NO generation via the nitrate and nitrite (NOS independent) pathways, during conditions associated with dysfunction of the NOS dependent pathway, as in IR injury.

7.3.1 Future work

Further studies using other human models of IR injury are required to characterise the protective role of NOS independent pathway and validate this potential therapeutic target.

7.4 Conclusion

This translational research project has been performed to obtain mechanistic information on aspects of ischaemic protection in humans. The ultimate goal is to identify therapeutic targets which can be developed for clinical application. The persisting gaps in knowledge necessitate further research.

References

1. WHO cardiovascular disease fact sheet 317. Jan 2011. http://www.who.int/mediacentre/factsheets/fs317/en/index.html
2. Falk E. Pathogenesis of Atherosclerosis. J Am Coll Cardiol. 2006; 47(8 Suppl):C7-12
3. Glass CK, Witzum JL. Atherosclerosis, the road ahead. Cell 2001; 104(4):503-16
4. Woolf N. The origins of atherosclerosis. Postgraduate Medical Journal 1978; 54:156-161
5. Stary HC. Evolution and Progression of Atherosclerotic lesions in Coronary Arteries of Children and Young Adults. Arteriosclerosis 1989; 9(1):19-32
6. Willeit J, Kiechl S. Biology of Arterial Atheroma. Cerebrovasc Dis 2000; 10(suppl 5):1-8
7. Hansson GK. Inflammation, Atherosclerosis and Coronary Artery disease. N Engl J Med 2005; 352(16):1685-95
8. Stary HC, Chandler B, Glagov S, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1994;89:2462-2478
9. Corti R, Hutter R, Badimon JJ, Fuster V. Evolving concepts in the triad of atherosclerosis, inflammation and thrombosis. J Thromb Thrombolysis 2004; 17(1):35-44

10. Jensen JS, Borch-Johnsen K, Jensen G, Feldt-Rasmussen B Atherosclerotic risk factors are increased in clinically healthy subjects with microalbuminuria. Atherosclerosis 1995; 112(2):245-52
11. Jennings RB, Reimer KA. The Cell Biology of Acute Myocardial Ischemia. Annu. Rev. Med. 1991; 42:225-46
12. Pasque MK, Wechsler AS. Metabolic intervention to affect myocardial recovery following ischemia. Ann Surg. 1984; 200(1):1-12
13. Katz AM. Quantification of myocardial contractility. Am J Cardiol 1970; 26(30:331-2
14. Jennings RB, Steenbergen C Jr. Nucleotide metabolism and cellular damage in myocardial ischemia. Annu Rev Physiol 1985; 47:727-49
15. Suleiman MS, Halestrap AP, Griffiths EJ. Mitochondria: a target for myocardial protection. Pharmacol Ther. 2001; 89(1);29-46
16. Duda M, Konior A, Klemenska E, Beresewicz A. Preconditioning protects endothelium by preventing ET-1-induced activation of NADPH oxidase and xanthine oxidase in post-ischemic heart. J Mol Cell Cardiol 2007; 42(2):400-10
17. Buja LM. Myocardial ischemia and reperfusion injury. Cardiovasc Pathol 2005; 14(4):170-5
18. Piper HM, Abdallah Y, Schafer C. The first minutes of reperfusion: a window of opportunity for cardioprotection. Cardiovasc Res. 2004; 61(3): 365-71
19. Rossi A, Kay L, Saks V. Early ischemia-induced alterations of the outer mitochondrial membrane and the intermembrane space: a potential cause for altered energy transfer in cardiac muscle? Mol Cell Biochem. 1998; 184(1-2):401-8

20. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. J Pathol 2000; 190(3):255-66

21. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. N Engl J Med. 2007; 357 (11): 1121-35

22. Javadov SA, Clarke s, Das M, Griffiths EJ, Lim KH, Halestrap AP. Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. J Physiol. 2003; 549(pt2):513-24

23. Crompton M. The mitochondrial permeability transition pore and its role in cell death. Biochem J. 1999; 341:233-49

24. Halestrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res. 2004; 61(3):372-85

25. Kinnally KW, Peixoto PM, Ryu SY, Dejean LM. Is mPTP the gatekeeper for necrosis, apoptosis or both? Biochim Biophys Acta 2011; 1813(4):616-22

26. Halestrap AP. A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection. Biochem.Soc.Trans 2010; 38(4): 841-60

27. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD. Loss of cyclophilin D reveals a crticial role for mitochondrial permeability transition in cell death. Nature 2005; 435 (7033):658-62

28. Nakgawa T, Shimizu S, Watanbe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 2005; 434 (7033):652-8

29. Schinzel AC, Takeuchi O, Huang Z, Fisher JK, Zhou Z, Rubens J, Hetz C, Danial NN, Moskowitz MA, Korsmeyer SJ. Cyclophilin D is a component of mitochondrial permeability transition and mediatesneuronal cell death after focal cerebral ischaemia. Proc Natl Acad Sci USA 2005; 102(34):12005-10

30. Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? Cardiovasc Res. 2002; 55(3):534-43

31. Argaud L, Gateau-Roesch O, Muntean D, Chalabreysse L, Loufouat J. Robert D, Ovize M. Specific inhibition of the mitochondrial permeability transition pore prevents lethal reperfusion injury. J Mol Cell Cardiol. 2005; 38(2):367-74

32. Griffiths EJ, Halestrap AP. Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open on reperfusion. Biochem J 1995; 307:93-8

33. Grankvist K, Marklung SL, Taljedal IB. CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidise in pancreatic islets and other tissues in the mouse. Biochem J. 1981; 199(2):393-8

34. Pigeleot E, Corbisier E, Houbion A, Lambert D, Michiels C, Raes M, Zachary MD, Remacle J. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. Mech Ageing Dev. 1990; 51(3):283-97

35. Zhan CD, Sindhu RK, Pang J, Ehdaie A, Vaziri ND. Superoxide dismutase, catalase and glutathione peroxidase in the spontaneously hypertensive rat kidney: effect of antioxidant rich diet. J Hypertens. 2004; 22(10):2025-33

36. Kunimatsu T, Kobayashi K, Yamashita A, Yamamoto T, Lee MC. Cerebral reactive oxygen species assessed by electron spin resonance spectroscopy in the initial

stage of ischemia-reperfusion are not associated with hypothermic neuroprotection. J Clin Neurosci. 2011; 18(4):545-8

37. Zweier JL, Duke SS, Kuppusamy P, Sylvester JT, Gabrielson EW. Electron paramagnetic resonance evidence that cellular oxygen toxicity is caused by the generation of superoxide and hydroxyl free radicals. FEBS Lett. 1989; 252(1-2):12-6
38. Garciarena CD, Caldiz CI, Correa MV, Schinella GR, Mosca SM, Chiappe de Cingolani GE, Ennis IL. Na+/H+ exchanger-1 inhibitors decrease myocardial superoxide production via direct mitochondrial action. J Appl Physiol. 2008; 105(6):1706-13
39. Pryzyklenk K, Kloner RA. Effect of oxygen-derived free radical scavengers on infarct size following six hours of permanent coronary artery occlusion: salvage or delay of myocardial necrosis. Basic Res Cardiol. 1987; 82(2):146-58
40. Jolly SR, Kane WJ, Bailie MB, Abrams GD, Lucchesi BR. Canine myocardial reperfusion injury: Its reduction by the combined administration of superoxide dismutase and catalase. Cir. Res. 1984; 54:277-285
41. Pleiner J, Schaller G, Mittermayer F, Marsik C, MacAllister RJ, Kapiotis S, Ziegler S, Ferlitsch A, Woltz M. Intra-arterial vitamin C prevents endothelial dysfunction caused by ischaemia-reperfusion. Atherosclerosis. 2008; 197:383-391
42. Moses AL, Champion HC, Claeys MJ, Tavazzi B, Kaminski PM, Wolin MS, Borgonjon DJ, Van Nassauw L, Haile A, Zviman M, Bedja D, Wuyts FL, Elsaesser RS, Cos P, Gabrielson KL, Lazzarino G, Paolocci N, Timmermans JP, Vrints CJ, Kass DA. High-dose folic acid pretreatment blunts cardiac dysfunction during ischemia coupled to maintenance of high-energy phosphates and reduces postreperfusion injury. Circulation. 2008; 117:1810-1819

43. Mueller CF, Laude K, McNally JS, Harrison DG. ATVB in focus: redox mechanisms in blood vessels. Arterioscler Thromb Vasc Biol. 2005; 25(2):274-8

44. Griendling KK. ATVB in focus: redox mechanisms in blood vessels. Arterioscler Thromb Vasc Biol. 2005; 25(2):272-3

45. Babior BM. NADPH oxidase. Curr Opin Immunol. 2004; 16(1):42-7

46. Lambeth JD. Nox enzymes and the biology of reactive oxygen. Nat Rev Immunol. 2004; 4(3): 181-9

47. Jordan JE, Zhao ZQ, Vinten-Johansen J. The role of neutrophils in myocardial ischemia-reperfusion injury. Cardiovasc Res. 1999; 43(4):860-878

48. Krijnen PA, Meischl C, Hack CE, Meijer CJ, Visser CA, Roos D, Niessen HW. Increased Nox2 expression in human cardiomyocytes after acute myocardial infarction. J Clin Pathol. 2003; 56:194-199

49. Fisher AB, Al Mehdi AB, Muzykantov V. Activation of endothelial NADPH oxidase as the source of reactive oxygen species in lung ischemia. Chest. 1999; 116:25S-26S

50. Kahles T, Luedke P, Endres M, Galla HJ, Steinmetz, Busse R, Neumann-Haefelin T, Brandes RP. NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. Stroke. 2007; 38:3000-3006

51. Looi YH, Grieve DJ, Siva A, Walker SJ, Anilkumar N, Marber M, Monaghan MJ, Shah AM. Involvement of Nox2 NADPH oxidase in adverse cardiac remodeling after myocardial infarction. Hypertension. 2008; 51:319-325

52. Harada H, Hines IN, Flores S, Gao B, McCord J, Scheerens H, Grisham MB. Role of NADPH oxidase-derived superoxide in reduced size liver ischemia and reperfusion injury. Arch Biochem Biophys. 2004; 423:103-108

53. Loukogeorgakis SP, van den Berg MJ, Sofat R, Nitsch D, Charakida M, Haiyee B, de Groot E, MacAllister RJ, Kuijpers TW, Deanfield JE. Role of NADPH oxidase in endothelial ischemia/reperfusion injury in humans. Circulation. 2010; 121:2310-6

54. Berry CE, Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. J Physiol. 2004; 555:589-606

55. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. N Engl J Med. 2007; 357:1121-35

56. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovasc Res. 2004; 61:481-497

57. Litt MR, Jeremy RW, Weisman HF, Winkelstein JA, Becker LC. Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 mninutes of ischemia. Evidence for neutrophil-mediated reperfusion injury. Circulation. 1989; 80:1816-1827

58. Jolly SR, Kane WJ, Hook BG, Abrams GD, Kunkel SL, Lucchesi BR. Reduction of myocardial infarct size by neutrophil depletion: effect of duration of occlusion. Am Heart J. 1986; 112:682-90

59. De Logeril M, Basmadjian A, Lavallee M, Clement R, Millette D, Rousseau G, Latour JG. Influence of leukopenia on collateral flow, reperfusion flow, reflow ventricular fibrillation, and infarct size in dogs. Am Heart J. 1989; 117:523-32

60. Weyrich AS, Ma XY, Lefer DJ, Albertine KH, Lefer AM. In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. J Clin Invest. 1993; 91:2620-9

61. Ma XL, Tsao PS, Lefer AM. Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. J Clin Invest. 1991; 88:1237-43

62. Vane JR, Angarrd EE, Botting RM. Regulatory functions of the vascular endothelium. N Engl J Med. 1990; 323:27-36

63. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature.1980; 288:373-6

64. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994; 298:249-58

65. Arnal JF, Dinh-Xuan A –T, Pueyo M, Darblade B, Rami J. Endothelium-derived nitric oxide and vascular physiology and pathology. Cell Mol Life Sci. 1999; 55:1078-1087

66. Arnal JF, Warin L, Michel JB. Determinants of aortic cyclic guanosine monophosphate in hypertension induced by chronic inhibition of nitric oxide synthase. J Clin Invest. 1992; 90:647-52

67. Moncada S. Biology and therapeutic potential of prostacyclin. Stroke. 1983; 14:157-68

68. Gryglewski RJ. Prostacyclin among prostanoids. Pharmacol Rep. 2008; 60:3-11

69. Luksha L, Agewall S, Klubickliene K. Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. Atherosclerosis. 2009; 202:330-44

70. Waldron GJ, Ding H, Lovren F, Kubes P, Triggle CR. Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase. Br J Pharmacol. 1999; 128: 653-8

71. Saito Y, Nakao K, Mukoyama M, Imura H. Increased plasma endothelin level in patients with essential hypertension. N Engl J Med. 1990; 322:205

72. Haak T, Jungmann E, Felber A, Hillman U, Usadel KH. Increased plasma levels of endothelin in diabetic patients with hypertension. Am J Hypertens. 1992; 5:161-5

73. Zouridakis EG, Schwartzman R, Garcia-Moll X, Cox ID, Fredericks S, Holt DW, Kaski JC. Increased plasma endothelin levels in angina patients with rapid coronary artery disease progression. Eur Heart J. 2001; 22:1578-84

74. Reriani M, Raichlin E, Prasad A, Mathew V, Pumper GM, Nelson RE, Lennon R, Rihal C, Lerman LO, Lerman A. Long-term administration of endothelin receptor antagonist improves coronary endothelial function in patients with early atherosclerosis. Circulation. 2010; 122:958-66

75. Halcox JP, Nour KR, Zalos G, Quyyumi AA. Endogenous endothelin in human coronary vascular function: differential contribution of endothelin receptor types A and B. Hypertension. 2007; 49:1134-41

76. Weber MA, Black H, Bakris G, Krum H, Linas S, Weiss R, Linseman JV, Wiens BL, Warren MS, Lindholm LH. A selective endothelin-receptor antagonist to reduce blood pressure in patients with treatment-resistant hypertension: a randomised controlled trial. Lancet. 2009; 374:1423-31

77. Ku DD. Coronary vascular reactivity after acute myocardial ischaemia. Science. 1982; 218:576-8

78. Reffelman T, Kloner RA. The no-reflow phenomenon: A basic mechanism of myocardial ischemia and reperfusion. Basic Res Cardiol. 2006; 101(5):359-72

79. Ito H, Maruyama A, Iwakura K et al. Clinical implications of the 'no reflow' phenomenon. A predictor of complications and left ventricular remodeling in reperfused anterior wall myocardial infarction. Circulation 1996; 93(2):223-8

80. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74(5):1124-36

81. Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. Circulation 1993; 88(3):1264-72

82. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. Circ Res. 1993; 72(6);1293-9

83. Baxter GF, Yellon DM. Time course of delayed myocardial protection after transient adenosine A1-receptor activation in the rabbit. J Cardiovasc Pharmacol 1997; 29(5):631-8

84. Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. Circulation. 1991; 84:350-6

85. Liu GS, Richards SC, Olsson RA, Mullane K, Walsh RS, Downey JM. Evidence that the adenosine A3 receptor mediate the protection afforded by preconditioning in the isolated rabbit heart. Cardiovasc Res. 1994; 28:1057-1061

86. Wall TM, Sheehy R, Hartman JC. Role of bradykinin in myocardial preconditioning. J Pharmacol Exp Ther. 1994; 270:681-9

87. Goto M, Liu Y, Yang XM, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. Circ Res. 1995; 77:611-21

88. Schultz JE, Rose E, Yao Z, Gross GJ. Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. Am J Physiol. 1995; 268:H2157-H2161

89. Schultz JJ, Hsu AK, Gross CJ. Ischemic preconditioning is mediated by a peripheral opioid receptor mechanism in the intact rat heart. J Mol Cell Cardiol. 1997; 29:1355-62

90. Bell SP, Sack MN, Patel A, Opie LH, Yellon DM. Delta opioid receptor stimulation mimics ischemic preconditioning in human heart muscle. J Am Coll Cardiol. 2000; 36:2296-2302

91. Bell RM and Yellon DM. The contribution of endothelial nitric oxide synthase to early ischaemic preconditioning: The lowering of the preconditioning threshold. An investigation in eNOS knockout mice. Cardiovasc Res. 2001; 52:274-280

92. Yao Z, Tong J, Tan X, Li C, Shao Z, Kim WC, Vanden-Hoek TL, Becker LB, Head CA, Schumacker PT. Role of reactive oxygen species in acetylcholine-induced preconditioning in cardiomyocytes. Am J Physiol. 1999; 277:H2504-9

93. Cohen MV, Yang XM, Liu GS, Heusch G, Downey JM. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K (ATP) channels. Circ Res. 2001; 89:273-8

94. Yellon DM, Downey JM. Preconditioning the myocardium: From cellular physiology to clinical cardiology. Physiol Rev. 2003; 83:1113-1151

95. Tritto I, D'Andrea D, Eramo N, Scognamiglio A, De Simone C, Violante A, Esposito A, Chiariello M, Ambrosio G. Oxygen radicals can induce preconditioning in rabbit hearts. Circ Res. 1997; 80:743-8

96. Baines CP, Goto M, Downey JM. Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. J Mol Cell Cardiol. 1997; 29:207-16

97. Banerjee A, Locke-Winter C, Rogers KB, Mitchell MB, Brew EC, Cairns CB, Bensard DD, Harken AH. Preconditioning against myocardial dysfunction after ischemia and reperfusion by an alpha 1-adrenergic mechanism. Circ Res. 1993; 73:656-70

98. Li YJ, Peng J. The cardioprotection of calcitonin gene-related peptide-mediated preconditioning. Eur J Pharmacol. 2002; 442:173-177

99. Tong H, Imahashi K, Steenbergen C, Murphy E. Phosphorylation of glycogen synthase-3β during preconditioning through a phosphatidylinositol-3-kinase-dependent pathway is cardioprotective. Circ Res. 2002; 90:377-9

100. Obame FN, Plin-Mercier C, Assaly R, Zini R, Dubois-Rande JL, Berdeaux A, Morin D. Cardioprotective effect of morphine and a blocker of glycogen synthase kinase 3 beta, SB216763 [3-(2,4-dichlorophenyl)-4(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione], via inhibition of the mitochondrial permeability transition pore. J Pharmacol Exp Ther. 2008; 326:252-8

101. Feng J, Bianchi C, Sandmeyer JL, Sellke FW. Bradykinin preconditioning improves the profile of cell survival proteins and limits apoptosis after cardioplegic arrest. Circulation. 2005; 112:I190-5

102. Xuan YT, Tang XL, Banerjee S, Takano H, Li RC, Han H, Qiu Y, Li JJ, Bolli R. Nuclear factor-kappaB plays an essential role in the late phase of ischemic preconditioning in conscious rabbits. Circ Res. 1999; 84:1095-109

103. Xuan YT, Tang XL, Banerjee S, Takano H, Li RC, Han H, Bolli R. Biphasic response of cardiac NO synthase isoforms to ischemic preconditioning in conscious rabbits. Am J Heart Circ Physiol. 2000; 279:H2360-71

104. Guo Y, Jones WK, Xuan YT, Tang XL, Bao W, Wu WJ, Han H, Laubach VE, Ping P, Yang Z, Qiu Y, Bolli R. The late phase of ischemic preconditioning is abrogated

by targeted disruption of the inducible NO synthase gene. Proc Natl Acad Sci USA. 1999; 96:11507-11512

105. Bolli R, Manchikalapudi S, Tang XL, Takano H, Qiu Y, Guo Y, Zhang Q, Jadoon AK. The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase. Evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. Circ Res. 1997; 81:1094-107
106. Inagaki N, Gonoi T, Clement JP 4th , Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J. Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. Science 1995; 270**:**1166–1170
107. McCully JD, Levitsky S. The mitochondrial K (ATP) channel and cardioprotection. Ann Thorac Surg 2003; 75:S667–S673
108. Gribble FM, Reimann F. Differential selectivity of insulin secretagogues Mechanisms, clinical implications, and drug interactions. J Diabetes Complications 2003; 17:11–15
109. Ashcroft FM, Gribble FM. Tissue-specific effects of sulfonylureas: lessons from studies of cloned KATP channels. J Diabetes Complications 2000; 14:192–196
110. Toller WG, Gross ER, Kersten JR, Pagel PS, Gross GJ, Warltier DC. Sarcolemmal and mitochondrial adenosine triphosphate- dependent potassium channels: mechanism of desflurane-induced cardioprtection. Anesthesiology. 2000; 92:1731-9
111. Suzuki M, Sasaki N, Miki T, Sakamoto N, Ohmoto-Sekine Y, Tamagawa M, Seino S, Marban E, Nakaya H. Role of sarcolemmal K(ATP) channels in cardioprotection against ischemia/reperfusion injury in mice. J Clin Invest. 2002; 109:509-16

112. Garlid KD, Dos Santos P, Xie ZJ, Costa AD, Paucek P. Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K(+) channel in cardiac function and cardioprotection. Biochim Biophys Acta. 2003; 1606:1-21

113. Halestrap AP, Clarke SJ, Khaliulin I. The role of mitochondria in protection of the heart by preconditioning. Biochim Biophys Acta. 2007; 1767:1007-1031

114. Stein AB, Tang XL, Guo Y, Xuan YT, Dawn B, Bolli R. Delayed adaptation of the heart to stress: late preconditioning. Stroke. 2004; 35:2676-9

115. Alkulaifi AM, Yellon DM, Pugsley WB. Preconditioning the human heart during aorto-coronary bypass surgery. Eur J Cardiothoracic Surg. 1994; 8:270-5

116. Lu EX, Chen SX, Yuan MD, Hu TH, Zhou HC, Luo WJ, Li GH, Xu LM. Preconditioning improves myocardial preservation in patients undergoing open heart operations. Ann Thorac. Surg. 1997; 64:1320-4

117. Lu EX, Chen SX, Hu TH, Xui LM, Yuan MD. Preconditioning enhances myocardial protection in patients undergoing open heart surgery. Thorac Cardiovasc Surg. 1998; 46:28-32

118. Solomon SD, Anavekar NS, Greaves S, Rouleau JL, Hennekens C, Pfeffer MA; HEART investigators. Angina pectoris prior to myocardial infarction protects against subsequent left ventricular remodelling. J Am Coll Cardiol. 2004; 43:1511-4

119. Rezkalla SH, Kloner RA. Ischemic preconditioning and preinfarction angina in the clinical arena. Nat Clin Pract Cardiovasc Med. 2004; 1:96-102

120. Ottani F, Galvani M, Ferrini D, Sorbello F, Limonetti P, Pantoli D, Rusticali F. Prodromal angina limits infarct size. A role for ischemic preconditioning. Circulation. 1995; 91:291-7

121. Kloner RA, Rezkalla SH. Preconditioning, postconditioning and their application to clinical cardiology. Cardriovasc Res. 2006; 70:297-307

122. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003; 285(2):H579-88

123. Kin H, Zhao HQ, Sun HY, Wang NP, Corvera JS, Halkos ME, Kerendi F, Guyton RA, Vinten-Johansen J. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res. 2004; 62(1):74-85

124. Loukogeorgakis SP, Panagiotidou AT, Yellon DM, Deanfield JE, MacAllister RJ. Postconditioning protects against endothelial ischemia-reperfusion injury in the human forearm. Circulation 2006; 113(7):1015-9

125. Vinten-Johansen J, Zhao ZQ, Zatta AJ, Kin H, Halkos ME, Kerendi F. Postconditioning--A new link in nature's armor against myocardial ischemia-reperfusion injury. Basic Res Cardiol. 2005; 100(4):295-310

126. Penna C, Mancardi D, Raimondo S, Geuna S, Pagliaro P. The paradigm of postconditioning to protect the heart. J Cell Mol Med 2008; 12(2):435-58

127. Halkos ME, Kerendi F, Corvera JS, Wang NP, Kin H, Payne CS, Sun HY, Guyton RA, Vinten-Johansen J. Myocardial protection with postconditioning is not enhanced by ischemic preconditioning. Ann Thorac Surg. 2004; 78(3):961-9

128. Hu X, Jiang H, Ma F, Xu C, Bo C, Wen H, Wu B, Lu Z. Similarities between ischemic preconditioning and postconditioning in myocardial ischemia/reperfusion injury. Int J Cardiol. 2010; 144: 135-6

129. Obal D, Dettwiler S, Favoccia C, Scharbatke H, Preckel B, Sclack W. the influence of mitochondrial KATP channels in the cardioprotection of preconditioning and postconditioning by sevoflurane in the rat in vivo. Anesth. Analg. 2005; 101:1252-1260

130. Wu BQ, Chu WW, Zhang LY, Wang P, Ma QY, Wang DH. Protection of preconditioning, postconditioning and combined therapy against hepatic ischemia/reperfusion injury. Chin J Traumatol. 2007; 10:223-7

131. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phoshatidylinocitol 3-kinase pathway. Circ. Res. 2004; 95:230-232

132. Zhao ZQ, Vinten-Johansen J. Postconditioning: reduction of reperfusion-induced injury. Cardiovasc Res. 2006; 70(2):200-11

133. Kin H, Zatta AJ, Lofye MT. Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. Cardiovasc Res. 2005; 67(1):124-33

134. Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol. 2005; 100(1):57-63

135. Philipp S, Yang XM, Cui L, Davis AM, Downey JM, Cohen MV. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2b receptor cascade. Cardiovasc Res. 2006; 70(2):308-14

136. Penna C, Mancardi D, Rastaldo R, Losano G, Pagliaro P. Intermittent activation of bradykinin B2 receptors and mitochondrial KATP channels trigger cardiac postconditioning through redox signaling. Cardiovascular Res. 2007; 75(1):168-77

137. Jang Y, Xi J, Wang H, Mueller RA, Norfleet EA, Xu Z. Postconditioning prevents reperfusion injury by activating delta-opioid receptors. Anesthesiology. 2008; 108(2):243-50

138. Zatta AJ, Kin H, Yoshishiqe D, Jiang R, Wang N, Reeves JG, Mykytenko J, Guyton RA, Zhao ZQ, Caffrey JL, Vinten-Johansen J. Evidence that cardioprotection by postconditioning involves preservation of myocardial opioid content and selective opioid receptor activation. Am J Physiol Heart Circ Physiol 2008; 294 (3): H144-51

139. Namiuchi S, Kagaya Y, Ohta J, Shiba N, Sugi M, Oikawa M, Kunii H, Yamao H, Komatsu N, Yui M, Tada H, Sakuma M, Watanabe J, Ichihara T, Shirato K. High serum erythropoietin level is associated with smaller infarct size in patients with acute myocardial infarction who undergo successful primary percutaneous coronary intervention. J Am Coll Cardiol. 2005; 45:1406-12

140. Bullard AJ, Govewalla P, Yellon DM. Erythropoietin protects the myocardium against reperfusion injury *in vitro* and *in vivo*. Basic Res Cardiol. 2005; 100:397-403

141. Parsa CJ, Kim J, Riel RU, Pascal LS, Thompson RB, Petrofski JA, Matsumoto A, Stamler JS, Koch WJ. Cardioprotective effects of erythropoietin in the reperfused ischaemic heart: a potential role for cardiac fibroblasts. J Biol Chem. 2004; 279:20655-20662

142. Suh JW, Chung WY, Kim YS, Kim KI, Jeon EJ, Cho YS, Youn TJ, Chae IH, Kim CH, Choi DJ. The effect of intravenous administration of erythropoietin on the infarct size in primary percutaneous coronary intervention. Int J Cardiol. 149:216-20

143. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res. 2004; 61(3):448-60

144. Hausenloy DJ, Yellon DM. Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. Heart Fail Rev. 2007; 12(3-4):217-34

145. Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signalling pathways. J Am Coll Cardiol. 2004; 44(5): 1103-10

146. Zatta AJ, Kin H, Lee G, Wang N, Jiang R, Lust R, Reeves JG, Mykytenko J, Guyton RA, Zhao ZQ, Vinten-Johansen. Infarct-sparing effect of myocardial postconditioning is dependent on protein kinase C signalling. Cardiovasc Res. 2006; 70(2):315-24

147. Fujita M, Asanuma H, Hirata A, Wakeno M, Takahama H, Sasaki H, Kim J, Takashima S, Tsukamoto O, Minamino T, Shinozaki Y, Tomoike H, Hori M, Kitakaze M. Prolonged transient acidosis during early reperfusion contributes to the cardioprotective effects of postconditioning. Am J Physiol Heart Circ Physiol.2007; 292:H2004-8

148. Wang JK, Yu LN, Zhang FJ, Yu J, Yan M, Chen G. Postconditioning with sevoflurane protects against focal cerebral ischemia and reperfusion injury via PI3K/Akt pathway. Brain Res. 2010; 1357:142-51

149. Yu LN, Yu J, Zhang FJ, Yang MJ, Ding TT, Wang JK, He W, Fang T, Chen G, Yan M. Sevoflurane postconditioning reduces myocardial reperfusion injury in rat isolated hearts via activation of PI3K/Akt signaling and modulation of Bcl-2 family proteins. J Zhejiang Univ Sci B. 2010; 11:661-72

150. Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gatullo D, Losano G, Pagliaro P. Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Res Cardiol 2006; 101(2):168-79

151. Abdallah Y, Gkatzoflia H, Pieper E, Zoga E, Walther S, Kasseckert S, Schäfer M, Schlüter KD, Piper HM, Schäfer C. Mechanism of cGMP-mediated protection in a cellular model of myocardial reperfusion injury. Cardiovasc Res. 2005; 66(1):123-31

152. Costa AD, Garlid KD, West IC et al. Protein kinase G transmits the cardioprotective signal from cytosol to mitochondria. Circ Res 2005; 97(4):329-36

153. Bolli R. Oxygen-derived free radicals and myocardial reperfusion injury: an overview. Cardiovasc Drugs Ther. 1991; 5 Suppl 2:249-68

154. Penna C, Rastaldo R, Mancardi D, Raimondo S, Capello S, Gatullo D, Losano G, Pagliaro P. Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K+ channel and protein kinase C activation. Basic Res Cardiol. 2006 101(2):180-9

155. Mykytenko J, Reeves JG, Kin H, Wang NP, Zatta AJ, Jiang R, Guyton RA, . Vinten-Johansen J, Zhao ZQ. Persistent beneficial effect of postconditioning against infarct size: role of mitochondrial K (ATP) channels during reperfusion. Basic Res Cardiol. 2008; 103(5):472-84

156. Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M. Postconditioning inhibits mitochondrial permeability transition. Circulation 2005; 111(2):194-7

157. Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti L, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet

G, Andre-Fouet X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, Ovize M. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med. 2008; 359(5):473-81

158. Cohen MV, Yang XM, Downey JM. Acidosis, oxygen, and interference with mitochondrial permeability transition pore formation in the early minutes of reperfusion are critical to postconditioning's success. Basic Res Cardiol. 2008; 103(5):464-71
159. Hausenloy DJ, Ong SB, Yellon DM. The mitochondrial permeability transition pore as a target for preconditioning and postconditioning. Basic Res Cardiol. 2009; 104(2) 189-202
160. Staat P, Rioufol G, Piot C, et al. Postconditioning the human heart. Circulation 2005; 112:2143–2148
161. Ma X, Zhang X, Li C, Luo M. Effect of postconditioning on coronary blood flow velocity and endothelial function and LV recovery after myocardial infarction. J Interv Cardiol. 2006; 19:367–375
162. Laskey WK, Yoon S, Calzada N, Ricciardi MJ. Concordant improvements in coronary flow reserve and ST-segment resolution during percutaneous coronary intervention for acute myocardial infarction: a benefit of Postconditioning. Catheter Cardiovasc Interv. 2008; 72(2):212–20
163. Luo W, Li B, Chen R, Huang R, Lin G. Effect of ischemic postconditioning in adult valve replacement. Eur J Cardiothoracic Surg. 2008; 33(2):203-8
164. Luo W, Li B, Lin G, Chen R, Huang R. Does cardioplegia leave room for postconditioning in paediatric cardiac surgery? Cardiol Young. 2008; 18(3):282-7
165. Thibault H, Piot C, Staat P, Bontemps L, Sportouch C, Rioufol G, Cung TT, Bonnefoy E, , Angoulvant D, Aupetit JF, Finet G, Andre-Fouet X, Macia JC,

Raczka F, Rossi R, Itti R, Kirkorian G, Derumeaux G, Ovize M. et al. Long-term benefit of postconditioning. Circulation 2008; 117(8):1037-44

166. Przyklenk K, Bauer B, Ovize M et al. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. Circulation 1993; 87(3):893-9
167. Takaoka A, Nakae I, Mitsunami K, Yabe T, Morikawa S, Inubushi T, Kinoshita M. Renal ischemia/reperfusion remotely improves myocardial energy metabolism during myocardial ischemia via adenosine receptors in rabbits: effects of “remote preconditioning”. J Am Coll Cardiol. 1999; 33:556-64
168. Patel HH, Moore J, Hsu AK, Gross GJ. Cardioprotection at a distance: mesenteric artery occlusion protects the myocardium via an opioid sensitive mechanism. J Mol Cell Cardiol. 2002; 34: 1317-23
169. Kharbanda RK, Mortensen UM, White PA, Kristiansen PA, Schmidt MR, Hoschtitzky JA, Vogel M, Sorensen K , Redington AN, MacAllister R. Transient limb ischemia induces remote ischemic preconditioning in vivo. Circulation. 2002; 106:2881-3
170. Kerendi F, Kin H, Halkos ME, Jiang R, Zatta AJ, Zhao ZQ, Guyton RA, Vinten-Johansen J. Remote postconditioning. Brief renal ischemia and reperfusion applied before coronary artery reperfusion reduces myocardial infarct size via endogenous activation of adenosine receptors. Basic Res Cardiol. 2005; 100(5):404-12
171. Loukogeorgakis SP, Williams R, Panagiotidou AT, Kolvekar SK, Donald A, Cole TJ, Yellon DM, Deanfield JE, MacAllister RJ. Transient limb ischemia induces remote preconditioning and remote postconditioning in humans by a K (ATP)-channel dependent mechanism. Circulation 2007; 116(12):1386-95

172. Wolfrum S, Schneider K, Heidbreder M, Nienstedt J, Dominiak P, Dendorfer A. Remote preconditioning protects the heart by activating PKCepsilon-isoform. Cardivasc Res. 2002; 55(3):583-9

173. Moses MA, Addison PD, Neligan PC, Ashrafpour H, Huang N, Zair M, Rassuli A, Forrest CR, Grover GJ, Pang CY. Mitochondrial KATP channels in hindlimb remote ischemic preconditioning of skeletal muscle against infarction. Am J Physiol Heart Circ Physiol. 2005; 288:H559-67

174. Shimizu M, Tropak M, Diaz RJ, Suto F, Surendra H, Kuzmin E, Li J, Gross G, Wilson GJ, Callahn J, Redington AN. Transient limb ischemia remotely preconditions through a humoral mechanism acting directly on the myocardium: evidence suggesting cross-species protection. Clin Sci (Lond). 2009; 117:191-200

175. Steensrud T, Li J, Dai X, Manlhiot C, Kharbanda RK, Tropak M, Redington A. Pretreatment with the nitric oxide donor SNAP or nerve transection blocks humoral preconditioning by remote limb. Am J Heart Circ Physiol.2010; 299:H1598-603

176. Brzozowski T, Kontureck PC, Kontureck SJ, Padjo R, Kwiecien S, Pawlik M, Drozdowicz D, Sliwoski Z, Pawlik WW. Ischemic preconditioning of remote organs attenuates gastric ischemia-reperfusion injury through involvement of prostaglandins and sensory nerves. Eur J Pharmacol. 2004; 499:201-13

177. Dickson EW, Lorbar M, Porcaro WA, Fenton RA, Reinhardt CP, Gysembergh A, Przyklenk K. Rabbit heart can be "preconditioned" via transfer of coronary effluent. Am J Physiol. 1999; 277:H2451-7

178. Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. Circulation. 1996; 94:2193-200

179. Schoemaker RG, Van Heijningen CL. Bradykinin mediates cardiac preconditioning at a distance. Am J Physiol Heart Circ Physiol. 2000; 278:H1571-H1576

180. Goto M, Liu Y, Yang XM, Ardell JL, Cohen MW, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. Circ Res. 1995; 77:611-21

181. Segond Von Banchet G, Petersen M, Heppelmann B. Bradykinin receptors in cultured rat dorsal root ganglion cells: influence of length of time in culture. Neuroscience. 1996; 75:1211-1218

182. Rydh-Rinder M, Kerekes N, Svensson M, Hökfelt T. Glutamate release from adult primary sensory neurons in culture is modulated by growth factors. Regul Pept. 2001; 102:69-79

183. Pell TJ, Baxter GF, Yellon DM, Drew GM. Renal ischemia preconditions myocardium: role of adenosine receptors and ATP-sensitive potassium channels. Am J Physiol. 1998; 275:H1542-7

184. Tang ZL, Dai W, Li YJ, Deng HW. Involvement of capsaicin-sensitive sensory nerves in early and delayed cardioprotection induced by a brief ischaemia of the small intestine. Naunyn Schmiedebergs Arch Pharmacol. 1999; 359:243-7

185. Kallner G, Gonon A, Franco-Cereceda A. Calcitonin gene-related peptide in myocardial ischaemia and reperfusion in the pig. Cardiovasc Res. 1998; 38:493-499

186. Li YJ, Peng J. The cardioprotection of calcitonin gene-related peptide-mediated preconditioning. Eur J Pharmacol. 2002; 442:173-7

187. Loukogeorgakis SP, Panagiotidou AT, Broadhead MW, Donald A, Deanfield JE, MacAllister RJ. Remote ischemic preconditioning provides early and late protection against endothelial ischemia-reperfusion injury in humans: role of the autonomic nervous system. J Am Coll Cardiol. 2005; 46:450-6

188. Birnbaum Y, Hale SL, Kloner RA. Reduction of Myocardial Infarct Size by Partial Reduction of Blood Supply Combined With Rapid Stimulation of the Gastrocnemius Muscle in the Rabbit. Circulation. 1997; 96:1641-1646

189. Candilio L, Hausenloy DJ, Yellon DM. Remote ischemic conditioning: A clinical trials update. J Cardiovasc Pharmacol Ther. 2011; 16:304-12

190. Botker HE, Kharbanda R, Schmidt MR, Bottcher M, Kaltoft AK, Terkelsen CJ, Munk K, Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH, Krusell LR, Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sorensen HT, Redington AN, Nielsen TT. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. Lancet. 2010; 375:727-34

191. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994; 298:249-58

192. Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Gödecke A, Schrader J, Schulz R, Heusch G, Schaub GA. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic Biol. Med 2003; 35(7): 790-6

193. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 1988; 333(6174):664-6

194. Patel RP, Hogg N, Kim-Shapiro DB. The potential role of the red blood cell in nitrite-dependent regulation of blood flow. Cardiovasc Res. 2011; 89:507-515

195. Kim-Shapiro DB, Schechter AN, Gladwin MT. Unraveling the Reactions of Nitric Oxide, Nitrite, and Hemoglobin in Physiology and Therapeutics. Arterioscler Thromb Vasc Biol. 2006; 26:697-705

196. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-ntric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov 2008; 7(2):156-67

197. Spiegelhalder B, Eisenbrand G, Preussman R. Influence of dietary nitrate on nitrite content of human saliva: Possible relevance to in vivo formation of N-nitroso compounds. Food Cosmet. Toxicol.1976; 14:549-552

198. Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, Smith L, Golden M, Benjamin N. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nat Med. 1995; 1(6):546-551

199. Lunberg JO, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide in humans: measurements in expelled air. Gut 1994; 35(11):1543-6

200. Ernst E. van Faassen, Soheyl Bahrami, Martin Feelisch, Neil Hogg et al. Nitrite as regulator of hypoxic signaling in mammalian physiology. Med Res Rev. 2009; 29(5): 683-741

201. Zweier JL, Li H, Samouilov A, Liu X. Mechanisms of nitrite reduction to nitric oxide in the heart and vessel wall. Nitric Oxide 2010; 22(2):83-90

202. Lundberg JO, Govoni M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. Free Radic Biol Med. 2004; 37(3): 395-400

203. Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. Nitric Oxide 2008; 19(4): 333-7

204. Rhodes P, Leone AM, Francis PL Struthers AD, Moncada S, Rhodes PM [corrected to Rhodes P. The L-arginine: nitric oxide pathway is the major source of plasma nitrite in fasted humans. Biochem Biophys Res Commun. 1995; 209(2):590-6

205. Zweier JL, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. Nat Med. 1995; 1(8):804-9

206. Mikula I, Durocher S, Martasek P, Mutus B, Slama-Schwok A. Isoform-specific differences in nitrite reductase activity of nitric oxide synthases under hypoxia. Biochem J 2009; 418(3):673-82

207. Calvert JW, Lefer DJ. Myocardial protection by nitrite. Cardiovasc Res. 2009; 83:195-203

208. Johnson G 3rd, Tsao PS, Mulloy D, Lefer AM. Cardioprotective effects of acidified sodium nitrite in myocardial ischemia with reperfusion. J Pharmacol Exp Ther. 1990; 252(1):35-41

209. Webb AJ, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. Proc Natl Acad Sci USA 2004; 101(37):13683-8

210. Jung KH, Chu K, Ko SY, Lee ST, Sinn DI, Park DK, Kim JM, Song EC, Kim M, Roh JK.. Early intravenous infusion of sodium nitrite protects brain against in vivo ischemia-reperfusion injury. Stroke 2006; 37(11): 2744-50

211. Lu P, Liu F, Yao Z et al. Nitrite-derived nitric oxide by xanthine oxidoreductase protects the liver against ischemia-reperfusion injury. Hepatobiliary Pancreat Dis Int. 2005; 4(3): 350-55

212. Tripatara P, Patel NSA, Webb A, Rathod K, Lecomte FM, Mazzon E, Cuzzocrea S, Yaqoob MM, Ahluwalia A, Thiemermann C. Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: role for xanthine oxidoreductase. J Am Soc Nephrol. 2007; 18(2);570-80

213. Shiva S, Sack MN, Greer JJ Duranski M, Ringwood LA, Burwell L, Wang X, MacArthur PH, Shoja A, Raghavachari N, Calvert JW, Brookes PS, Lefer DJ,

Gladwin MT. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. J Exp Med. 2007; 204(9):2089-2102

214. Bryan NS, Calvert JW, Gundewar S, Lefer DJ. Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice. Free Rad Biol Med. 2008; 45(4): 468-74

215. Duranski MR, Greer JJ, Dejam A Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. J Clin Invest. 2005; 115(5):1232-40

216. Baker JE, Su J, Fu X Hsu A, Gross GJ, Tweddell JS, Hogg N. Nitrite confers protection against myocardial infarction: role of xanthine oxidoreductase, NADPH oxidase and K (ATP) channels. J Mol Cell Cardiol. 2007; 43(4):437-44

217. Bryan NS, Calvert JW, Elrod JW, Gundewar S, Ji SY, Lefer DJ. Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. Proc Natl Acad Sci USA. 2007; 104(48): 19144-9

218. Jung KH, Chu K, Lee ST, Park HK, Kim JH, Song EC, Kang KM, Kim M, Lee SK, Roh JK. Augmentation of nitrite therapy in cerebral ischaemia by NMDA receptor inhibition. Biochem Biophys Res Commun. 2009; 378(11): 507-12

219. Jung KH, Chu K, Lee ST, Sun-Woo JS, Park DK, Kim JH, Kim S, Lee SK, Kim M, Roh JK. Effects on long term nitrite therapy on functional recovery in experimental ischemia model. Biochem Biophys Res Commun. 2010; 403:66-72

220. Shiva S, Gladwin MT. Nitrite mediates cytoprotection after ischemia/reperfusion by modulating mitochondrial function. Basic Res Cardiol 2009; 104(2):113-9

221. Munzel T, Daiber A, Mulsch A. Explaining the Phenomenon of Nitrate Tolerance. Circ Res. 2005; 95:618-628

222. ISIS-4: a randomised factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulphate in 58,050 patients with suspected acute myocardial infarction. ISIS-4 (Fourth International Study of Infarct Survival) Collaborative Group. Lancet. 1995; 345:669-85

223. Fox-Robichaud A, Payne D, Hasan Su, Otrovsky L, Fairhead T, Reinhardt T, Kubes P. Inhaled NO as a Viable Antiadhesive Therapy for Ischemia/Reperfusion Injury of Distal Microvascular Beds. J Clin Invest. 1998; 101:2497-2505

224. Lange JD, Teng X, Chumley P, Crawford JH, Isbell TS, Chacko BK, Liu Y, Jhala N, Crowe DR, Smith AB, Cross RC, Frenette L, Kelley EE, Wilhite DW, Hall CR, Page GP, Fallon MB, Bynon JS, Eckhoff DE, Patel RP. Inhaled NO accelerates restoration of liver function in adults following orthoptic liver transplantation. J Clin Invest. 2007; 117: 2583-2591

225. Liu X, Huang Y, Pokreisz P, Vermeersch P, Marsboom G, Swinnen M, Verbeken E, Santos J, Pellens M, Gillijns H, Van de Werf F, Bloch KD, Janssens S. Nitric Oxide Inhalation Improves Microvascular Flow and Decrease infarction Size after Myocardial Ischemia and Reperfusion. J Am Coll Cardiol 2007; 50:808-817

226. Nagasaka Y, Fernandez BO, Garcia-Saura MF, Petersen B, Ichinose F, Bloch KD, Feelisch M, Zapol WM. Brief periods of nitric oxide inhalation protect against myocardial ischemia-reperfusion injury. Anesthesiology 2008; 109(4); 675-682

227. Thabut G, Brugiere O, Leseche G, Stern JB, Fradj K, Herve P, Jebrak G, Marty J, Fournier M, Mal H. Preventive effect of inhaled nitric oxide and pentoxifylline on ischemia/reperfusion injury after lung transplantation. Transplantation 2001; 71(9):1295-300

228. Mathru M, Huda R, Solanki DR, Hays S, Lang JD. Inhaled Nitric Oxide Attenuates Reperfusion Inflammatory Responses in Humans. Anesthesiology 2007; 106:275–82

229. Griffiths MJD, Evans TW. Inhaled Nitric Oxide Therapy in Adults. N Engl J Med 2005; 353:2683-95

230. Germann P, Braschi A, Della Rocca G, Dinh-Xuan AT, Falke K, Frostell C, Gustafsson LE, Herve P, Jolliet P, Kaisers U, Litvan H, Mcrae DJ, Maggiorini M, Marczin M, Mueller B, Payen D, Ranucci M, Schranz D, Zimmermann R, Ullrich R. Inhaled nitric oxide therapy in adults: European expert recommendations. Intensive Care Med 2005; 31:1029–1041

231. Wang T, El Kebir D, Blaise G. Inhaled nitric oxide in 2003: a review of its mechanism of action. Can J Anaesth 2003; 50(8):839–846

232. Gonzalez FM, Siva S, Vincent PS, Ringwood LA, Hsu LY, Hon YY, Aletras AH, Cannon RO 3rd, Gladwin MT, Arai AE. Nitrite Anion Provides Potent Nitrite Anion Provides Potent Cytoprotective and Antiapoptotic Effects as Adjunctive Therapy to Reperfusion for Acute Myocardial Infarction. Circulation 2008;117:2986-2994

233. Broadhead MW, Kharbanda RK, Peters MJ, MacAllister RJ. KATP channel activation induces ischemic preconditioning of the endothelium in humans in vivo. Circulation. 2004; 110:2077-82

234. Nerem RM, Alexander RW, Chappell DC, Medford RM, Varner SE, Taylor WR. The study of the influence of flow on vascular endothelial biology. Am J Med Sci. 1998; 316:169-75

235. Vallance P, Calver A, Collier J. The vascular endothelium in diabetes and hypertension. Hypertens Suppl. 1992; 10:S25-9

236. Corretti MC, Anderson TJ, Benjamin EJ, Celemajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R, International Brachial Artery Reactivity Task Force. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. J Am Coll Cardiol. 2002; 39(2):257-65

237. Benjamin N, Calver A, Collier J, Robinson B, Vallance P, Webb D. Measuring forearm blood flow and interpreting the responses to drugs and mediators. Hypertension. 1995; 25:918–923

238. Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, Lüscher TF. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation. 1995; 91(5):1314-9

239. Mullen MJ, Kharbanda RK, Cross J, Donald AE, Taylor M, Vallance P, Deanfield JE, MacAllister RJ. Heterogenous nature of flow-mediated dilatation in human conduit arteries in vivo: relevance to endothelial dysfunction in hypercholesterolemia. Circ Res. 2001; 88:145-51

240. Sorensen KE, Celermajer DS, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Thomas O, Deanfield JE. Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility. Br Heart J. 1995; 74:247–253

241. Donald AE, Charakida M, Cole TJ, Friberg P, Chowienczyk PJ, Millasseau SC, Deanfield JE, Halcox JP. Non-invasive assessment of endothelial function: which technique? J Am Coll Cardiol. 2006; 48:1846-50

242. Donald AE, Halcox JP, Charakida M, Storry C, Wallace SM, Cole TJ, Friberg P, Deanfield JE. Methodological approaches to optimize reproducibility and power in clinical studies of flow-mediated dilation. J Am Coll Cardiol. 2008; 51:1959-64

243. Wilkinson IB, Webb DJ. Venous occlusion plethysmography in cardiovascular research: methodology and clinical applications. Br J Clin Pharmacol. 2001; 52:631-46

244. Pallares, L.C., C.R. Deane, S.V. Baudouin, and T.W. Evans. Strain guage plethysmography and Doppler ultrasound in the measurement of limb blood flow. Eur J Clin Invest. 1994; 24:279-86

245. Petrie JR, Ueda S, Morris AD, Murray LS, Elliot HL, Connell JM. How reproducible is bilateral forearm plethysmography? Br J Clin Pharmacol. 1998; 45:131-9

246. Walker HA, Jackson G, Ritter JM, Chowienczyk PJ. Assessment of forearm vasodilator responses to acetylcholine and albuterol by strain gauge plethysmography reproducibility and influence of strain gauge placement. Br J Clin Pharmacol. 2001; 51:225-9

247. Lind L, Sarabi M, Millgard J. Methodological aspects of the evaluation of endothelium-dependent vasodilatation in the human forearm. Clin Physiol. 1998; 18:81-7

248. Oldroyd KG, Harvey K, Gray CE, Beastall GH, Cobbe SM. Beta endorphin release in patients after spontaneous and provoked acute myocardial ischaemia. Br Heart J. 1992; 67:230-5

249. Wang Y, Van Bockstaele EJ, Liu-Chen L. In vivo trafficking of endogenous opioid receptors. Life Sci.2008; 83:693-699

250. Fredricksen RC, Geary LE. Endogenous opioid peptides: review of physiological, pharmacological and clinical aspects. Prog. Neurobiol. 1982; 19:19-69

251. Pugsley MK. The diverse molecular mechanisms responsible for the actions of opioids on the cardiovascular system. Pharmacol Ther. 2002; 93:51-75
252. Li CH, Chung D. Isolation and structure of an untriakontapeptide with opiate activity from camel pituitary glands. Proct Natl Acad Sci U.S.A. 1976; 73:1145-8
253. Dalayeun JF, Nores JM, Bergal S. Physiology of beta-endorphins. A close-up view and a review of the literature. Biomed Pharmacother. 1993; 47:311-20
254. Yasunaga F, Morikawa N, Takeyama M. Enzyme linked immunosorbent assay for β-endorphin in human plasma. Chem Pharm Bull (Tokyo). 1992; 40:1951-1953
255. Sarma JK, Hoffmann SR, Houghten RA. Enzyme linked immunosorbent assay (ELISA) for beta-endorphin and its antibodies. Life Sci. 1986; 38:1723-32
256. Hochhaus G, Sadee W. A biotin-avidin-based enzyme immunoassay for beta h-endorphin. Pharm Res. 1988; 5:232-5
257. Braman RS, Hendrix SA. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium (III) reduction with chemilumenescence detection. Anal Chem. 1989; 61:2715-2718
258. Nagababu E, Rifkind JM. Measurement of Plasma Nitrite by Chemiluminescence without Interference of S-, *N*-nitroso and Nitrated Species. Free Radic Biol Med. 2007; 42:1146-1154
259. Pelletier MM, Kleinbongard P, Ringwood L, Hito R, Hunter CJ, Schechter AN, Gladwin MT, Dejam A. The measurement of blood and plasma nitrite by chemiluminescence: Pitfalls and solutions. Free Radic Biol Med. 2006; 41:541-548
260. Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. Proct Natl Acad Sci U.S.A. 1993; 90:8103-8107

261. Kharbanda RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, Vallance P, Deanfield J, MacAllister R. Ischaemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischaemia-reperfusion in humans in vivo. Circulation 2001; 103:1624–1630

262. Lee TM, Chou TF. Impairment of myocardial protection in type 2 diabetic patients. J Clin Endocrinol Metab 2003;88:531–7

263. Klepzig H, Kober G, Matter C, Luus H, Schneider H, Boedeker KH, Kiwoski W, Amann FW, Gruber D, Harris S, Burger W. Sulfonylureas and ischemic preconditioning. A double-blind, placebo-controlled evaluation of glimepiride and glibenclamide. Eur Heart J 1999;20:439–446

264. Argaud L, Garrier O, Loufouat J, Gomez L, Couture-Lepetit E, Gateau-Roesch O, Robert D, Ovize M. Second-generation sulfonylureas preserve inhibition of mitochondrial permeability transition by the mitochondrial K+(ATP) opener nicorandil in experimental myocardial infarction. Shock 2009;32:247–252

265. Gateau-Roesch O, Argaud L, Ovize M. Mitochondrial permeability transition pore and postconditioning. Cardiovasc Res 2006; 70:264–273

266. Ji Y, Pang QF, Xu G, Wang L, Wang JK, Zeng YM. Exogenous hydrogen sulfide postconditioning protects isolated rat hearts against ischaemia-reperfusion injury. Eur J Pharmacol 2008; 587:1–7

267. Donato M, D'Annunzio V, Berg G, Gonzalez G, Schreier L, Morales C, Wikinski RL, Gelpi RJ. Ischaemic postconditioning reduces infarct size by activation of A1 receptors and K^+ (ATP) channels in both normal and hypercholesterolemic rabbits. J Cardiovasc Pharmacol 2007; 49:287–292

268. Mocanu MM, Maddock HL, Baxter GF, Lawrence CL, Standen NB, Yellon DM. Glimepiride, a novel Sulfonylurea, Does not abolish Myocardial Protection

Afforded by Either Ischaemic Preconditioning or Diazoxide. Circulation 2001; 103:3111–3116

269. Geisen K, Vegh A, Krause E, Papp JG. Cardiovascular effects of conventional sulfonylureas and glimepiride. Horm Metab Res 1996; 28:496–507
270. Ueba H, Kuroki M, Hashimoto S, Umemoto T, Yasu T, Ishikawa SE, Saito M, Kawakami M. Glimepiride induces nitric oxide production in human coronary artery endothelial cells via a PI3- kinase dependent pathway. Atherosclerosis 2005; 183:35–39
271. Nishida H, Sato T, Nomura M, Miyazaki M, Nakaya H. Glimepiride Treatment upon Reperfusion Limits Infarct size via the Phophatidylinositol 3-Kinase/Akt Pathway in Rabbit Hearts. J Pharmacol Sci 2009; 109:251–256.
272. Halestrap A.P. What is the mitochondrial permeability transition pore? J Mol Cell Cardiol 2009;46:821–31
273. Griffiths EJ, Halestrap AP. Protetection by cyclosporine A of ischaemia reperfusion induced damage in isolated rat hearts. J Mol Cell Cardiol 1993; 25:1461–9
274. Shanmuganathan S, Hausenloy DJ, Duchen MR, Yellon DM. Mitochondrial permeability transition pore as a target for cardioprotection in the human heart. AmJ Physiol Heart Circ Physiol 2005;289:H237–42
275. Jacobson J, Duchen M. Mitochondrial oxidative stress and cell death in astrocytes – requirement for the stored Ca2+ and sustained opening of the permeability transition pore. J Cell Sci 2001;115:1175–118
276. Saotome M, Katoh H, Yaguchi Y, Tanaka T, Urushida T, Satoh H, Hayashi H. Transient opening of the mitochondrial permeability transition pore by reactive

oxygen species protects myocardium form ischaemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2009;296:H1125–H1132

277. Lim YS, Davidson SM, Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: The essential role of the mitochondrial transition pore. Cardiovasc Res 2007; 75:530–5

278. Gomez L, Thibault H, Gharib A, Dumont JM, Vuagniaux G, Scalfaro P, Derumeaux G, Ovize M. Inhibition of mitochondrial permeability transition improves functional recovery and reduces mortality following acute myocardial infarction in mice. Am J Physiol Heart Circ Physiol 2007; 293:H1654–61

279. Dickson EW, Reinhardt CP, Renzi FP, Becker RC, Porcaro WA, Heard SO. Ischemic preconditioning may be transferable via whole blood transfusion: preliminary evidence. J Thromb Thrombolysis. 1999; 8:123-9

280. Dickson EW, Blehar DJ, Carraway RE, Heard SO, Steinberg G, Przyklenk K. Naloxone blocks transferred preconditioning in isolated rabbit hearts. J Mol Cell Cardiol. 2001; 33: 1751–1756

281. Tomai F, Crea F, Gaspardone A, Versaci F, Ghini AS, Ferri C, Desideri G, Chiariello L, Gioffre PA. Effects of naloxone on myocardial ischemic preconditioning in humans. J Am Coll Cardiol. 1999; 33(7): 1863-9

282. Mayahi L, Heales S, Owen D, Casas JP, Harris J, MacAllister RJ, Hingorani AD. (6R)-5,6,7,8-tetrahydro-L-biopterin and its stereoisomer prevent ischemia reperfusion injury in human forearm. Aterioscler Thromb Vasc Biol. 2007; 27(6):1334-9

283. Pernow J, Bohm F, Beltran E, Gonon A. L-arginine protects from ischaemia reperfusion induced endothelial dysfunction in humans in vivo. *J Appl Physiol* 2003; 95:2218–2222

284.Lim YS, Yellon DM, Hausenloy DJ. The neural and humoral pathways in remote limb ischemic preconditioning. Basic Res Cardiol. 2010; 105:651-655

285.Konstantinov IE, Li J, Cheung MM, Shimizu M, Stokoe J, Kharbanda RK, Redington AN. Remote ischemic preconditioning of the recipient reduces myocardial ischemia-reperfusion injury of the denervated donor heart via a KATP channel-dependent mechanism. Transplantation. 2005; 79:1691-5

286.Hausenloy DJ, Yellon DM. Remote ischaemic preconditioning: underlying mechanisms and clinical application. Cardiovasc Res. 2008; 79: 377-86

287.Tomai F, Crea F, Gaspardone A, Versaci F, Ghini AS, De Paulis R, Chiariello L, Gioffrè PA. Phentolamine prevents adaptation to ischemia during coronary angioplasty: role of alpha adrenergic receptors in ischemic preconditioning. Circulation 1997; 96:2171-2177

288.Scheinin H, Helminen A, Huhtala S, Grönroos P, Bosch JA, Kuusela T, Kanto J, Kaila T.Spectral analysis of heart rate variability as a quantitative measure of parasympatholytic effect--integrated pharmacokinetics and pharmacodynamics of three anticholinergic drugs. Ther. Drug Monit. 1999; 21(2):141-51

289.Penttila J, Vesalainen R, Helminen A, Kuusela T, Hinkka S, Rahi K, Kaila T, Scheinin H. Spontaneous baroreflex sensitivity as a dynamic measure of cardiac anticholinergic drug effect. J. Auton. Pharmacol. 2001; 21(2):71-8

290.Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol 2006; 40(1):16-23

291. Joshipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Hennekens CH, Spiegelman D, Willett WC. Fruit and vegetable intake in relation to risk of ischemic stroke. JAMA 1999; 282(13):1233-9

292. Joshipura KJ, Hu FB, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Colditz G, Ascherio A, Rosner B, Spiegelman D, Willett WC. The effect of fruit and vegetable intake on risk for coronary heart disease. Ann Intern Med. 2001; 134(12):1106-14

293. Hord NG, Tang Y, Bryan NS. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. Am J Clin Nutr. 2009; 90(1):1-10

294. Lundberg JO, Weitzburg E. NO-synthase independent NO generation in mammals. Biochem Biophys Res Commun. 2010; 396:39-45

295. Gori T, Dragoni S, Di Stolfo G, Sicuro S, Liuni A, Luca MC, Thomas G, Oelze M, Daiber A, Parker JD. Tolerance to nitroglycerin-induced preconditioning of the endothelium: a human in vivo study. Am J Physiol Heart Circ Physiol. 2010; 298:H340-5