The Role of Adenosine in Remote Ischaemic Conditioning

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A thesis submitted for the degree of Doctor of Philosophy

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Abstract

Strategies to reduce infarct size in ischaemia-reperfusion (IR) syndromes such as acute myocardial infarction are of high clinical and scientific interest. Remote ischaemic preconditioning (rIPC) is one such strategy but its mechanisms remain incompletely understood. Multiple lines of evidence from animal studies suggest that the endogenous purine nucleoside adenosine is a key mediator of preconditioning pathways but no evidence exists as to adenosine's role in the more complex physiology of humans. The work in this thesis aims to elucidate the role of endogenous adenosine in the physiological phenomenon of rIPC and to examine the role of exogenous adenosine in triggering preconditioning-like states.

In a randomised, placebo controlled study using healthy volunteers and the human forearm model of ischaemia-reperfusion injury, I demonstrate that delivery of the adenosine receptor antagonist caffeine, prior to the initiation of a rIPC stimulus abrogates the protective effect of rIPC on IR. By then selectively infusing caffeine to achieve high local but low systemic concentrations, I also demonstrate that adenosine receptor activation is important in the 'trigger' phase of rIPC rather than in the 'effector' phase and that blockade of the trigger phase effectively inhibits the release of a circulating humoral protective factor. These studies provide evidence of the crucial role of adenosine receptor activation in human rIPC, demonstrating their sites of action and illuminating their potential mechanism of action.

To study whether exogenously delivered adenosine can recapitulate preconditioning-like states, in initial studies in a large mammal model of acute myocardial infarction, I demonstrate that adenosine, given after the onset of ischaemia, but prior to reperfusion, significantly reduces myocardial infarct size. In a subsequent study, translating these findings to humans with coronary disease, I demonstrate that the delivery of adenosine in a range of concentrations is able to illicit the release of a circulating preconditioning factor which is transferrable across species and can reduce infarct size in a murine model of myocardial IR.

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List of Abbreviations

8-SPT	8-(p-Sulfophenyl)theophylline
AAA	Aortic abdominal aneurysm
Ach	Acetylcholine
Ado	Adenosine
AdoHcy	Adenosylhomocysteine
ADP	Adenosine diphosphate
AK	Adenosine kinase
Akt	Protein Kinase B
alb	Albumin
AMP	Adenosine monophosphate
ATP	Adenosine Tri-Phosphate
AUC	Area under curve
AV	Atrio-ventricular
BMI	Body mass index
CABG	Coronary artery bypass grafting
cAMP	cyclic AMP
CGRP	Calcitonin gene-related peptide
CKMB	Creatine kinase (muscle-brain isotype)
Cr	Creatinine
ECG	Electrocardiogram
ENT	Equilibrative nucleoside transporters
ENTPD	Ectonucleotidase triphosphate diphosphohydrolase
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
FMD	Flow-mediated dilation
Glc	Glucose
GPCR	G protein-coupled receptor
GSK3β	Glycogen synthase kinase 3 beta
GTN	Glyceryl trinitrate
Hcy	Homocysteine
HR	Heart rate
Ino	Inosine
IPC	Ischaemic preconditioning
IR	Ischaemia-reperfusion
IS	Infarct size
ITU	Intensive therapy unit
JAK	Janus kinase
kDa	kilo-Dalton
KH	Krebs-Henseleit
KO	Knockout
LAD	Left anterior descending artery
LC-MS/MS	Liquid chromatography–mass spectrometry
LCx	Left circumflex artery
LDH	Lactate dehydrogenase
L-NMMA	Asymmetric dimethylarginine

LV	Left ventricle
LVDeP	Left ventricular developed pressure
LVEF	Left ventricular ejection fraction
MAP	Mean arterial pressure
MI	Myocardial Infarction
mPTP	Mitochondrial permeability transition pore
NAdr	Noradrenaline
NGAL	Neutrophil gelatinase-associated lipocalin
NMD	Nitrate-mediated (endothelium independent) dilation
NO	Nitric oxide
NOS	Nitric oxide synthase
PCI	Percutaneous coronary intervention
PI3K	Phosphoinositide 3-kinase
PKCε	Protein kinase C epsilon
PPARδ	Peroxisome proliferator-activated receptor delta
rIPC	Remote ischaemic preconditioning
RISK	Reperfusion injury survival kinase
ROS	Reactive oxygen species
SAFE	Survivor activating factor enhancement
SPECT	Single-photon emission computed tomography
STAT	Signal transducers and activators of transcription
SWOP	Second window of preconditioning
TNFα	Tumour necrosis factor-alpha
TnI	Troponin I
TnT	Troponin T
TTC	2,3,5-Triphenyltetrazolium chloride
VF	Ventricular fibrillation
VOP	Venous occlusion plethysmography
VT	Venticular tachycardia

CHAPTER 1: INTRODUCTION

1.1 Ischaemia-Reperfusion Syndromes

Ischaemia-reperfusion syndromes are characterised by the occlusion of arterial blood flow to a tissue or organ, followed by the subsequent restoration of perfusion and rapid reoxygenation. They encompass a wide variety of pathological conditions, the most clinically important of which are acute myocardial infarction and stroke which are the two leading causes of death worldwide. In total, these ischaemia-reperfusion syndromes accounted for approximately 13.5 million deaths globally in 2008 or 23.6% of total all-cause mortality[1]. In the UK alone, in 2009, there were close to 80,000 hospital admissions with acute myocardial infarction with a pooled age adjusted 30 day mortality rate of approximately 8% and direct costs to the NHS of over £3 billion[4]. In the US there are approximately 1 million presentations with acute myocardial infarction annually with an estimated cost to the healthcare system of $475.3 billion [5]. In addition to unpredictable episodes of ischaemia presenting as emergencies, iatrogenic ischaemia through various forms of vascular and cardiac surgery likewise lead to episodes of predictable ischaemia-reperfusion injury with potentially deleterious consequences in the peri- and post-operative periods.

The degree of ischaemic injury in acute myocardial infarction increases as a function of time[6], although this relationship is non-linear[7] with the greatest myocardial loss occurring during the first two or three hours after the onset of symptoms. Importantly, the extent of myocardial injury correlates with future mortality and morbidity[8] and so strategies to limit infarct size are of direct prognostic benefit. It was only in the 1970s however that accumulating experimental evidence suggested that tissue in the ischaemic zone may still be viable and that reperfusion may limit the extent of infarction[9], laying the foundations for the modern treatment of acute myocardial infarction by coronary reperfusion either by pharmacological[10-12] or mechanical[13-15] means.

Whilst the implementation of reperfusion therapy as the primary treatment modality in acute myocardial infarction, and to a more limited degree in acute ischaemic stroke, has been highly successful and a major contributor to the on-going decline in cardiovascular mortality rates[5], reperfusion itself causes paradoxical tissue injury, with the restoration of blood supply leading to the loss of previously viable tissue. Although initially considered by some to be an experimental artefact[16, 17], it has become clear that this paradoxical damage is relevant to human disease[18] and has been termed 'reperfusion injury'[19].

1.1.1 Reperfusion Injury

Ischaemia ensues when the arterial blood supply is insufficient to meet the metabolic demands of a tissue, and in acute myocardial infarction this is classically due to the abrupt occlusion of an epicardial coronary vessel, most commonly secondary to the rupture of an atherosclerotic plaque. At a molecular level, ischaemia involves complex pathophysiological changes with the accumulation of sodium, hydrogen and calcium ions causing intracellular acidosis and subsequent cell necrosis or the triggering of apoptosis[20]. In particular, extremely high mitochondrial matrix calcium concentrations in conjunction with conditions of high oxidative stress and adenine nucleotide depletion can trigger the opening of a non-selective pore known as the mitochondrial permeability transition pore, leading to mitochondrial swelling, breakdown of proton motive force, loss of ATP production and cell death[21].

Although prompt reperfusion salvages myocardial tissue, this in it itself leads to the paradoxical phenomenon of reperfusion injury, a multifaceted phenomenon that remains incompletely understood. The restoration of blood flow elicits rapid alterations in ion flux with activation of the Na^+-H^+ exchanger and the Na^+-HCO_3^- transporter leading to increased intracellular sodium accumulation[20]. These high sodium concentrations in turn drive increases in sarcoplasmic reticular Ca^{2+} by Na^+-Ca^{2+} exchange[22]. The resultant Ca^{2+} overload leads to myofibrillar hypercontractility, ATP depletion and ultrastructural damage to mitochondria.

In addition, reperfusion also leads to a deleteriously rapid normalisation in pH[23], the generation of large amounts of highly reactive free radicals, most likely through the action of xanthine oxidase[24], complement activation[25], immune cell infiltration[26], endothelial dysfunction and dissipation of the mitochondrial membrane potential through opening of mitochondrial permeability transition pore (mPTP)[27] (see Fig. 1.1). These changes in the intra and extracellular environments hasten cell death in already lethally injured cells but also leads to the loss of otherwise viable tissue, increasing overall infarct size. As such, adjuncts which limit the extent of reperfusion injury have elicited a great deal of scientific and clinical interest due to their potential to reduce myocardial injury and improve clinical outcomes.

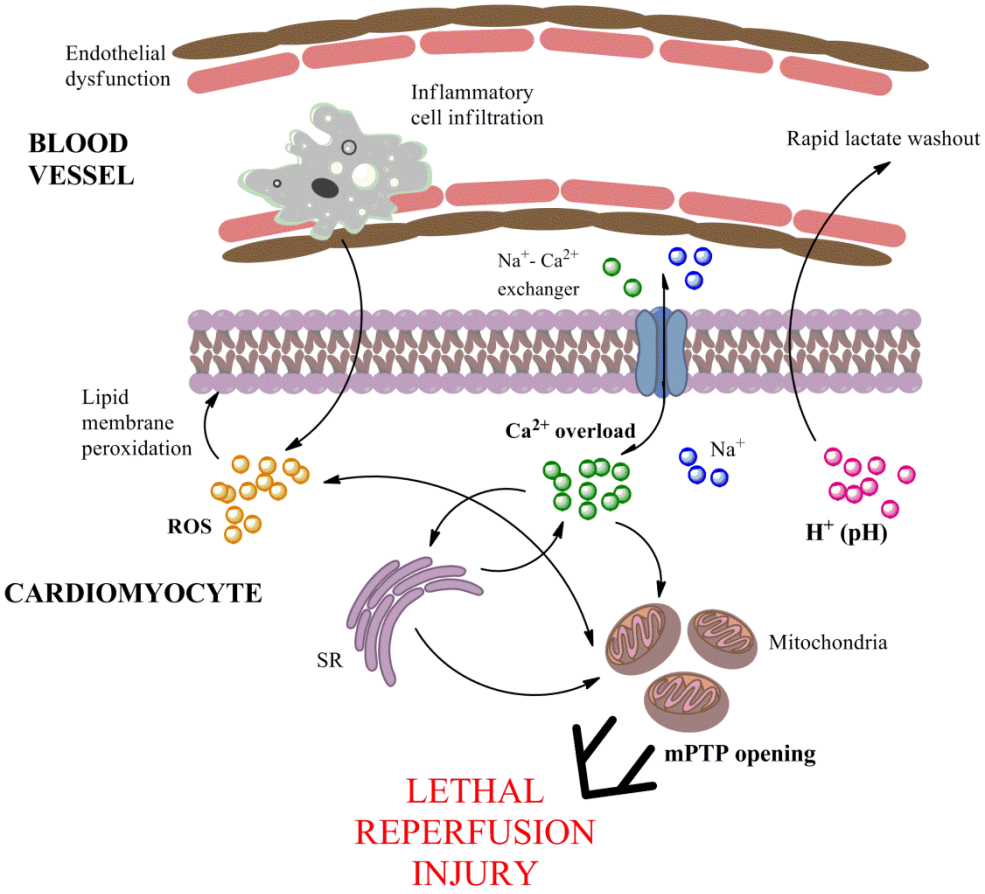


Figure 1.1 Mechanisms of ischaemia-reperfusion injury leading to tissue injury and cell death. In this simplified scheme, multiple abrupt biochemical and metabolic changes occur at the time of reperfusion including Ca^{2+} overload, ROS generation and later inflammatory cell activation. Many of these changes potentiate permeability transition pore opening and result in lethal cellular injury (adapted from[28]).

1.1.2 Reperfusion Injury in Man

As alluded to previously, the concept of lethal reperfusion injury as a distinct form of cardiomyocyte death independent to that occurring from the initial ischemic injury has been the matter of some debate particularly as pertains to human disease[28]. Myocardial reperfusion injury was initially postulated by the work of Jennings *et al* [29] reporting on the histological features of reperfused myocardium in a canine infarct model and the majority of the mechanistic work described above relates to animal

models. Whilst the reperfusion phenomena of myocardial stunning[30], microvascular dysfunction[31] and reperfusion arrhythmias[32] are widely recognised and seen in routine clinical practice, it is much more difficult to demonstrate lethal reperfusion injury in humans due to the inability to accurately assess necrosis *in-situ* as the heart is reperfused after an ischemic insult. Consequently, the most convincing evidence of the existence of this phenomenon in man has come not from demonstrating the injury itself but from interventions that are able to modify the extent of infarction, initiated only *after* the commencement of reperfusion. Although still only in small scale studies, both mechanical[33, 34] and pharmacological interventions[35] in humans initiated after the end of ischemia, in the early moments of reperfusion have been demonstrated to reduce infarct size. By their very temporality, these interventions cannot be modifying ischemic injury and so provide evidence for reperfusion injury as a real phenomenon in man. Known as postconditioning, this concept is discussed in greater detail below, and provides the best evidence of a modifiable injury phenomenon occurring during the phase of reperfusion.

1.2 Strategies of Cardioprotection

1.2.1 Local Ischaemic Preconditioning

Multiple experimental strategies of cardioprotection have now been described but the first to be robustly and widely reproducible was that of ischaemic preconditioning (IPC). Although an IPC like phenomenon was reported to be protective against arrhythmias as early as 1977[36] it was not until the serendipitous discovery by Murry *et al* in 1986[37], that IPC was found to reduce myocardial infarct size and even 25 years later remains the most powerful cardioprotective strategy yet described. Working in a canine model of myocardial infarction, the authors ascertained that four successive five minute cycles of non-lethal ischaemia and reperfusion, achieved by tightening a ligature around the left circumflex artery (LCx), prior to a prolonged episode of ischaemia designed to simulate myocardial infarction, greatly reduced infarct size in that territory. The description of IPC demonstrated the presence of endogenous cardioprotective mechanisms which could be activated by a simple physical stimulus and precipitated sustained research into the mediators and mechanisms of this phenomenon. IPC itself however has been limited in its clinical use. Due to its inapplicability in episodes of unpredictable ischaemia such as myocardial infarction, due to the need to apply the stimulus before the onset of ischaemia coupled with the inherent difficulty involved in accessing the arterial supply to an organ, it has failed to enter widespread clinical practice. Even in situations such as cardiac surgery where the heart is exposed, it has failed to demonstrate consistent clinical benefits [38-40]. Despite this, IPC remains the

bench mark comparator for other cardioprotective strategies and due to its simplicity and reliability in models of IR injury has allowed the study of molecular pathways and mechanisms pertinent to cytoprotective strategies in general.

1.2.2 Remote Ischaemic Preconditioning

The description by Przyklenk et al in 1993[41] of the phenomenon that has become known as remote ischaemic preconditioning (rIPC), served as a conceptual leap forward in the field of cytoprotection. Using a similar canine experimental model as used by Murry in his initial descriptions of IPC, the authors described cardioprotection in the territory of the left anterior descending (LAD) coronary artery when a similar stimulus of short repeat cycles of non-lethal ischaemia were applied in the LCx territory. Initially termed 'preconditioning at a distance', subsequent work went on to demonstrate that a similar cardioprotective phenotype could be elicited by applying a comparable stimulus to a distant non-cardiac organ such as the intestine[42], kidney[42, 43] and also the hind limb[44]. In fact, rIPC appears to provide systemic protection and has been shown to protect not only the heart but the kidneys[45, 46], liver[47, 48], muscle[49] and brain[50-52]. Now almost exclusively elicited by limb ischaemia, easily achieved using a tourniquet or pressure cuff, rIPC has become a much more practical and non-invasive means of eliciting cellular protective responses against ischaemia-reperfusion injury and broadened the clinical scope of cardioprotective stimuli. The nature and mechanism of rIPC are the primary focus of the work contained within this thesis, principally

concentrating on the role of the purine nucleotide adenosine. The purpose of the majority of the remainder of this chapter is to contextualise rIPC within the broader framework of other cardioprotective strategies, to describe its utility in preventing IR injury and to describe its putative mechanisms and mediators, including adenosine.

1.3 Perconditioning and Post-Conditioning

By its very nature, preconditioning is only useful when applied prior to the onset of ischaemia. In the more common clinical situation where patients present acutely with no prior warning, other strategies are needed. In 2003 a group led by Vinten-Johansen described the phenomenon of local ischaemic post-conditioning. Using a similar open-chest canine model as Murry and Przyklenk, they demonstrated that short 30 or 60 second episodes of ischaemia interspersed with reperfusion, immediately following the offset of the index ischaemic insult, reduced infarct size to a degree comparable with that of preconditioning[53]. This was the first description of a cytoprotective strategy that might be implemented after the onset of ischaemia, significantly broadening the range of IR syndromes that might benefit from a conditioning stimulus and adding further evidence to support the concept of reperfusion injury. Post-conditioning has been validated in numerous further studies in both animal models[54, 55] and humans[33, 56] and has also been extended from local post-conditioning strategies to the demonstration of remote post-conditioning[57, 58].

Post-conditioning was followed in 2007 by the demonstration of remote ischaemic perconditioning by Schmidt *et al* [59]. In this study, conducted in a porcine model, four five minute cycles of hind limb ischaemia interspersed with reperfusion, begun after the onset of cardiac ischaemia but prior to reperfusion reduced infarct size through a K^+ATP sensitive channel dependent mechanism.

This initial experimental validation has been followed up in subsequent work by Botker *et al[60]* who have demonstrated that this same stimulus is active and applicable in humans presenting with acute myocardial infarction where four cycles of upper limb ischaemia-reperfusion immediately prior to percutaneous coronary intervention significantly reduced infarct size as assessed by SPECT imaging in comparison with a contemporaneous control group. A smaller perconditioning study in acute myocardial infarction published soon after demonstrated a reduction in troponin release with perconditioning although this only reached significance when supplemented with opiates as a second cytoprotective agent[61].

1.4 Pharmacological and other preconditioning stimuli

As well as short cycles of ischaemia and reperfusion, multiple other agents and stimuli have been described as being able to trigger a preconditioning-like state. These include nicorandil[62], propofol[63], rosuvastatin[64], glceryl trinitrate[65], cyclosporine[35], sevoflurane[66], erythropoietin[67], ketamine[68], morphine[69], ethanol[70],

hydrogen sulfide[71], pain[72], both hyperthermia[73] and hypothermia[74], and exercise[75]. This far from exhaustive list gives some illustration of the wide array of cardioprotective strategies that have been demonstrated in pre-clinical models and small scale clinical studies. Despite this broad array of candidates with early promise, only a handful have been shown to be effective when attempting to translate initial findings to the more complex situation of human physiology and disease. Nonetheless, many of these models have been useful in allowing exploration of the multiple pathways and mechanisms involved in triggering preconditioning and deepened our understanding of the cellular responses to cytoprotective stimuli.

1.5 Translation of rIPC to the clinical setting

Early work on rIPC concentrated on pre-clinical animal models to investigate the magnitude and mechanisms of the elicited cytoprotection, but its applicability in the more complicated pathobiology of humans was unclear. However, in 2002 Kharbanda *et al*[76] demonstrated rIPC's protective effect in healthy human volunteers, using a forearm model of endothelial IR injury, with 3 cycles of 5 minutes of ischaemia and 5 minutes of reperfusion of the contralateral arm using a pressure cuff, as the stimulus. These findings have been rapidly translated to the field of human disease with early pilot studies in children[77, 78] and adults[79, 80] undergoing cardiac surgery, elective abdominal aortic surgery[46] and elective coronary angioplasty[81]. For the most part these trials have demonstrated a beneficial effect of rIPC, predominantly using biochemical markers of injury such as Troponin or Creatine Kinase release as end-

points. It should be noted however that the majority of these studies have been small in size and the largest study to date in patients undergoing coronary artery bypass surgery has failed to show a benefit in any of its primary or secondary outcome measures[82]. Indeed, there are an increasing number of other recent neutral clinical studies [83-86]. Taken in their entirety, although these studies suggest that rIPC is a biologically effective strategy in humans and is capable of reducing the incidence and magnitude of human myocardial IR injury as well as preventing injury in other organs and tissues (see Table 1.1 for summary), its clinical relevance remains uncertain.

Study	Type of Surgery	No of Patients	rIPC Protocol	Outcome	Ref
Gunaydin *et al*	CABG+/-valve	8	2x3 (arm)	Decreased LDH	[87]
Cheung *et al*	Paediatric cardiac surgery	37	4x5 (leg)	Decreased TnI AUC, inotrope requirements and airway pressures	[77]
Ali *et al*	Elective AAA repair	82	2x10 (iliac arteries)	Decreased TnI AUC, Cr AUC	[46]
Hausenloy *et al*	CABG+/-valve	57	3x5 (arm)	Decreased TnT AUC	[79]
Venugopal *et al*	CABG+/-valve	45	3x5 (arm)	Decreased TnT AUC	[80]
Hoole *et al*	Elective PCI	242	3x5 (arm)	Decreased median TnI	[81]
Zhou *et al*	Paediatric cardiac surgery	60	3x5 (arm)	Decreased CKMB and TnT	[88]
Thielmann *et al*	CABG	53	3x5 (arm)	Decreased TnI AUC	[89]
Rahman *et al*	CABG	162	3x5 (arm)	No difference in TnT AUC or LVEF	[82]
Walsh *et al*	Elective AAA repair	40	2x10 (iliac arteries)	No difference in retinol binding protein or alb:Cr	[90]
Walsh *et al*	Endovascular AAA repair	40	2x10 (leg)	Decreased alb:Cr ratio	[91]
Walsh *et al*	Carotid endarterectomy	70	2x10 (leg)	No difference in saccadic latency	[92]
Li *et al* (perconditioning)	Valve surgery	81	4x4 (leg)	Decreased TnI and defibrillation use	[93]

Study	Type of Surgery	No of Patients	rIPC Protocol	Outcome	Ref
Wagner *et al* (2nd window)	CABG+/-valve	101	3x5 (arm)	Decreased TnI	[94]
Hong *et al*	CABG	130	4x5 (arm)	No difference in TnI AUC	[95]
Ali *et al*	CABG	100	3x5 (arm)	Decreased CKMB and TnT	[96]
Karuppasamy *et al*	CABG	54	3x5 (arm)	No difference in TnI AUC	[97]
Choi *et al*	Valve surgery	76	3x10 (leg)	Reduced CKMB, reduced ITU stay	[98]
Pedersen *et al*	Paediatric cardiac surgery	113	4x5 (arm)	No difference in cystatin C or NGAL	[99]
Jin *et al*	Valve surgery	32	3x8 (arm)	Decreased CKMB and TnI	[100]
Zimmerman *et al*	CABG	120	3x5 (leg)	Decreased acute kidney injury score	[101]

Table 1.1 Summary of major published cardiac and vascular surgical trials using remote preconditioning to elicit cytoprotection in episodes of predictable ischaemia-reperfusion injury. End points and preconditioning protocols vary between studies with the majority showing benefit in primary end-points. rIPC protocol denotes number of cycles x inflation time in minutes. CABG, coronary artery bypass grafting; AAA, aortic abdominal aneurysm; PCI, percutaneous coronary intervention; LDH, lactate dehydrogenase; TnI, troponin I; TnT, troponin T; AUC, area under curve; CKMB, creatine kinase (muscle-brain isotype); Cr, creatinine; alb, albumin; LVEF, left ventricular ejection fraction; ITU, intensive therapy unit; NGAL, neutrophil gelatinase-associated lipocalin.

1.6 Mechanisms of rIPC

Intensive efforts to study the pathways through which conditioning strategies operate has elucidated many insights into the mechanisms of cytoprotection and holds the promise of optimising conditioning strategies to maximise clinical benefits. Despite this progress, many questions remain unanswered.

The mechanisms through which pre- per and post-conditioning strategies operate appear to be interrelated with similar functional pathways present in each stimulus. However, despite this overlap, there are differences in the mode of action of each conditioning strategy and delineation of one does not necessarily denote universality. In addition, important model and species differences also contribute to the difficulties in describing underlying mechanisms. Moreover, multiple parallel pathways appear to be operating simultaneously, presumably providing biological redundancy, but somewhat paradoxically, blockade of individual constituents of single pathways has been shown by multiple groups under experimental conditions to be able to abrogate preconditioning responses.

In rIPC the stimulus may be thought of as acting in two phases – a transmission of the stimulus from the ischaemic limb or tissue to the target organ ('trigger') and a second phase in which signals from the cell surface are transmitted to intracellular target organelles ('effector'). In addition to this, as well as the immediate protection provided

by rIPC against IR injury, known as the first window of preconditioning, which wanes after 2-3 hours, a second window of preconditioning (SWOP) was described in 1993 by Marber and colleagues [102] and has subsequently been demonstrated in man[103]. This SWOP appears approximately 24-48 hours after the initial window, lasts for 2-3 days and is associated with the transcription of multiple cardioprotective factors [104]. A so-called 'third-window' of preconditioning initiated by multiple repeat preconditioning episodes over an extended time course has recently been postulated as being characteristically and molecularly distinct from other forms of preconditioning[105], but further work to confirm and expand on these initial studies is necessary.

1.6.1 Trigger Phase - Evidence for a Neural Mechanism

Several lines of evidence point to a neural mechanism being involved in transmission of the rIPC signal from the initiating tissue to the target organ. Initial evidence for such a mechanism was presented by Gho *et al* [42] in 1996 who demonstrated that in a rat model, the ganglion blocking agent hexamethonium was able to abrogate the cytoprotective effect of mesenteric artery occlusion and in multivariate analysis this was deemed to be independent of its blood pressure lowering effect. Other authors have confirmed similar effects with femoral nerve transection in rat and rabbit models leading to at least partial blockade of rIPC when using limb ischaemia as the stimulus [106-108].

Adding to this body of evidence, the neurotransmitter calcitonin gene-related peptide (CGRP) has been implicated as potential mediator of rIPC, with the release of CGRP from sensory nerves seemingly able to trigger preconditioning through a PKCε dependent pathway [109]. Moreover, evidence for a neural mechanism has been translated to human models of rIPC where treatment with the ganglion blocking agent trimetaphan was effective in reducing the protective effect of rIPC in an ischaemia-reperfusion model in the forearm [103]. However, as evidenced by Konstantinov *et al,* this pathway may not be vital for rIPC to be effective, with cardioprotection apparent in denervated transplanted hearts when subjected to IR injury following rIPC[110].

1.6.2 Trigger Phase - Evidence for a Humoral Mechanism

The necessity of a reperfusion period in the rIPC tissue or organ to be effective has been taken to suggest that a 'washout' period is important in conferring protection, allowing the release and circulation of a cytoprotective substance to take place. Several groups have demonstrated that ischaemic preconditioning stimulates the release of factor(s) into the circulation which are able to protect other tissues against ischaemia-reperfusion injury [111, 112]. In these studies, the transfer of coronary effluent or whole blood following a preconditioning stimulus, to naïve animals, led to cardioprotection in these subjects and argued strongly for the release of a circulating factor by preconditioning. In a similar manner, blocking venous return from a limb where a preconditioning stimulus is applied has been shown to be able to attenuate cardioprotection [108] (see Fig 1.2).

Several authors have made attempts to characterise this transferrable factor(s) by techniques including proteomics [113] and C18 fractionation [114] but unfortunately its identity remains unknown. Recent experimental work has demonstrated that it is dialyzable across a 15kDa cut-off membrane, will bind to a C18 hydrophobic column and furthermore is transferrable between mammalian species [115] with dialysate generated from human subjects undergoing rIPC able to protect cardiac tissue from mouse and rabbit hearts. On-going work by a number of techniques including LC-MS/MS is attempting to elucidate the identity of this factor(s) but until positive identification has occurred, simple and robust assays to detect its presence or absence are lacking, as is the ability to quantify this agent. However, as its protective activity can be demonstrated in a variety of models of ischaemia-reperfusion including isolated perfused heart Langendorf apparatus[107] and isolated cardiac myocytes [115], the factor can be utilised as a surrogate marker for the effective triggering of a preconditioning stimulus.

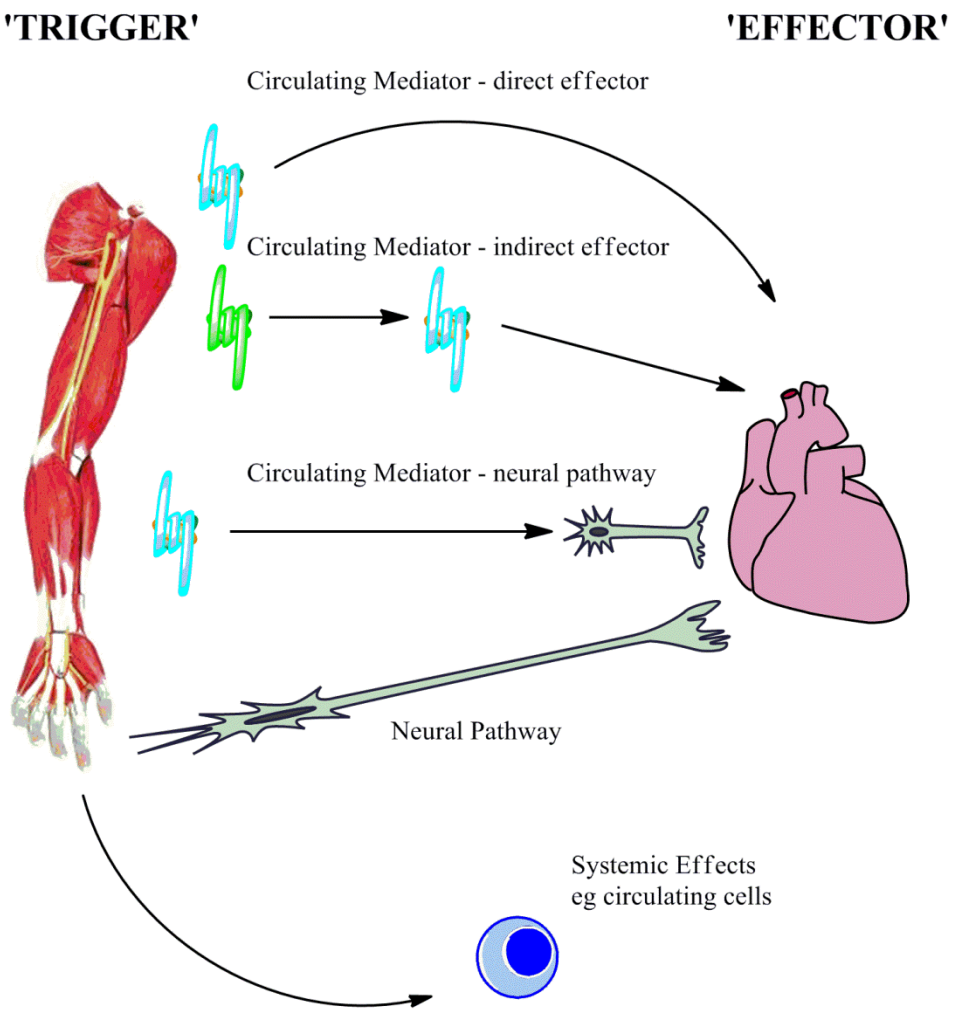


Figure 1.2 Potential mechanisms of transmission of the rIPC signal to the target organ. Multiple potential pathways of the rIPC signal have been suggested with both neurally conducted and humorally transmitted mediators having been described. It is not unlikely that rIPC acts through a combination of these pathways with multiple redundant mechanisms being present.

1.6.3 Effector phase – from Cell surface to Intracellular Pathways and Target Organelles

After the triggering of a rIPC stimulus, and conveyance to the target organ, a number of steps occur with initial cell surface receptor activation, activating intracellular signal transduction cascades which target a number of intracellular molecules and organelles

(particularly the mitochondria), resulting in a switch to a protected phenotype. The identity of many of the participants in this process in rIPC remains incompletely defined, with multiple potential candidates identified in the literature and with evident "cross-talk" between pathways. However, the basic paradigm as outlined is pervasive and multiple lines of evidence point to its veracity (see Fig 1.3 for cartoon of possible sub-cellular pathways).

At the cell surface an array of G-protein coupled receptors (GPCRs) have been identified as being triggered by an rIPC stimulus with a number of lines of evidence implicating both the κ [116] and δ [117] opioid receptors, the endocannabinoid (CB(2)) receptor [118], adenosine receptors [119, 120] and bradykinin [121] although the relevance of this last receptor in human preconditioning has not been substantiated [122].

In addition, a number of second messenger systems have been described as being triggered by conditioning stimuli including the PI3K, Akt, ERK1/2, GSK3β 'Reperfusion Injury Survival Kinase' (RISK) pathway[123], the TNFα, JAK-STAT 'Survivor Activating Factor Enhancement' (SAFE) pathway[124, 125], PKCε and ROS signalling. The relevance of many of these systems to rIPC is largely unexplored but recent data from Li et al [126]has demonstrated the PI3K/AKT pathway is activated in the myocardium by a rIPC stimulus with pharmacological blockade of PI3K by Wortmannin abrogating protection. In this model, RISK activation led to translocation of β-catenin to the nucleus with up-regulation of cell survival genes including E-cadherin and PPARδ.

Similarly, the role of PKCε in rIPC has been implied by the use of the pharmacological inhibitor chelerythrine which has been shown to block remote preconditioning effects [127, 128], with other work demonstrating that exposure to dialysate containing the humoral preconditioning factor inducing subcellular PKCε redistribution[115]. Other evidence for pathways is sparse although the use of free radical scavengers to block rIPC has implicated ROS[129] signalling, although it is unclear whether this is through a myocardial or peripheral action.

The sub-cellular targets of cytoprotective stimuli that are affected by these second messenger systems have also received a great deal of attention. The most prominent amongst them is the mitochondrion and the non-specific transmembrane pore known as the mitochondrial permeability transition pore (mPTP). The mPTP is regarded by many researchers as a 'final common pathway' for preconditioning strategies with inhibition of opening crucial to cell survival [27, 130, 131]. However, other targets may also include the mitochondrial K^+ATP channel [59, 132], translocation of pro-survival factors to the nucleus[126], cell volume regulatory elements [133], mitochondrial aldehyde dehydrogenase 2[70] and the cell membrane component MG53 [134]. Again, the specific relevance of these targets in rIPC is largely unexplored with the best researched element being the mitochondrial K^+ATP channel which has been shown to be pertinent not only in lab based models [135] but also in humans [136], where the sulphonylurea glibenclamide was used as a pharmacological inhibitory agent. More recently, work by Cao *et al* has demonstrated that mPTP opening is inhibited by rIPC in reperfusion [95], as in other forms of cytoprotection. Further clarification of the sub-

cellular pathways and targets specific to rIPC will be essential in increasing our understanding of this important protective mechanism.

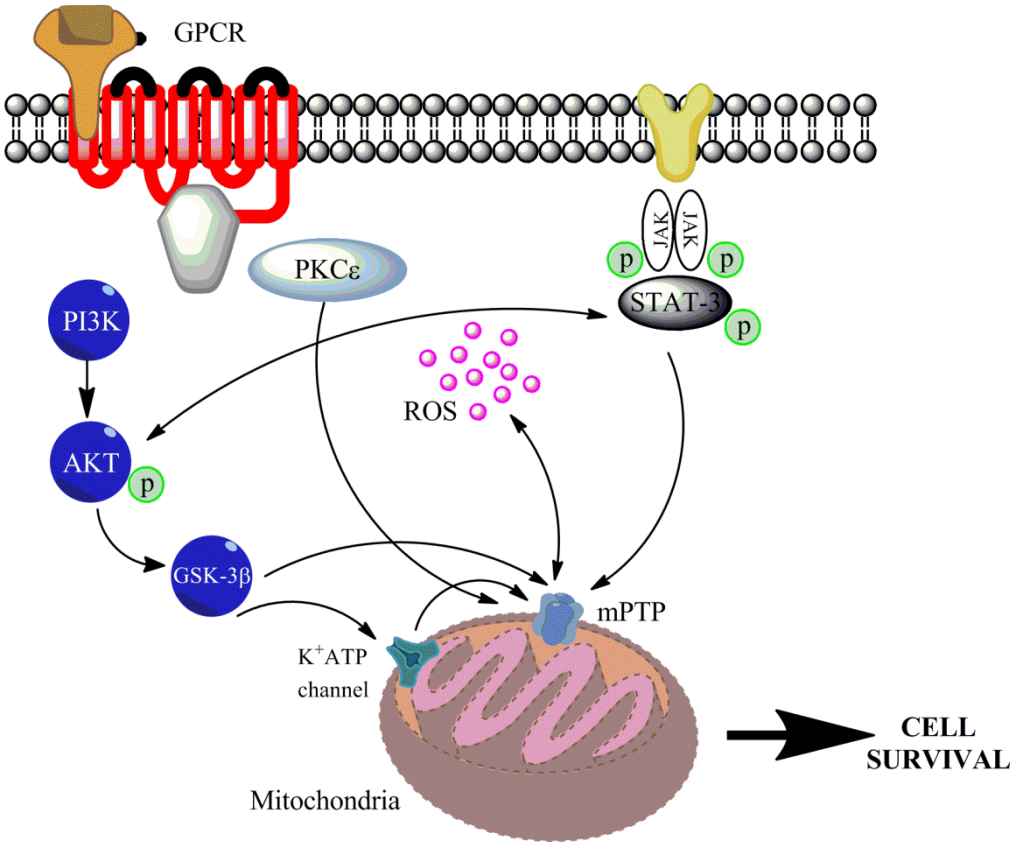


Figure 1.3 Multiple sub-cellular pathways in preconditioning. The activation of a variety of cell surface GPCRs appear to be able to trigger intra-cellular second-messenger systems which convey the preconditioning signal to target organelles, notably the mitochondria, conferring a switch to a protected phenotype. PI3K, phosphoinositide 3-kinase; GSK3β, glycogen synthase kinase 3 beta; PKCε, protein kinase C epsilon; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

1.6.4 Evidence for a systemic response

As well as direct effects on the target organ, several lines of evidence suggest that rIPC also has systemic effects leading to down-regulation of inflammatory responses. In a feline model of IR injury, preconditioning has been shown to reduce vascular permeability and neutrophil adhesion[137] while in a rat model of hepatic IR injury it was reported to down-regulate TNF-induced p-selectin up-regulation. This initial work was translated to human preconditioning with a microarray approach demonstrating a down-regulation in circulating leukocytes of a number of proteins including regulators of cytokine synthesis, chemotaxis, adhesion and migration[138]. More recently these preliminary findings were confirmed to modify neutrophil function, with reductions in adhesion, exocytosis, phagocytosis and inflammatory cytokines observed following rIPC[139].

The pathways of rIPC are multiple and complex and further work is necessary to clarify its mechanisms, particularly as pertains to human health and disease. One of the key steps in several forms of preconditioning responses is thought to be the release of adenosine, and this thesis will explore the role of adenosine as pertains to human rIPC.

1.7 Adenosine

The first description of adenosine dates back to 1929 when the physiologists Drury and Szent-Gyorgi, working in Cambridge, published their observations that an extract from bullock hearts when injected into dogs, caused a transient and reproducible decrease in heart rate that was not inhibited by atropine[140]. After a series of purification steps they were able to identify the biologically active substance as an 'adenine compound'. Nearly 50 years later adenosine was recognised for its ability to control arrhythmias [141, 142] and today its primary clinical use is in the emergency treatment of supraventricular tachycardias. However, as demonstrated all those years ago, adenosine is an intrinsic biological substance and continued research has shown it to be active in a wide array of physiological processes.

Adenosine is a highly abundant endogenous purine nucleotide and is principally formed by the intra and extra-cellular hydrolysis of adenosine monophosphate (AMP) [3]. It is composed of a molecule of adenine attached to a ribose moiety via a glycosidic bond (see Fig 1.4) and plays important roles in a variety of important homeostatic processes including energy transfer though ADP and ATP[143], the sleep-wake cycle[144] and a variety of intracellular signalling pathways through cyclic AMP (cAMP). Adenosine is also known to act as an extracellular signalling molecule and acts by stimulation of membrane bound G-protein coupled adenosine receptors of which there are four sub-types; the A_1, A_{2A}, A_{2B} and A_3 receptors.

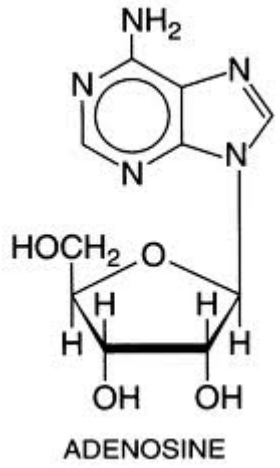


Figure 1.4 Molecular structure of adenosine. The molecule consists of an adenine purine ring attached to a single ribose carbohydrate moiety by a β-N_9-glycosidic bond (from[3]).

1.7.1 Adenosine Metabolism

Extracellular adenosine is principally formed from phospho-ester hydrolysis of its precursor molecules AMP, ADP and ATP (see Fig 1.5) catalysed by the widely expressed surface bound ecto-apyrase enzyme CD39, a member of the ectonucleotidase triphosphate diphosphohydrolase family (ENTPD), also referred to as ENTPD-1 (EC 3.6.1.5). CD39 acts by catalysing the degradation of ATP and ADP to AMP and in a second step the ectonucleotidase CD73, (5-prime-ribonucleotide phosphohydrolase; EC 3.1.3.5), a membrane-bound glycoprotein, catalyses the conversion of AMP to adenosine.

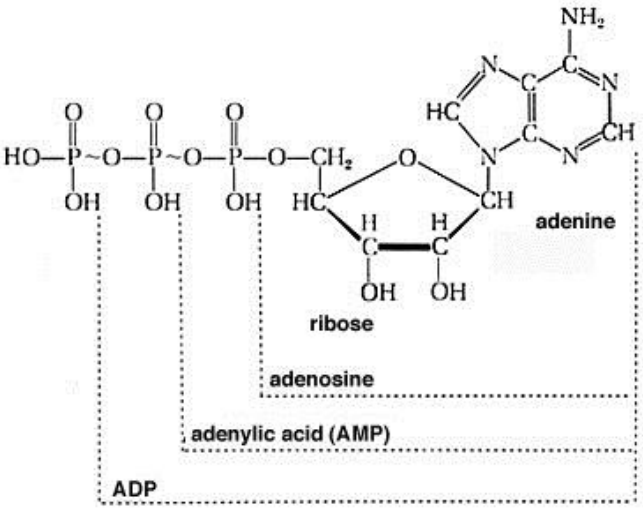


Figure 1.5 The relationship of the molecular structure of adenosine to ADP and AMP. ADP and ATP breakdown is catalysed by CD39

Free adenosine may then undergo several processes. As well as activating cell surface receptors, adenosine can traverse the cell membrane through the equilibrative nucleoside transporters ENT1 and ENT2 which allow the free flow of adenosine across a concentration gradient so that in normal physiological conditions intra- and extracellular adenosine concentrations differences are very small. The ENT transporters can be inhibited by dipyridamole which leads to an increase in extracellular adenosine concentrations.

Once inside the cell, adenosine is rapidly metabolised. Two alternative metabolic pathways principally control free adenosine's fate, adenosine deaminase which irreversibly deaminates adenosine to inosine, and adenosine kinase which phosphorylates adenosine to AMP. An alternative and less important pathway is the conversion of adenosine to adenosylhomocysteine which is included here for completeness (see Fig 1.6). Due to the rapid equilibration of adenosine, these

pathways consequently play a large role in determining the extracellular adenosine concentration with the circulating half-life being less than 5 seconds.

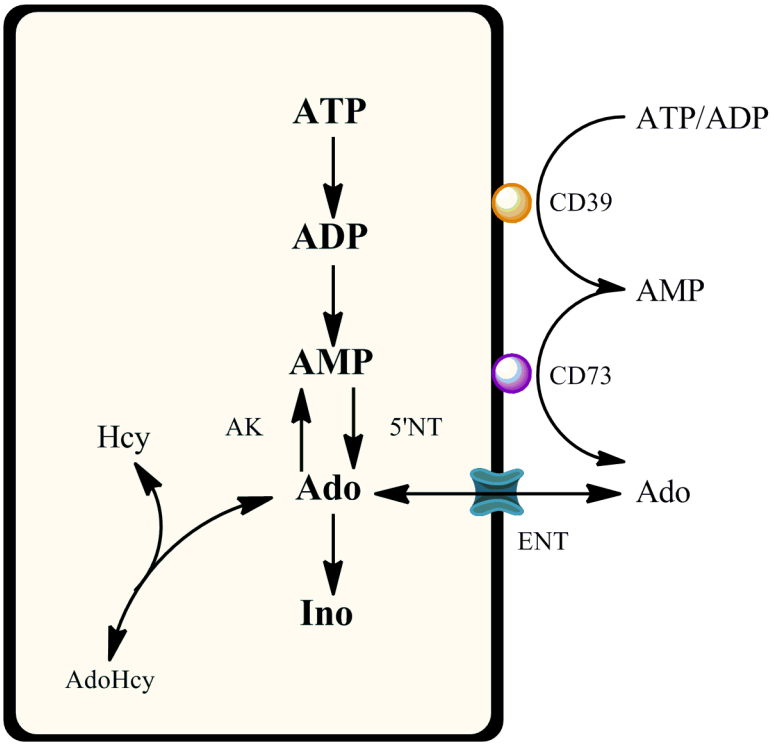


Figure 1.6 Pathways of Adenosine Metabolism (adapted from[3]). Intra and extracellular adenosine concentrations are rapidly equilibrated through the cell surface ENTs (equilibrative nucleoside transporters). Intracellular adenosine is principally deaminated to inosine or phosphorylated to form AMP although can also be converted to adenosylhomocysteine.

The quantification of free adenosine is extremely challenging due to its rapid uptake and metabolism in whole blood, principally by erythrocytes, with rapid degradation continuing after blood withdrawal. Attempts have been made to use vacutainers with blockers of adenosine transmembranous transport, extracellular formation and degradation[145], but these systems do not reflect interstitial concentrations of adenosine *per se* as the endothelium acts as a highly active metabolic barrier to

adenosine, due to uptake and degradation in endothelial cells[146]. Alternative systems using microdialysis catheters, corrected for relative recovery profiles, are able to determine concentrations of adenosine, by blocking passage of cells and degradative enzymes without inhibiting the passage of adenosine. However, these systems require direct access and imbedding in tissue for prolonged periods to allow initial equilibration, making human use difficult.

1.7.2 Adenosine Receptor Subtypes

Four adenosine receptor subtypes encoded by distinct genes have been characterised; the A_1, A_{2A}, A_{2B} and A_3 receptors all of which mediate a number of discrete physiological responses. The A_1 and A_2 receptors were characterised over 25 years ago through their interaction with methylxanthines but it was not until 1992 that the A_3 receptor was characterised after cloning of its cDNA from a rat brain library revealed its high sequence homology to the two previously cloned receptors.

The A_1, A_{2A} and A_3 receptors all have high affinity for adenosine activating at concentrations between 10nM and 1µM. In contrast, the A_{2B} has a relatively low affinity, requiring concentrations that exceed 10µM of adenosine, and while it is very difficult to determine the adenosine concentration at the cell surface, it is thought to generally only activate during pathological conditions.

It should also be considered that the effects of adenosine depend greatly on the relative expression of adenosine receptors on the extracellular surface of individual cell types or a specific tissue. While the heart expresses all receptor subtypes their distribution throughout the myocardium as a whole may vary, leading to varied effects throughout the tissue as a whole.

All adenosine receptors are G-protein coupled, with the A_1 and A_3 receptors acting through $G_{i/0}$ (inhibiting cAMP activity), while the A_{2A} receptor acts though G_s and the A_{2B} acts though both G_s and $G_{q,}$ both increasing levels of cAMP. Each adenosine receptor subtype has been implicated in preconditioning responses with the A_1 and A_3 receptors seemingly particularly prominent in this response. However, evidence for their specific involvement in rIPC and particularly in human rIPC is sparse.

The actions of the adenosine receptors are further complicated by their ability to heterodimerise with other GPCRs. In addition, there is also evidence of significant 'cross-talk' between receptor subtypes making adenosine receptor function extremely difficult to unravel (see Table 1.2).

Receptor	Adenosine A_1	Adenosine A_{2A}	Adenosine A_{2B}	Adenosine A_3
Gene	*ADORA1*	*ADORA2A*	*ADORA2B*	*ADORA3*
G-protein coupling	$G_{i,}$ G_o	G_s	G_s, G_q	G_iG_o
Physiological Functions	• Bradycardia • ↓lipolysis • ↓GFR • ↓Autonomic activity • ↓Pain	• ↓Platelet aggregation • Vasodilatation • ↑Sensory nerve activity	• Relaxation of vascular smooth muscle • ↓Monocyte and macrophage function • ↑Mast cell release	• ↑ Mast cell release
KO phenotype	• Anxiety • Hyperalgesia • ↓Hypoxic tolerance • Altered insulin secretion • ↑Lipolysis	• Anxiety • Hypoalgesia • ↑Ischaemic tolerance • ↓Platelet aggregation	• Hyperinflammation • Vascular adhesion	• Decreased oedema • Altered inflammatory reactions

Table 1.2 The actions of adenosine receptor subtypes, adapted from [147]. Adenosine receptor subtypes are responsible for a variety of actions in health and disease. The overall response in a tissue is determined by the relative expression of each subtype and the current extracellular adenosine concentrations. KO, knockout.

1.7.3 Systemic Response to Adenosine

The systemic response to adenosine is dependent on opposing actions on the vascular and nervous systems and is dose and host dependent. Adenosine acts as a potent vasodilator through an endothelial dependent process, most likely mediated through release of NO as it can be at least partially inhibited by concomitant infusion of the NOS inhibitor L-NMMA[148]. In conjunction with this, adenosine has a dual action on the sympathetic nervous system, causing both systemic activation by stimulation of adenosine–sensitive chemoreceptors in the carotid body [149], while concurrently

inhibiting the sympathetic release of NAdr in local tissue. In essence, this dual action in conjunction with its intrinsic vasodilating properties leads to systemic vasoconstriction and local vasodilation, effectively redistributing blood flow to areas where adenosine is present in a high concentration. While adenosine also has direct negative chronotropic and dromotropic effects on the heart, depending on dose, it is the vascular effects that predominate and systemic adenosine infusion generally elicits a typical haemodynamic response consisting of an increase in heart rate and systolic blood pressure and a slight decrease in diastolic pressure [3].

Physiologically, the concentrations of extracellular adenosine are generally in the range of 20-200 nM but these can rise extremely rapidly to over 30 μM during periods of hypoxia or ischaemia. During ischaemia, the breakdown of ATP and ADP accelerates while the rephosphorylation by adenosine kinase is inhibited resulting in immediate and dramatic increases in the extracellular concentration of adenosine. Adenosine can therefore be considered as a metabolic signal, released from cells in response to energy perturbations or imbalances in O_2 supply and demand, acting through adenosine receptors to increase blood flow, decrease oxygen demand and trigger cytoprotective preconditioning mechanisms (see Fig 1.7).

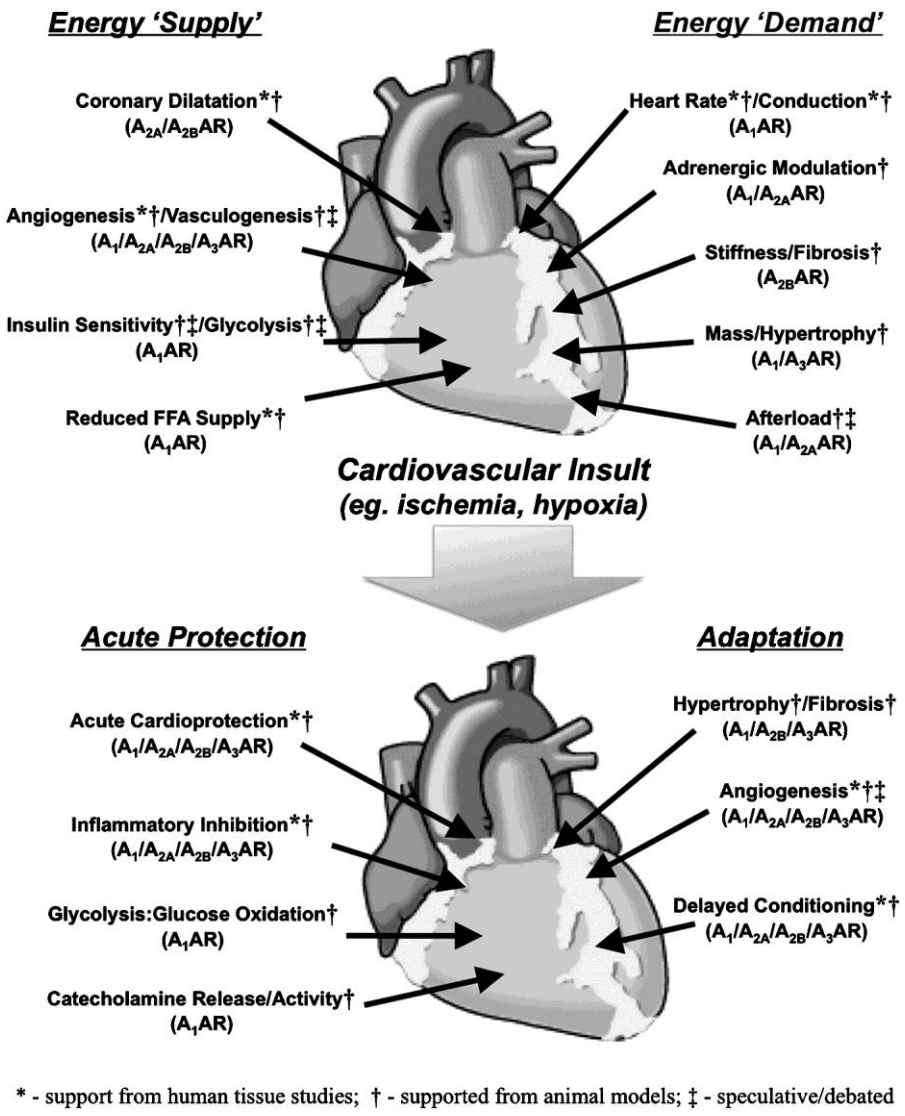


Figure 1.7 Adenosine receptors in the cardiovascular system, taken from [150]. Adenosine receptors play a variety of roles in both physiological and pathophysiological states and are in intrinsic to the normal function of the cardiovascular system.

1.7.4 Adenosine as a Preconditioning Agent

Multiple lines of evidence support adenosine as being important in endogenous preconditioning responses as well as being able to act as a pharmacological preconditioning agent. Initial evidence for the role of endogenous adenosine in

mediating preconditioning was provided by Liu *et al* [151] who demonstrated that the reduction in infarct size secondary to preconditioning could be blocked by the adenosine receptor blocker 8-SPT and a specific A_1 receptor antagonist, while both exogenous adenosine and an A_1 agonist were able to mimic the cardioprotection of preconditioning. This work has been confirmed [152, 153] and extended by other groups with evidence of involvement of other adenosine receptor subtypes in both models of pharmacological inhibition and genetic knockout models [154-156]. The role of adenosine has been subsequently translated to models of human preconditioning including evidence for adenosine receptor activation in superfused atrial trabeculae [157, 158], isolated paediatric cardiomyocytes [159], and a balloon angioplasty model of adult preconditioning [160]. Despite this wealth of data supporting the role of adenosine in preconditioning, much less evidence exists regarding the role of adenosine in rIPC and in human rIPC in particular.

1.7.5 Adenosine in rIPC

A limited number of studies have examined the role of adenosine in rIPC. Initial evidence for the role of adenosine came in the study by Pell *et al* which demonstrated blockade of renally induced remote preconditioning by the non-specific adenosine antagonist, 8-(p-Sulfophenyl)theophylline (8-SPT)[119]. In a subsequent study by Takaoka *et al* [161], the infusion of 8-SPT after the completion of the rIPC stimulus was still able to block cardiac preconditioning suggesting a role for adenosine receptor

activation in the effector phase. This was further explored by Liem *et al* [120] utilising a rat model where mesenteric artery ischaemia was used as a preconditioning stimulus to induce cardioprotection. While this intervention was successful, the prior infusion of 8-SPT into the mesenteric artery blocked this affect. Interestingly, intramesenteric artery but not portal vein infusion of adenosine was equally effective in inducing cardiac preconditioning suggesting adenosine could recapitulate the effects of rIPC. Finally, when 8-SPT was infused into the heart following reperfusion, once more the effects of rIPC was blocked, reinforcing the suggestion of not only a peripheral role of adenosine in initiating rIPC but also a central role for the activation of adenosine receptors in the target organ. This work has been followed up by several groups with the demonstration by Dong *et al* that femoral arterial and not femoral vein adenosine infusion was able to precondition rat hearts and that adenosine preconditioning could be inhibited by femoral nerve transection [106]. Similarly Steensrud *et al* [107] demonstrated in a rabbit model that femoral arterial and not femoral vein infusion of adenosine was able to release a humoral cardioprotective factor into the circulation which was able to reduce infarct size when transferred to a naïve Langendorf heart. In this range of animal models, adenosine therefore appears to be acting at multiple points in the rIPC pathway and may play important roles both in the 'trigger' and 'effector' arms of the stimulus (see Fig 1.8.).

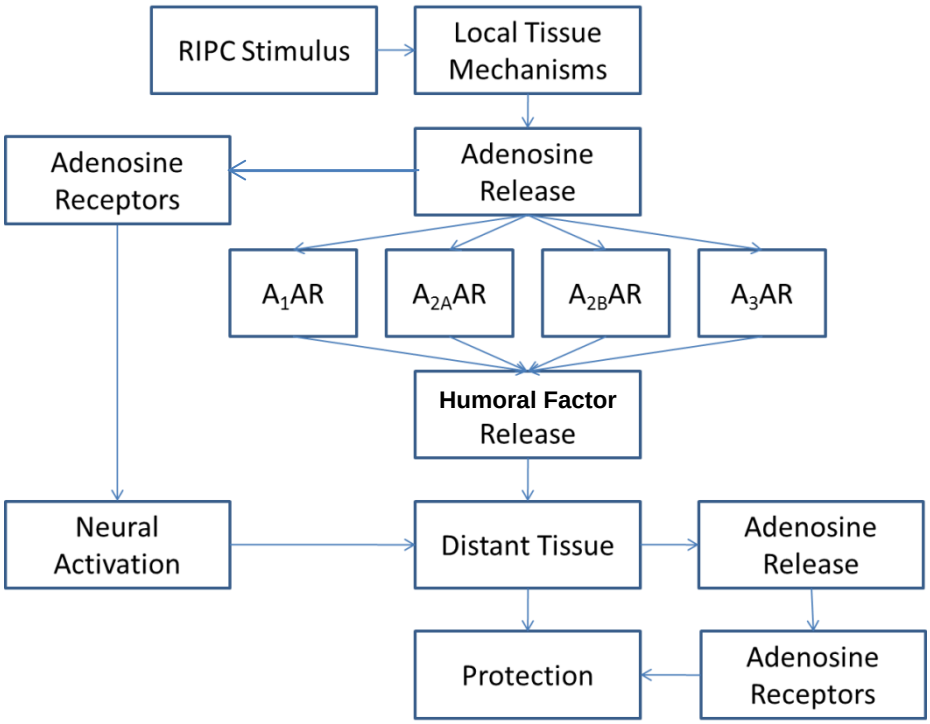


Figure 1.8 Schematic of the possible roles of adenosine in rIPC. Adenosine release may be crucial in both the activation of the stimulus ('trigger' stage) and then later in the distant target of the stimulus where cell surface adenosine-receptor activation appears to be important in the switch to a protected phenotype ('effector' stage).

1.7.6 Adenosine as a Cardioprotective Agent - Pre-Clinical Studies

Whilst adenosine has consistently and reproducibly shown benefit as a pharmacological preconditioning agent and has also been demonstrated to be involved in the intrinsic physiological mechanisms of ischemic preconditioning, its efficacy when delivered peri-reperfusion is less certain. Pre-clinical models have been used to investigate this important, clinically relevant question but have produced mixed results. In early work by Olafsson *et al[162]*, using a canine infarct model, a 60 minute infusion of intracoronary adenosine at reperfusion had marked salutary effects, including

significant improvements in myocardial fractional shortening and a reduction in infarct size.

Despite this initial promise, similar results have been difficult to reproduce and despite working in a similar canine infarct model, a subsequent study was unable to show benefit from adenosine infusion at reperfusion although on this occasion the adenosine was infused intravenously peripherally[163]. Similarly, a third canine study failed to show a reduction in infarct size with intracoronary adenosine unless it was co-administered with lidocaine[164] and a subsequent rabbit study was unable to reproduce even this, demonstrating no benefit from adenosine either alone or in conjunction with lignocaine[165]. However, later work by Budde *et al* in a similar canine model was once again able to demonstrate a reduction in infarct size with adenosine though only if the infusion was given episodically or for less than 2 hours post infarction[166].

Despite these inconsistent pre-clinical results for adenosine administered at the time of reperfusion, translational clinical studies and large scale outcome trials have been undertaken in human myocardial infarction and these are discussed in further detail below.

1.7.7 Adenosine as a Cardioprotective agent - Clinical Trials in Man

Several clinical trials have examined the role of adenosine in the treatment of acute ST segment elevation myocardial infarction and these are listed in Table 1.3 below.

Study	No. of Patients	Study Type	Reperfusion Strategy	Dose of Adenosine	Infarct Size (SPECT)	LV Function	Reference
Garratt *et al*	82	Historical Controls	PCI	70µg/kg/min IV for 60 mins	Reduced		[167]
Claeys *et al*	279	Historical Controls	PCI	60 or 90 µg/kg/min IC for 20 mins	Reduced (ECG)		[168]
AMISTAD I	236	Prospective Randomised	Lytics	70µg/kg/min IV for 3 hours	Decreased (anterior)		[169]
Marzilli *et al*	56	Prospective Randomised	PCI	4mg IC	Decreased (CK)	Improved	[170]
ATTAC	608	Prospective Randomised	Lytics	10µg/kg/min IV for 6 hours		Unchanged	[171]
AMISTAD II	2118	Prospective Randomised	PCI or Lytics	50 or 70µg/kg/min IV for 3 hours	Decreased (70 dose)		[172]
Fokkema *et al*	448	Prospective Randomised	PCI	2 x 120µg IC	Unchanged (CK)		[173]
Desmet *et al*	112	Prospective Randomised	PCI	4mg IC	Unchanged (T_2CMR)	Unchanged	[174]

Table 1.3 Clinical trials of adenosine as a cardioprotective agent. Various doses and routes of administration have been utilised with varying results.

By far the largest of these studies was the AMISTAD II trial which reported in 2005[172]. In 2,118 patients presenting with acute anterior myocardial infarction adenosine was

infused intravenously either immediately prior to or immediately after reperfusion therapy which could be by thrombolysis or PCI. Although there was a significant reduction in median infarct size with high dose adenosine (70 μg/kg/min) this failed to translate into significant clinical benefit with no improvement in the primary end-point of new congestive heart failure >24 hours after randomisation, re-hospitalisation with heart failure or all cause death within 6 months. Despite its large size, the reasons for this disappointment may be due to underpowering with the trial designed on a sample size calculation based on a reduction of events in the pooled adenosine group of 25%. The reduction observed was only 11% [172]. Despite this relative disappointment, interest in adenosine as a cardioprotective agent has remained current. At this time however there is little consensus on the optimal timing, route or dose of adenosine to trigger cardioprotection and further work is needed to explore these important issues.

In particular, recent observations in animal models suggesting that the intra-arterial rather than intravenous delivery of adenosine is critical in determining whether cytoprotective mechanisms are triggered [106, 107], may be a further possible explanation for the lack of consensus generated by the results of clinical trials to date which would achieve varying arterial concentrations with their different delivery protocols. In this work, I focus on the intrinsic role of adenosine in remote preconditioning pathways and also explore the observation that intra-arterial adenosine administration may be important in triggering preconditioning effects.

1.8 Models of IR injury

In order to study the effects of preconditioning and IR injury, a number of model systems have been developed including isolated cardiomyocytes, isolated perfused animal hearts, whole animal infarct models, models utilising human tissue including the isolated atrial trabeculae model and finally experimental models using whole human volunteers. As with all model systems, each has its advantages and limitations and results should be contextualised in light of these issues. Although the models used in this thesis will be more fully described later in the methods section it is useful to outline them here.

1.8.1 Human Endothelial Forearm Model

The endothelium is a monolayer of cells that lines the luminal surface of blood vessels and plays a key role in regulating vascular tone. In response to a variety of stimuli including increased flow [175] and endothelial dependent vasodilators such as acetylcholine, the endothelium is able to produce locally active mediators such as nitric oxide which act on the surrounding smooth muscle and induce dilation. This phenomenon is well described and can be measured by a variety of means including ultrasound, magnetic resonance imaging and invasive plethysmography. As this is an active, energy requiring biological process, this phenomenon can be used to investigate ischaemia-reperfusion injury in humans in a safe and non-injurious manner. First

described by Kharbanda *et al* [176], 20 minutes of ischaemia (usually achieved by inflating a proximal pressure cuff to supra-systolic pressures) followed by 15 minutes of reperfusion has been repeatedly demonstrated to reduce endothelial-dependent vasodilation acting as a readout for IR injury [64, 65, 103]. The magnitude of the reduction is large and is modifiable by preconditioning stimuli [103, 176] providing a pertinent model of human ischaemia-reperfusion injury and preconditioning responses. While the human forearm model has now been widely used to investigate a number of preconditioning stimuli it should be noted that endothelial responses and mechanisms of preconditioning may not precisely translate to human myocardial mechanisms of IR injury and cytoprotection. Secondly, it should be noted that the IR injury induced by 20 minutes of ischaemia appears to be fully reversible with spontaneous resolution of injury within 60 minutes of reperfusion [176]. This implies that the model is actually one of 'endothelial stunning' rather than one of necrosis/apoptosis as occurs in true IR syndromes. Finally, due to the heterogeneity of study volunteers there can be large inter-individual variations in endothelial responses, however as the model generally involves assessing the same parameter in the same individual on the same day before and after ischaemia-reperfusion injury, studies can still be sufficiently powered with relatively small numbers of individuals due to the paired nature of the subsequent analysis. Despite these limitations, the model provides a valuable testing ground for preconditioning stimuli in humans allowing relevant conclusions to be drawn, the translation of pre-clinical laboratory data to humans and the ability to study mechanisms of IR and preconditioning in humans.

1.8.2 Porcine Infarct model

While rodent models have been widely adopted throughout the scientific community due to their rapid breeding cycles, ease of housing, general low cost and the ability to generate transgenic animals, and have undoubtedly been instrumental in elucidating key biological and physiological processes in a living organism, questions remain over the translational applicability of these models to the more complex physiology of humans [177-179]. A number of large mammal models have therefore been developed to investigate cardiovascular physiology in a potentially more representative fashion including canine and porcine models. Porcine hearts have a similar coronary distribution to humans with a low coronary collateral circulation [180] and a pattern of infarction and repair of the myocardium that is almost identical to humans [181]. Furthermore like humans their blood vessels have vasa vasorums, their cardiac conduction systems are similar and they demonstrate similar responses to a wide variety of cardiovascular drugs suggesting their receptor distributions are alike [180]. In general their comparable size, anatomy and cardiovascular physiology makes cardiovascular investigation in pigs a good translational model and they have been widely utilised in previous cardiovascular studies. For studies of cardiac ischaemia-reperfusion injury two general models have been described. By performing a median or lateral thoracotomy, the coronary circulation can be revealed and a snare used to occlude the arterial supply (usually the LAD) to induce ischaemia. Disadvantages of this model include the stress response induced by surgery and the physiological compromise of having an open chest and divided pericardium. Alternatively, arterial

occlusion can be achieved by means of a standard angioplasty balloon delivered percutaneously by the femoral or carotid route. Whilst potentially more physiological, the delivery of angioplasty balloons can be technically demanding and there is no access to cardiac tissue during the procedure should sampling be required. Nevertheless, the porcine model of IR is a valuable tool to directly study cardiac preconditioning mechanisms and has been widely utilised in studies of cytoprotection [59, 76, 182].

1.8.3 Mouse Langendorf Model

Isolated perfused heart models have been used to investigate cardiac physiology for over a hundred years now. The ability to isolate a beating heart which can maintain a steady state of isovolumetric pressure generation for several hours is valuable in separating effects on heart physiology from those induced by systemic effects of interventions and is a convenient and rapid model in which to test the cardiac specific pharmacology of substances. Relying on retrograde perfusion of the coronary arteries through an aortic cannula, a ventricular balloon is also inserted to measure ventricular pressures and record heart rate and allows the derivation of parameters such as LVDeP and dp/dT. Ischaemia may be regional (by use of a ligature around the LAD) or more commonly global, achieved by stopping flow though the aortic cannula. After reperfusion, hearts are frozen, sliced and then stained with 1% 2,3,5-Triphenyltetrazolium chloride (TTC). The colourless TTC is enzymatically reduced to a

red formazan dye by dehydrogenases which are most abundant in intact mitochondria[183], staining viable tissue brick red and leaving infarcted tissues pale. Computerised planimetry then allows delineation of infarcted from non-infarcted areas and is corrected for heart weight slice allowing an estimation of infarct size as a % of heart mass. A similar technique is used in the porcine model with the addition of an additional step with Evans blue dye used to delineate the area at risk first. Despite the obvious limitations of investigating cardiovascular physiology in an isolated heart, the Langendorf model remains widely utilised in cardiovascular science and is a useful tool in assessing cardioprotective interventions.

1.9 Hypothesis and Aims

The literature summarised in the sections above has described how preconditioning is a powerful and clinically applicable phenomenon in the reduction of harm from ischaemia-reperfusion syndromes and demonstrated remote preconditioning in particular may be an attractive and useful stimulus to induce cytoprotection. Pivotal in the mechanism of rIPC appears to be the purine nucleoside adenosine (the so-called 'retaliatory' metabolite) with adenosine receptor activation either by adenosine or specific adenosine receptor agonists able to trigger rIPC-like effects. Evidence from animal models suggests that adenosine receptor activation may also be a crucial in the effector or target organ where blockade of adenosine receptors can abrogate the protection of rIPC. Furthermore, growing data suggests that the arterial delivery of adenosine as opposed to the intravenous delivery may be important in recapitulating the effects of rIPC and provides a possible confounder to explain the disparate findings in trials of adenosine cardioprotection.

The pertinence of these mechanisms to human rIPC are unknown and the overarching hypothesis of this thesis therefore is that adenosine is a key mediator in the endogenous pathway of human remote ischaemic preconditioning and that the intra-arterial delivery of adenosine can re-capitulate preconditioning-like effects. The key aims explored in the following studies are :–

1. Whether blockade of adenosine abrogates the protective effects of rIPC in human models of rIPC

2. To delineate where in the signalling cascade adenosine receptor activation is important (i.e. 'trigger' or 'effector' or both)

3. To discover whether adenosine infusion can act as a cardioprotective stimulus in a peri-infarct model of myocardial ischaemia – i.e. can adenosine mimic perconditioning

4. To ascertain whether infusion of adenosine releases a cardioprotective factor into the circulation

These aims are described and approached in the following structure:-

Chapter 3

i. Does infusion of an adenosine receptor antagonist block the protective effect of rIPC in humans

Chapter 4

i. Does blockade of adenosine in the 'trigger' pathway inhibit rIPC in humans and what affect does this have on the circulating humoral factor
ii. Does blockade of adenosine in the 'effector' pathway inhibit rIPC in humans and what affect does this have on the circulating humoral factor

Chapter 5

i. Does infusion of adenosine after the onset of ischaemia and prior to reperfusion afford cardioprotective effects

Chapter 6

i. Does infusion of adenosine in the human arterial system induce the release of a circulating cardioprotective factor

CHAPTER 2: MATERIALS AND METHODS

2.1 Introduction

Very few models exist to study ischaemia-reperfusion injury in intact humans under experimental conditions. While human myocardial models of ischaemia-reperfusion have been described, they are extremely challenging to utilise, and have not become widely established [184, 185]. A surrogate model of ischaemia-reperfusion that has gained wide acceptance is the human forearm model. First described by Kharbanda *et al* in 2001 [176] it has gone on to be used by multiple other groups who have validated its initial findings and have used it to explore a variety of preconditioning stimuli [64, 65, 103]. Utilising a pressure cuff to induce a twenty minute period of ischaemia in the forearm and then measuring endothelial function as the primary end-point, the protocol is safe, efficient and well-tolerated by subjects. Endothelial function can be assessed in a number of ways and in my studies I utilised flow-mediated dilation and venous occlusion plethysmography. Also described here are the animal models utilised to explore further aspects of rIPC

2.2 Human Vascular Preconditioning Models

All procedures were carried out in the dedicated vascular laboratory, a quiet, temperature controlled room maintained at 22-24°C. The vast majority of studies were carried out in the morning with a very small number of individuals being studied later in the day due to scheduling issues. In protocols where subjects returned for further studies after a period of time had elapsed, care was taken to schedule an appointment at the same time of day as the original study. All participants were asked to abstain from caffeine containing drinks for a minimum of 24 hours, alcohol for 24 hours and from food for at least 4 hours before participation in each study session.

2.2.1 Assessment of Flow-mediated Dilation

Flow-mediated dilation (FMD) was assessed according to guidelines from the International Brachial Artery Reactivity Task Force [186]. Test subjects were asked to lie recumbent on an imaging couch for a period of 10 minutes prior to the start of each study. The brachial artery was located by palpation and then imaged longitudinally with high resolution B-mode ultrasound approximately 5-10cm above the antecubital fossa. A 7-10Hz linear array transducer probe was used for all studies along with a Philips Sonos 5500 (Philips Medical Systems, Bothell, Washington). The forearm was stabilised in a pre-formed support channel and once an adequate image was obtained allowing clear identification of vascular boundaries, the probe was fixed in position with a

stereotactic clamp (see Fig 2.1). The vessel was imaged for 1 minute to record baseline diameter. All images were recorded to magnetic tape for later digitisation and offline analysis. A 7.5cm pneumatic cuff (Hokanson Inc. Bellevue, WA, USA) placed around the forearm, immediately distal to the elbow was inflated to 200mmHg for 5 minutes. On cuff release, recording was recommenced and the artery imaged for a further 5 minutes. At the end of this period, a second 1 minute baseline was recorded and then the subject given 25mcg of sublingual GTN. Imaging was continued for a further 3 minutes to assess nitrate-mediated (endothelium independent) dilation (NMD). All scans were digitised using Vascular Converter (Medical Imaging Applications LLC, Coralville, Iowa) at an image density of 2 frames-per-second.



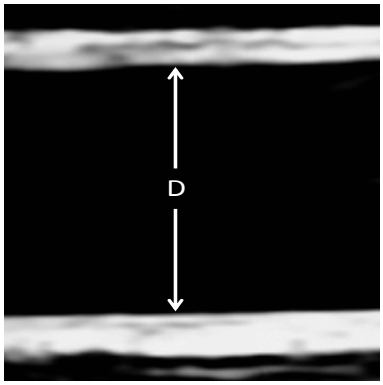


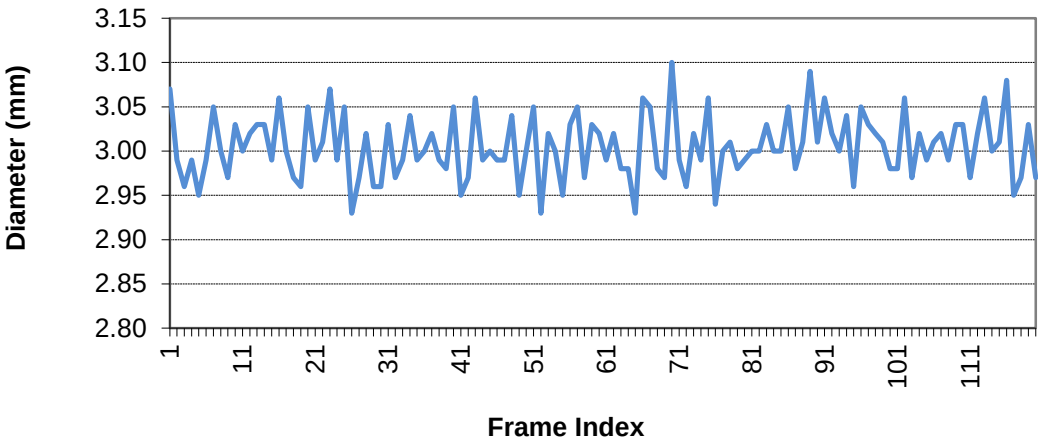
Figure 2.1 Ultrasound acquisition of FMD. The brachial artery is imaged proximal to a lower arm cuff. After a five minute inflation the cuff is released causing reactive hyperaemia and flow-mediated dilation of the proximal artery. The change in diameter (D) from baseline of the artery is used to calculate the peak percentage FMD. The top image demonstrates the typical set-up with the ultrasound probe fixed with a stereotactic clamp and the upper and lower pneumatic arm cuffs utilised for inducing ischaemia (see section 2.1.2). The bottom image demonstrates a typical acquired brachial artery image.

During the digitisation step, scans were divided into four distinct periods (baseline, cuff down, GTN baseline and GTN) according to pre-registered time points and each file saved and analysed separately in order to minimise movement artefacts and increase overall scan fidelity. Saved files were subsequently analysed using the automatic B-mode edge detection software Brachial Analyser for Research v 4.0.6 (Medical Imaging Applications LLC, Coralville, Iowa) with computer analytical errors corrected manually on a frame by frame basis (see Fig 2.2). End-diastolic values were used to mitigate the potential influence of vascular compliance on diameter and FMD calculated as the proportional change in brachial artery diameter in response to hyperaemic shear stress corrected for resting vessel diameter using the formula

$$\% \text{ FMD} = \frac{(\mathrm{B}\beta - \mathrm{B}\alpha)}{\mathrm{B}\alpha * 100}$$

where Bα = baseline brachial artery diameter and Bβ = peak brachial diameter in response to hyperaemia following cuff deflation. NMD was calculated in the same way, simply substituting the appropriate values.

(A)



(B)

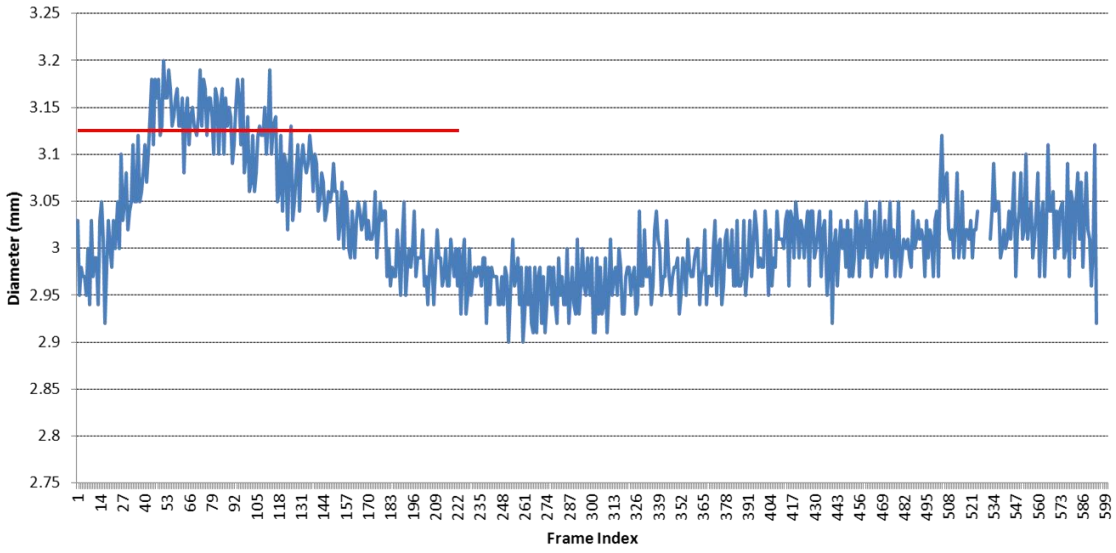


Figure 2.2 Representative traces of a brachial artery FMD scan (A) baseline diameter and (B) following release of the distal cuff. The processed graph demonstrates the characteristic peak in diameter seen after the release of the distal cuff with the

maximum change from baseline representing the FMD. With off-line processing the scan can be divided into separate segments reducing problems with movement artefact. The x-axis is labelled frame index rather than time as the images are digitised at rate of 2 frames per second to attempt to achieve high data fidelity. Therefore, simply dividing the frame index by 2 will give time in seconds.

2.2.2 Induction of IR

In protocols inducing IR injury, a 12 cm pneumatic cuff (Hokanson Inc. Bellevue, WA, USA) was placed around the upper arm just distal to the shoulder joint and inflated to a pressure of 200mmHg for a period of 20 minutes. Ischaemia was confirmed by loss of radial artery pulsation and increasing pallor of the distal limb. At 20 minutes the cuff was deflated and reperfusion commenced with immediate hyperaemia apparent confirming the return of arterial in-flow to the limb. Reperfusion continued for a period of 15 minutes during which the subject rested supine, prior to re-assessment of endothelial function.

2.2.3 Remote Ischaemic Preconditioning

In protocols studying rIPC, rIPC was induced by a 12 cm pneumatic cuff (Hokanson Inc. Bellevue, WA, USA) placed around the contralateral upper arm (to that undergoing subsequent ischaemia-reperfusion) just distal to the shoulder joint and inflated to a pressure of 200mmHg for a period of 5 minutes. This was followed by 5 minutes of

reperfusion and a total of three cycles of ischaemia-reperfusion were used in each preconditioning phase.

2.2.4 Venous Occlusion Plethysmography

In studies where venous occlusion plethysmography was utilised, procedures were carried out in the vascular laboratory as previously and individuals were subject to the same conditions as described above.

Subjects were studied lying recumbent with the hands elevated above the level of the heart by means of bolsters. 10 cm pneumatic cuffs were placed around the upper arms bilaterally and 7.5cm cuffs around both wrists. The circumference of each forearm was measured approximately 5 cm below the ante-cubital fossa and appropriately sized mercury-in-silastic strain gauges positioned. In protocols where drugs were infused according to forearm volume this was estimated as being the volume of a truncated cone using the formula

$$V = \tfrac{1}{3}\pi(R_1^2+R_1*R_2^2+R_2)h$$

where V=volume, R_1 = radius of the base, R_2 = radius of the top and h = height.

For each study, the brachial artery of the non-dominant forearm was localised by manual palpation and then cannulated under local anaesthesia (3 ml of 1% lignocaine) with a 27-gauge needle (Cooper's Needle Works, Birmingham, UK) attached to an infusion line (see section 2.1.5) under standard aseptic technique. Drugs or normal saline (sodium chloride 0.9% wt/vol) were infused continuously during the remainder of the study to insure line patency at 1 ml/min. After cannulation, the subject was allowed to rest for 10 minutes prior to initiating recordings. Each recording period lasted 12 minutes during which the subject was asked to remain quiet and still. During recording, the hands were excluded from the circulation by inflation of wrist cuffs to 200 mmHg (see Fig. 2.3). The proximal arm cuffs were set to automatically inflate and deflate to a pressure of 40mmHg with a period of 9 seconds of inflation followed by 6 seconds of deflation in a total cycle time of 15 seconds.



Figure 2.3 Typical venous occlusion plethysmography set-up. With the subject lying recumbent, the hands are raised above the level of the heart to aid venous return. The hands are excluded from the arterial supply by means of a pneumatic wrist cuff inflated to supra-systolic pressures for the period of each 12 minute recording. Around the upper arm is a second pneumatic cuff, set to automatically inflate and deflate to 40mmHg to cyclically restrict venous outflow from the arm. Also visible is the mercury-in-silastic strain gauge around the forearm and the brachial needle in the antecubital fossa.

In all studies, responses to both acetylcholine (Ach) in incremental doses of 25, 50 and 100 nmol/min, and glyceryltrinitrate (GTN) in incremental doses of 4, 8 and 16 nmol/min were assessed. Each dose was given for three minutes with a three minute baseline recording with saline making up the whole recording period, and a 10 minute washout period was allowed between drugs, during which all cuffs were deflated. In studies in which drugs were co-infused, all drugs were made up in double concentrations and then mixed with an equal volume of the co-infused drug to achieve the final desired concentrations. All recordings and analysis were made using LabChart v.6 (AD Instruments, Chalgrove, UK). Software was used to calculate the mean slope of

the final three inflation and deflation cycles at each drug infusion dose along with the contemporaneous slope of the non-infused arm which acted as a real time control (see Fig 2.4).

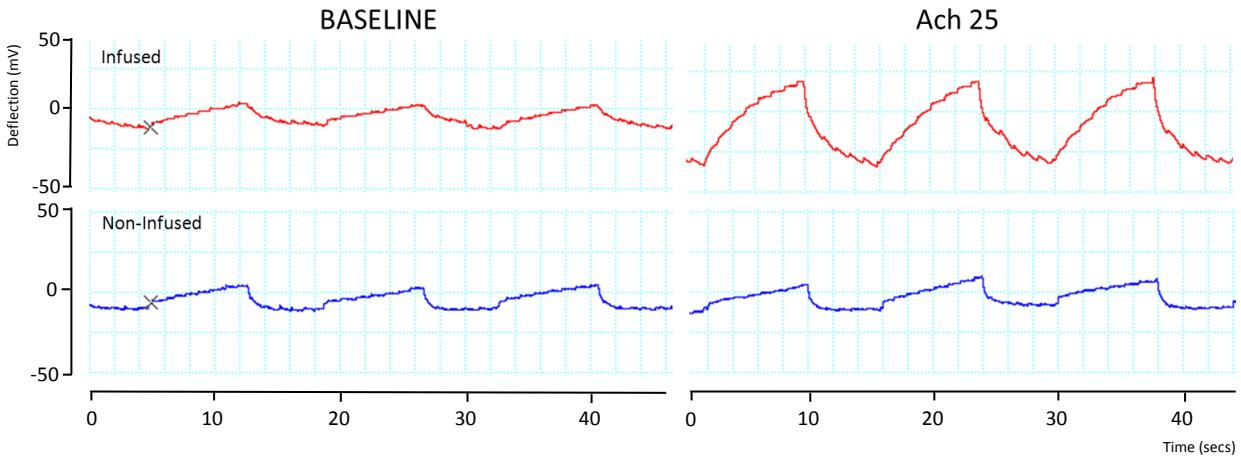


Figure 2.4 Typical venous occlusion plethysmography trace. When the upper arm cuff is inflated, venous outflow is obstructed while arterial in-flow continues. Hence, forearm volume increases proportional to forearm blood flow and is measured by the mercury in-silastic strain gauges (see above). Forearm blood flow responses increase in response to intrarterial infusion of the vasodilator acetylcholine (Ach 25) while contemporaneous blood flow in the non-infused arm remains at baseline.

Percentage increase in forearm blood flow was calculated using the formula

$$\% \text{ increase in FBF} = \frac{(\bar{x}\,F\gamma \,/\, \bar{x}\,F\delta) - (\bar{x}\,F\alpha \,/\, \bar{x}\,F\beta)}{(\bar{x}\,F\alpha \,/\, \bar{x}\,F\beta) * 100}$$

where Fα = baseline slope of infused arm, Fβ = baseline slope of non-infused arm, Fγ = slope of infused arm and Fδ = slope of non-infused arm.

2.2.5 Brachial Needle

No commercially available brachial needles with infusion lines are available. A new needle was constructed for each study under aseptic technique. A radiation sterilised 27-gauge blade without a hub (Cooper's Needle Works, Birmingham, UK), was sealed to the cut end of a 16G epidural catheter using heated sticky dental wax. After drying the catheter was flushed with saline to remove air and test for leaks before being inserted as above (see Fig 2.5).

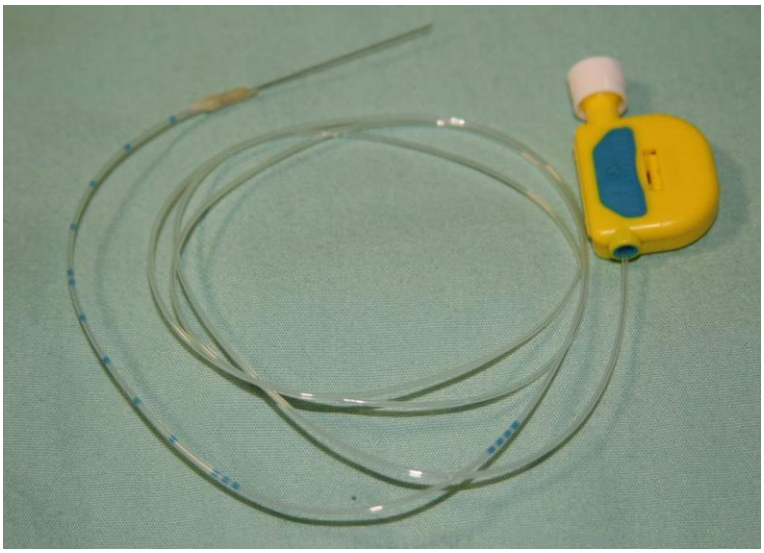


Figure 2.5 Brachial needles were constructed by sealing a radiation sterilised needle blade to the cut end of an epidural catheter.

2.3 Porcine Model of Ischaemia-Reperfusion

Due to the lack of availability of a large animal facility locally, this work was conducted by myself at the University Hospital Skejby, Aarhus, Denmark. All studies were conducted on healthy 30kg mixed Danish Landrace and Yorkshire pigs. Animals were fasted overnight prior to study and were preanesthetized with midazolam (0.2mg/kg) (Dormicum®,Roche, Basel, CH) and azaperone (7mg/kg) (Stresnil®, Janssen, Birkerod, DK) by subcutaneous injection. An ear vein was cannulated and animals induced with intravenous etomidate (0.4mg/kg) (Hypnomidate®, Janssen, Birkerod, DK) the trachea intubated and the animals subsequently ventilated at 4.5 L/min with a 50/50 mixture of atmospheric air and oxygen maintaining a PEEP of 3.5 cmH_2O and a tidal volume of 400ml. Anaesthesia was maintained with intravenous pentobarbital (6mg/kg/hr) (Mebumal®, Amgros I/S, Copenhagen, DK).

By means of a cut-down procedure the jugular vein and carotid arteries were identified and cannulated with 6Fr introducer sheaths using a standard Seldinger technique. A median sternotomy was performed, the pericardial sack divided and the heart exposed. A snare was placed around the LAD just distal to the first diagonal artery but not tightened at this stage. CMA 20 microdialysis catheters (CMA, Solna, SE) with a molecular cut off of 20000 Da were placed using an introducer cannula (0.45 mm × 12 mm) and fixed with tissue glue in 5 areas – the right atrium, the left ventricular free wall

in the non-risk area, 2 probes were placed in the area at risk of the LV and a fifth probe placed in the muscle of the hind limb.

Microdialysis catheters were continuously infused with a Krebs-Henseleit buffer (NaCl 118.5; KCl 4.7; $NaHCO_3$ 25.0; glucose monohydrate 11.1; $MgSO4{\cdot}7H_2O$ 1.2; $CaCl_2$ 2.4; KH_2PO_4 1.2 mmol/L) using a Univentor 801 syringe pump (Univentor, Zejtun, MT) set at a continuous flow rate of 1µL/minute. The buffer was deoxygenated before use with 95%N_2 and %CO_2 to avoid any confounding issues associated with the delivery of an O_2 saturated solution to the myocardium. Outlet tubing (internal volume 1.2 µL/10cm) length was minimized and was connected to a Univentor 820 fraction collector (Univentor, Zejtun, MT) with collection cycle times adjusted through the protocol to focus on periods of interest. Vials were cooled within the fraction collector to 4˚C, then removed and immediately frozen on dry ice and stored at -80˚C until analysis (see Fig. 2.6 and 2.7).

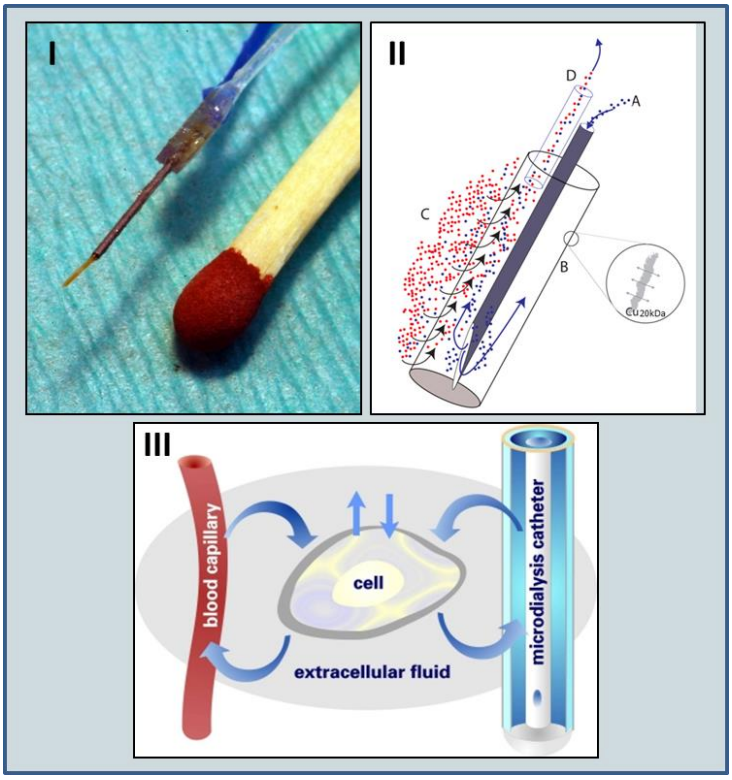


Figure 2.6 CMA 20 microdialysis probe with match head for comparison (I). The probe head is inserted into the tissue of interest using the Seldinger technique and stabilised with tissue glue. The probe is continually perfused with a modified KHB solution at 7µl/min which had been gassed with 95%N_2 and 5%CO_2 to achieve a low 0_2 concentration. The interstitial:luminal gradient drives compounds into the KHB which is then collected by an automated fraction collector (II and III).

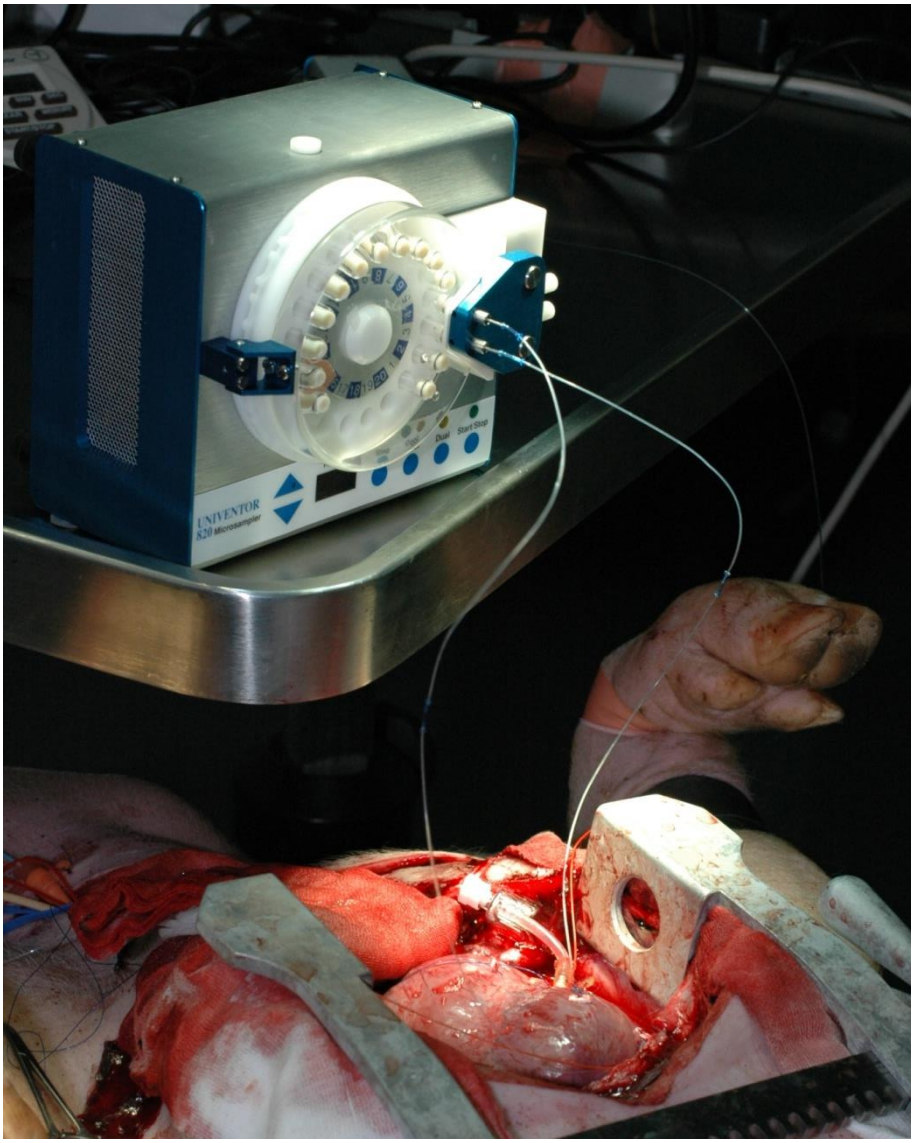


Figure 2.7 Microdialysis fraction collector and microdialysis probes in the myocardium. KH buffer solution is infused through the microdialysis probes then returns to the rotary collector. At pre-specified time points the collector automatically switches to the next fraction vial allowing a time course to be constructed for metabolic fluctuations in the myocardium. The collector automatically cools all fractions to 4˚C which are then stored at -80˚C until analysis.

A 5000u loading bolus of heparin (Leo Pharma, Copenhagen, DK) was administered following completion of surgical procedures and a further 2000u of heparin were given hourly during the study. Core temperature was carefully maintained between 36.5 and 38˚C with the use of heating blankets. Hydration was maintained by saline infusion at

200ml/hr. Hourly blood gas analysis allowed alterations in ventilation and electrolytes to maintain parameters within physiological norms. After a 60 minute stabilisation period the LAD snare was tightened and a 40 minute ischaemia period commenced. Ischaemia was confirmed by visual inspection of the LV wall which rapidly appeared cyanosed (see Fig. 2.8). Five minutes prior to the end of ischaemia a standard 6Fr pigtail catheter was passed into the distal descending aorta and a bolus of 1.5mg/kg of adenosine (Astellas, Deerfield, IL, USA) or 10ml of saline (placebo) was administered. At the completion of 40 minutes of ischaemia, the snare was loosened and reperfusion commenced. During ischaemia and reperfusion episodes of VF or haemodynamically unstable VT were treated with DC cardioversion but no antiarrhythmic drugs or inotropes were utilised with only fluid supplementation allowed to maintain blood pressure if necessary. Reperfusion was for 120 minutes after which the snare was retightened around the LAD and the heart perfusion stained with 10% Evans Blue (Sigma-Aldrich, Brondby, DK) by injecting 30ml of solution into the left atrial appendage. The animal was euthanized and the heart excised. The right atrium and ventricles were removed and the heart placed in a -80˚C freezer for approximately thirty minutes.

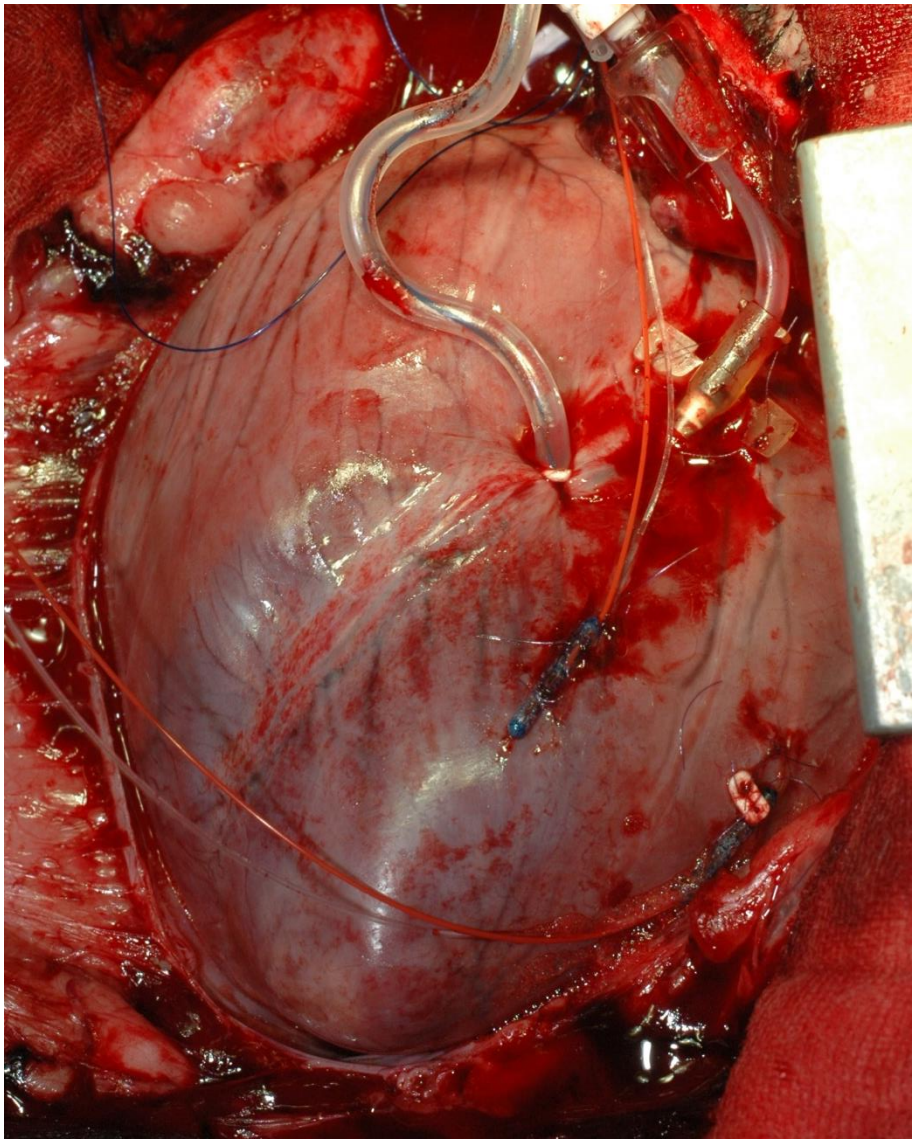


Figure 2.8 Typical myocardial ischaemia-reperfusion set-up. A snare is tightened around the LAD artery causing anterior wall myocardial ischaemia and infarction, clearly visible as the area of pallor in the image above. Also demonstrated are microdialysis catheters in the area-at risk and the non-risk area.

The left ventricle was then sliced into 8 mm slices from the apex to the base perpendicular to the septum and slices incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) (pH 7.4) (Sigma-Aldrich, Brondby, DK) at 37°C for 15 minutes. All slices were then scanned on a flat-bed digital scanner and weighed. Computerised planimetry (Image Pro Analyser v7.0, Bethseda, MD, USA) was used to assess area at

risk, area not at risk and infarct size allowing calculation of final infarct size as a percentage of mass of area at risk (see Fig. 2.9). Using the formula

$$\text{Total IS as \% AAR} = \sum_{k=1}^{n} \frac{M(n)}{AAR(n)} * \frac{IS(1)}{AAR(1)}$$

where n=number of slices, k=slice number, M(n) = total mass of all slices, AAR(n) = total area at risk, IS(1) = infarct size of slice, AAR (1) = area at risk on slice.



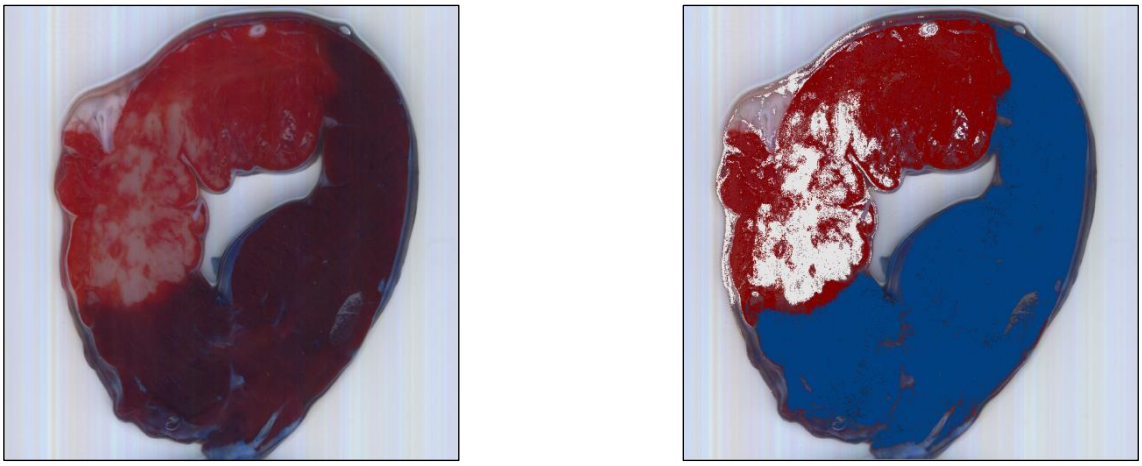


Figure 2.9 Typical left ventricular slices pre and post processing. The left ventricle has been sliced into 8 mm sections perpendicular with the apex after being perfusion stained *in-situ* by Evans blue dye to delineate the area not at risk of infarction (upper image). After incubation in TTC this area stains a dark red-brown (left lower image) while viable tissue in the area at risk stains red. Infarcted tissue remains pale. In the right-hand lower image the slice has been processed by computerised planimetry and the various size regions delineated by pixel counts. As the weight of the slice is known, the mass of each area can be calculated and final infarct size expressed as a percentage of area at risk.

2.4 Analysis of Microdialysis Samples

Samples were analysed in the Department of Forensic Medicine, Aarhus University by Dr R Birkler, using a newly developed protocol for the profiling of intermediary metabolites in microdialysis samples [190]. Given the low molecular weight of samples no pretreatment or derivatization was necessary. Samples were thawed and 15µL of deuterated internal standard added to each 10 µL sample. After vortex mixing and centrifugation, samples were placed into a 96 well Waters 2 mL Square Collection Plate that could be directly used in the mass spectrophotometric analyser. An ultra-performance liquid chromatography/electronspray-tandem mass spectrometry (UPLC-ESI-MS/MS) method was used using a Waters Xevo triple quadropole tandem mass

spectrometer (Waters Corp., Manchester, UK). The instrument's Z-spray electro-ionisation source was set to operate in both positive and negative ion mode. All analytes were verified using two daughter ions when possible. Analysis results were integrated using the software package MassLynx v4.1 (Waters Corp., Manchester, UK) before export to Microsoft Excel and correction for relative recovery rates. Relative recovery had been previously established from *in-vitro* experiments, with an inverse non-linear relation existing between flow rates and concentration of recovered metabolites.

2.5 Manufacture of Preconditioning Dialysate

Despite the continuing difficulties in identifying the circulating preconditioning factor(s), it can be assayed for from its ability to limit infarct size in models of ischaemia-reperfusion. In order to achieve a convenient format for the testing of activity it can be simply and easily dialysed out of plasma and into a KH solution. By taking blood before and after a preconditioning stimulus, samples of dialysate can be compared in a paired fashion and the presence or absence of the humoral preconditioning agent ascertained.

Heparinised whole blood (5u/ml) was taken and transported on ice for immediate centrifugation. Blood was centrifuged at 4640g and the plasma fraction pipetted off. A 15cm length of 15kDa dialysis membrane which had been washed overnight in RO water was taken and one end clamped. Plasma volume was measured and the plasma

transferred into the dialysis membrane, the open end sealed with a second clamp. Twenty times the volume of plasma was measured of a modified KH buffer containing (all in mM/L) NaCl 118.5, KCL 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.19 and D-glucose 11.0 and the sealed dialysis membrane placed into this. The buffer solution was covered and transferred to a cold room (4˚C) with continuous stirring using a magnetic stirrer. After 48 hours the plasma and dialysis membrane were discarded and the dialysate solution decanted into bottles and stored at -80˚C until used (see Fig. 2.10). Before use $NaHCO_3$ 25 mM/L and $CaCl_2$ 2.4mM/L was added to the buffer and which was saturated 95% O_2 and 5%CO_2 as previous.

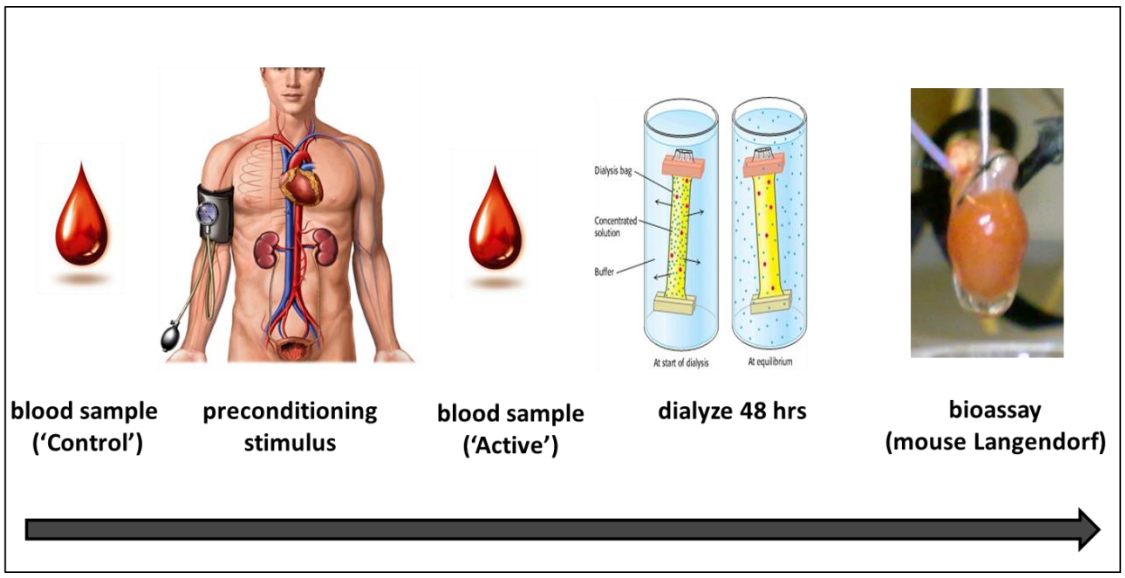


Figure 2.10 Method for making dialysate. Blood taken prior to the preconditioning stimulus is used to make 'control' dialysate. The stimulus is then applied and a repeat blood sample taken for 'active'dialysate. Blood is dialysed in 15kDa cut-off membrane for 48hrs against a modified KH buffer which is then used to perfuse a bioassay (mouse Langendorf) and assessed for its protection against ischaemia-reperfusion injury.

2.6 Mouse Langendorf Ischaemia-Reperfusion Model

Samples were analysed by Dr J Li at the Division of Cardiology, Hospital for Sick Children, Toronto. All studies were carried out on 25-30g C57BL/6 mice (8-10 weeks of age) who were housed under controlled conditions with a 12:12-hour light-dark cycle. Animals were fed standard chow and provided with food and water *ad libitum*. Mice were euthanized with an IP injection of pentobarbital (300mg/kg) also containing heparin (150 IU) and surgery commenced when pedal and corneal reflexes were no longer apparent. A transverse laparotomy just under the diaphragm and then a midline sternotomy were performed, the great vessels identified and the heart rapidly excised and placed into cold (4˚C) Krebs-Henseleit (see below) buffer acting as a cardioplegic solution.

The heart was quickly transferred to the Langendorf apparatus and the aorta cannulated under direct vision allowing retrograde perfusion of the heart to commence. The heart was secured to the perfusion cannula by circumferential ties around the aorta and the pulmonary arteries were ligated. The heart was perfused at a constant pressure of 80mmHg under non-recirculating conditions with a modified Krebs-Henseleit (KH) buffer containing (in mM/L) NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.75, KH_2PO_4 1.18, $MgSO_4$ 1.19, D-glucose 11.0, insulin 100 mU/l and $CaCl_2$ 2.4, at pH 7.4, saturated with 95% O_2 and 5% CO_2. All perfusate was passed through a 5-25 µm in-line filter before perfusing the heart. During the course of each study, hearts were kept immersed in a water-jacketed chamber containing KH buffer which was carefully maintained at 37^{o}C throughout the procedure (see Fig 2.11).

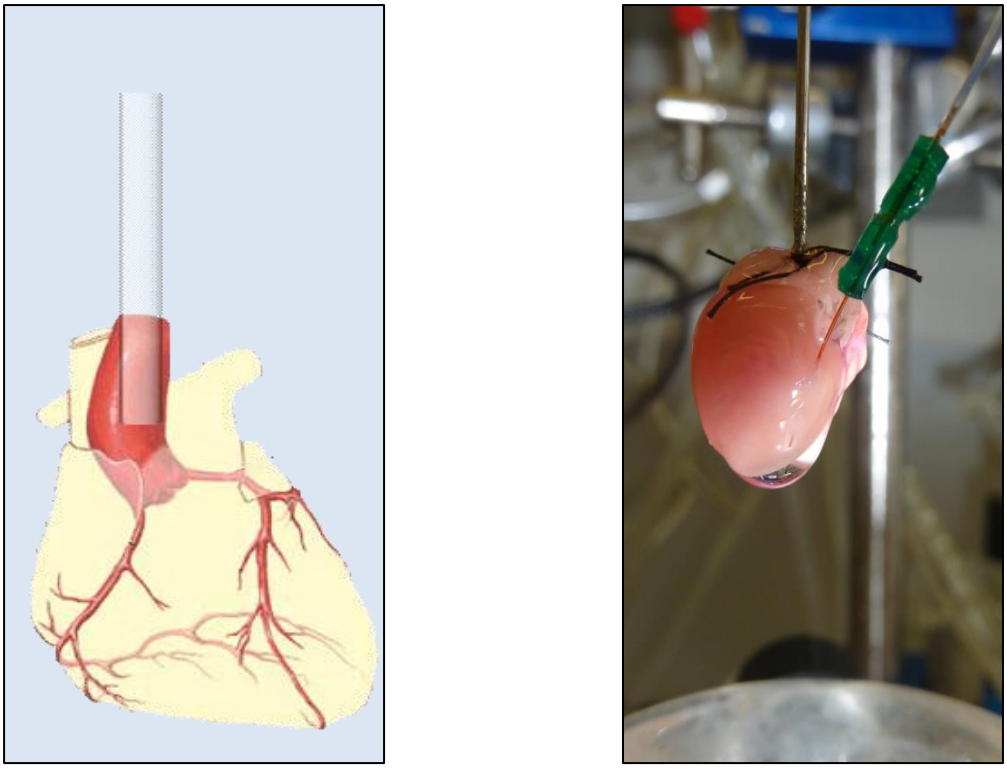


Figure 2.11 Mouse Langendorf heart set-up. Left-hand image demonstrates schematic of retrograde perfusion of coronary arteries through the aorta with right-hand image demonstrating typical study set-up.

The left atrium was excised and left ventricular (LV) pressure transduced through a fluid-filled balloon (made by attaching Saran wrap to a blunt ended cannula) inserted into the LV cavity and left ventricular end-diastolic pressure (LVEDP) was adjusted to between 5 and 10 mmHg. In all studies, after a baseline 20 minute stabilization period, the test dialysate was infused for 30 mins followed by a 5 mins wash-out period. Global no-flow ischaemia was established by suspending flow through the aortic cannula. Ischaemia was for 30 minutes followed by a 1 hour reperfusion period. Hearts were decannulated, covered in Saran wrap to avoid desiccation and placed in the -80˚C freezer overnight. Still frozen hearts were sliced into 1 mm sections using a mouse heart slicer (Zivic Instruments, Pittsburgh, PA, USA). Hearts were immersed in 1% TTC for 15 minutes as above, before fixing overnight in 10% neutral buffered formalin

scanning and weighing. Scanned images were processed using computerised planimetry as previously. As this was a model of global ischaemia the whole heart acts as the 'area at risk' thus simplifying calculations and infarct size is presented as a percentage of whole heart mass.

$$\text{Total IS as \% heart mass} = \sum_{k=1}^{n} \frac{IS(1)}{AAR(1)} * M(1)$$

where IS = infarct size of slice, AAR = total slice area, M = mass of slice.

2.7 Caffeine assay

Caffeine assays were carried out by technicians in the Dept. of Clinical Biochemistry, John Radcliffe Hospital. Briefly, heparinised blood samples collected using a standard vacutainer system were centrifuged for 5 mins at 4640g, the plasma fraction pipetted off and stored at -80˚C till use. A Syva™ EMIT spectrophotometric assay kit was used as per manufacturer's instructions utilizing a Thermo-Fisher Kone 30 analyser.

2.8 General Statistical Methods

All statistical analyses were carried out using SPSS v17.0 (IBM, Armonk, NY, USA) or GraphPad Prism v5.0 (GraphPad Software, La Jolla, CA, USA). Categorical variables were compared (where appropriate) using the chi-squared test. Continuous variables were tested for normality by the Kolmogorov-Smirnov and the Shapiro-Wilk tests.

Paired or unpaired (as appropriate) student's t-testing was used to compare two groups affected by one single variable. One-way analysis of variance (ANOVA) was used to compare more than two data groups and 2-way repeated measures ANOVA used to compare multiple data groups affected by more than 1 independent variable. When using ANOVA, if a significant difference was found, *post hoc* Bonferroni analysis correction was used to compare individual groups.

CHAPTER 3: ADENOSINE RECEPTOR ACTIVATION IN rIPC

3.1 Introduction

Despite numerous animal studies suggesting adenosine is an important mediator of endogenous preconditioning responses, there is sparse evidence to support this suggestion in humans and no evidence to support this in human rIPC. The objective of this study was therefore to test the hypothesis that human rIPC utilised adenosine signalling in its pathway, using the human forearm model of rIPC as a representative system.

3.2 Adenosine Receptors in rIPC Study

This study was a randomised double-blind placebo controlled study utilising the human forearm model of ischaemia-reperfusion injury in healthy volunteers. The study protocol was approved by Oxfordshire Research Ethics Committee A (ref 08/H0604/152).

3.2.1 Establishing the IR Model

In initial work it was necessary for me to establish and confirm the human forearm model of IR injury, using FMD as readout. Briefly, in five healthy volunteers the brachial artery was imaged longitudinally with high resolution B-mode ultrasound using a linear array transducer and Philips Sonos 5500 (Philips medical Systems, Bothell, Washington). After a 1 minute baseline recording period a pressure cuff was inflated on the distal forearm for five minutes to 200mmHg. At release, hyperaemia was induced causing flow-mediated dilation in the proximal vessel. The vessel was imaged for a further five minutes to ascertain the vessel's peak diameter and its return to baseline. Endothelium-independent dilation or nitrate mediated dilation (NMD) was induced by 25μg of sublingual GTN with the brachial artery re-imaged 3 minutes post administration. Following assessment of baseline FMD, IR was induced by proximal arm pressure cuff inflation to 200mmHg for 20 minutes followed by 15 minutes of reperfusion. FMD was then re-measured as described above (see Fig 3.1). The results of this initial study are presented below.



Figure 3.1 Schematic representation of study design for establishing IR model. After baseline measurement of FMD, the subject undergoes a twenty minute period of ischaemia (I) achieved by inflation of a proximal arm cuff to supra-systolic pressure. Following fifteen minutes of reperfusion (R) FMD is re-assessed.

3.2.2 Establishing rIPC in the IR model

In addition to establishing the IR model it was also necessary to demonstrate that rIPC was protective against endothelial IR injury. In eight additional healthy volunteers a similar protocol to that described above was undertaken with FMD/ NMD recorded in an identical manner. However, in this protocol the additional step of rIPC was undertaken, consisting of 3 cycles of five minutes of ischaemia interspersed with five minute periods of reperfusion on the contralateral arm to that in which FMD was being assessed and the standard IR protocol was undertaken. The protocol is demonstrated in Fig 3.2 below.



Figure 3.2 Schematic of study protocol for establishing rIPC protocol. Similar to the initial protocol for establishing IR injury, this protocol added rIPC on the contralateral arm to attempt to demonstrate the effects of this cytoprotective stimulus.

3.2.3 Pharmacological Blockade of Adenosine Receptors using Caffeine

Caffeine and other methylxanthines such as theophylline have a similar purine ring structure to that of adenosine (see Fig. 3.3) and are known to be effective inhibitors of the adenosine A_1AR, A_{2A}AR and A_{2B}AR, although the A_3AR is not significantly affected by them[191]. It has been demonstrated that infusion of caffeine at 4mg/kg provides effective steady-state blockade of these adenosine receptors without significantly

affecting cAMP levels (through phosphodiesterase inhibition) or intracellular calcium concentrations, in adults allowing investigation of the physiological effects of adenosine. Using a similar protocol, caffeine has already been used at this dose to investigate the role of adenosine in human IPC [2]. I elected to try and utilise this effect to attempt to block the protective effects of rIPC and thereby demonstrate whether rIPC was dependent on adenosine receptor activation.

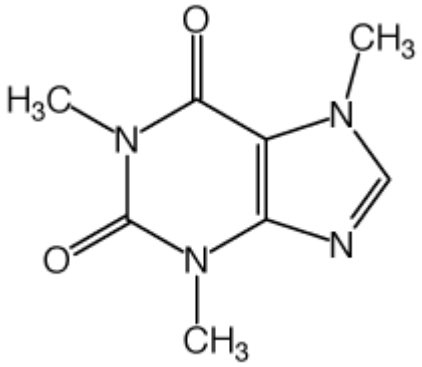


Figure 3.3 Molecular structure of caffeine. Adenosine and caffeine possess similar purine ring structures (see Fig. 1.4 above for structure of adenosine). This allows caffeine to antagonise adenosine binding to its receptors (from[2])

3.2.4 Design and subjects for main study cohort

Individuals who were non-smokers with no known chronic diseases, who were not currently using prescribed medications (except the oral contraceptive pill) and who had abstained from caffeine containing drinks for a minimum of 24 hours, alcohol for 24 hours and from food for at least 4 hours were recruited. All studies were carried out in a dedicated vascular laboratory, a quiet temperature controlled room maintained at 22-24°C. By means of a random number table, participants were allocated to placebo (5% dextrose vehicle only) or caffeine infusion (4mg/kg). The investigator and participants were blinded to group allocation and infusion solutions provided by a third party.

Baseline blood was taken to assess compliance with caffeine abstinence and then FMD and NMD were measured. Intravenous caffeine or matching placebo was infused over 10 minutes and a repeat blood test for caffeine levels was taken thirty minutes after the start of the infusion. A repeat FMD and NMD to assess caffeine's effect on FMD/NMD was then performed. This was followed by a rIPC protocol consisting of 3 cycles of five minutes of ischaemia interspersed with five minute periods of reperfusion of the contralateral arm followed immediately by 20 minutes of ischaemia/15 minutes of reperfusion in the imaged arm. Completing the protocol, a final FMD/NMD was undertaken to assess the effects on endothelial function of rIPC + IR (see Fig. 3.4). In an initial smaller group the protocol was carried out as demonstrated below but without the initiation of the rIPC protocol. This group acted to demonstrate the effects of caffeine infusion on IR injury and to gain initial data as to whether caffeine itself significantly affected FMD.

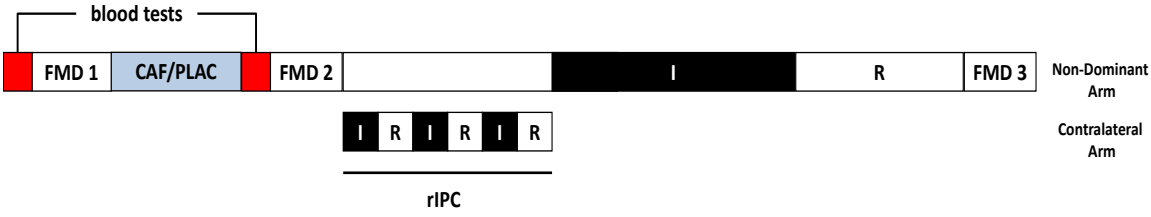


Figure 3.4 Representation of study design for the adenosine receptors in rIPC study. Following a baseline FMD caffeine or placebo solution was infused in a double blind fashion over 10 mins. A repeat FMD was followed by rIPC (3x5 min cuff inflation on the opposite arm) and then IR. The final (third) FMD acted as the primary end-point of the study.

All studies were analysed by a single observer blinded to group allocation using a semi-automated computer analysis programme to further reduce sources of potential error.

3.2.5 Power Calculation and Statistical Analysis

Power calculations were based on initial pilot studies and previous data from our group. By assuming that the difference in response of FMD in sequential studies is Normally distributed with a standard deviation of 5% it was estimated that with eight subjects per group it would be possible to detect a true difference in mean response of 6% with power of 90% and an α=0.05.

For initial studies in which before and after intervention comparisons of FMD were made I used paired Student t-tests. In the full protocol with repeated FMDs, I used repeated measures ANOVA testing. When a significant difference was observed, post-hoc Bonferroni testing was undertaken to check the validity of multiple comparisons.

3.3 Results

3.3.1 Ischaemia-Reperfusion Model

In these preliminary studies 20 minutes of ischaemia, followed by 15 minutes of reperfusion reduced FMD from a mean of 9. 9+/-2.3 to 1.8+/-1.8 (P=0.002 – paired t test) thus confirming the previously described model of IR injury (see Fig. 3.5). As demonstrated in previous studies there was no significant change in NMD with IR (data not shown).

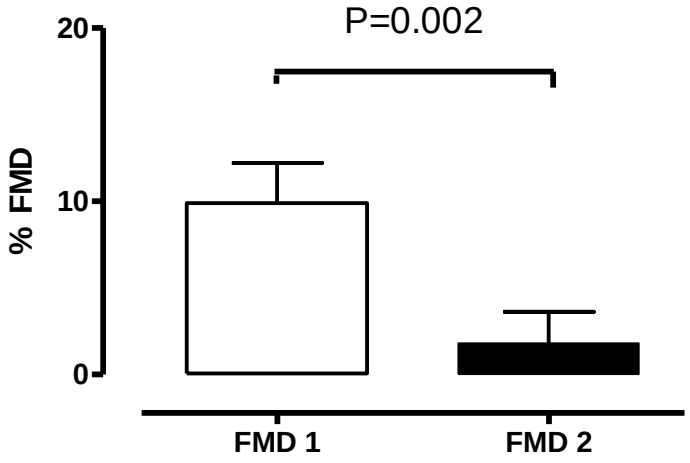


Figure 3.5 IR injury significantly reduces FMD as measured by high resolution ultrasound. FMD 1 (pre-ischaemia) demonstrates normal resting baseline FMD in a group of healthy volunteers (n=5). After 20 minutes of ischaemia followed by 15 minutes of reperfusion there is a marked decrease in FMD (FMD 2) demonstrating endothelial IR injury. Columns represent mean + SEM.

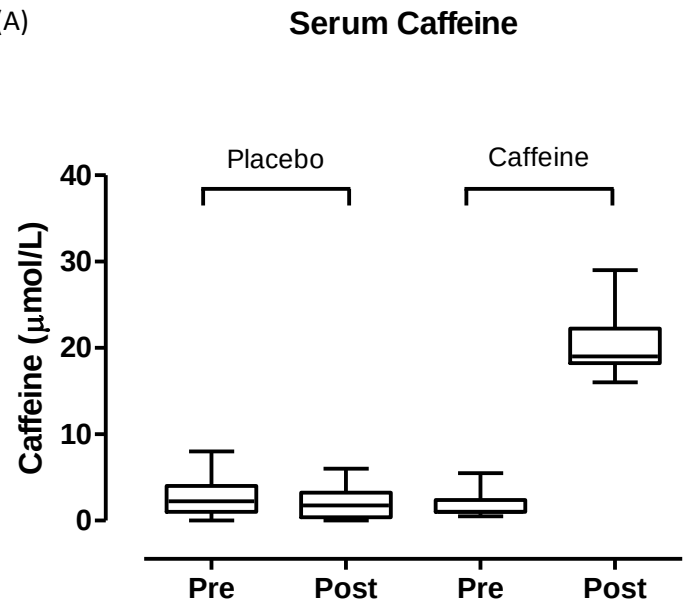
3.3.2 rIPC studies

26 healthy volunteers (including those used in the preliminary studies) were recruited and underwent a total of 78 FMD studies. All studies were analysed blinded to study group allocation. Demographic characteristics of all participants in the two groups were similar and caffeine did not significantly increase blood pressure (see Table 3.1). Caffeine levels rose appropriately in all participants, confirming correct group allocations after unblinding and were comparable to values previously described in the literature[2]. All baseline caffeine measurements suggested that participants had adhered to caffeine avoidance. Results are displayed graphically in Fig 3.6.

	Caffeine Group (n=10)	Placebo Group (n=10)
Age (yrs)	26.5	29.5
Male	7	5
Est Caff Intake (mg/day)	210	231
Fasting Glc (mmol/l)	5	5.2
BMI (kg/m^2)	23.7	25
Baseline MAP/HR	92.9/66	87.2/67
Post Infusion MAP/HR	91.8/57.5	86.5/56
Caffeine Pre (µmol/l)	1.7	3
Caffeine Post (µmol/l)	20	2.2

Table 3.1 Demographic characteristics of main cohort study participants. There were no significant differences in between group characteristics.

(A)



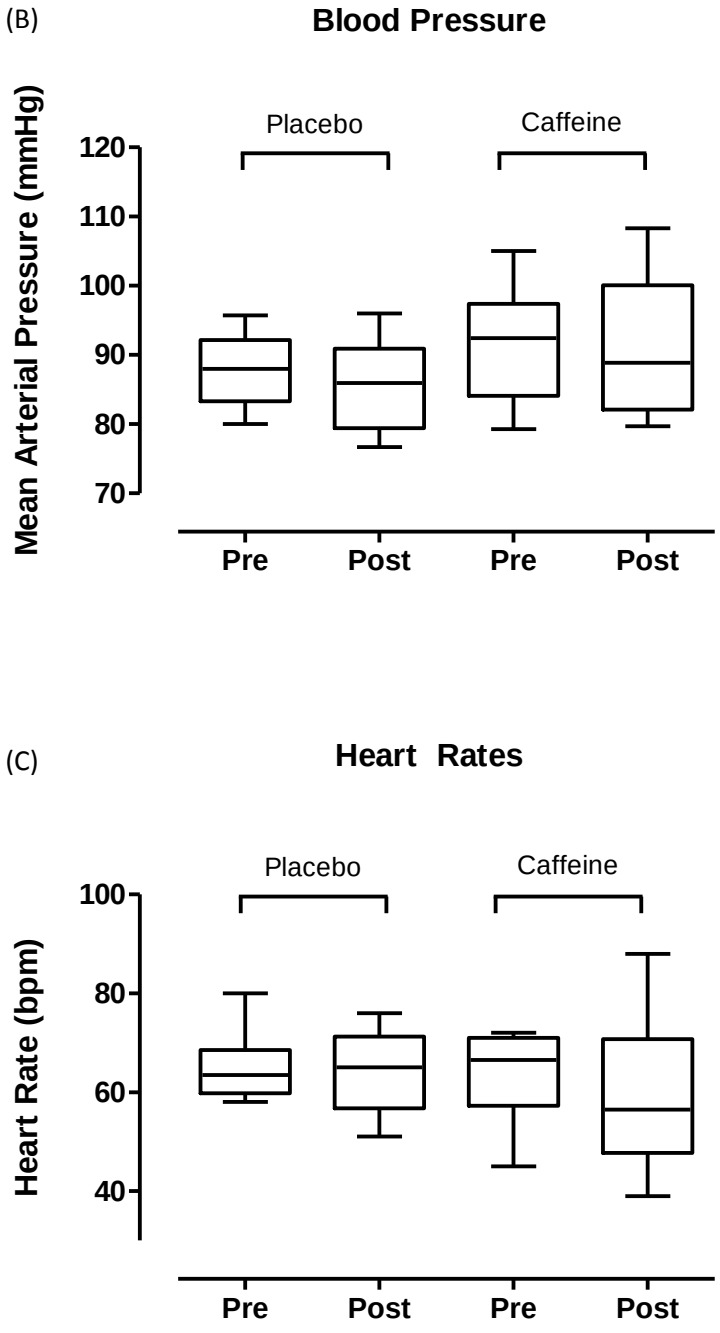


Figure 3.6 Serum caffeine levels and haemodynamic changes. An expected significant increase was seen in systemic caffeine levels following caffeine infusion from 1.7+/-0.5 to 20.3+/-1.2 (graph (A)) ($P<0.0001$ – paired t-test). This had no significant effect on heart rate or blood pressure graph (B) and (C). Box and whiskers represent median and 5/95 percentiles.

Baseline brachial artery diameters were similar in both cohorts across all three FMDs performed (Fig. 3.7).

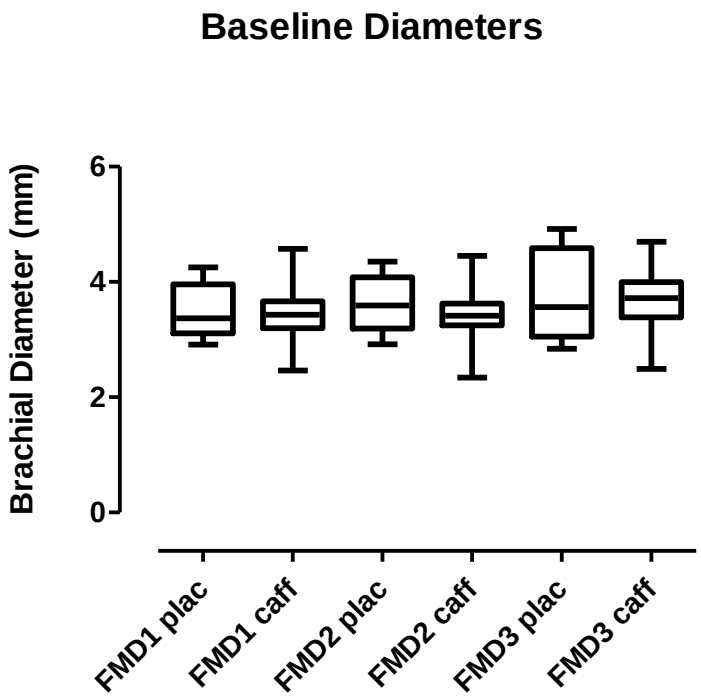


Figure 3.7 Baseline brachial artery diameters did not vary throughout the study period with no significant differences demonstrated. Box and whiskers represent median and 5/95 percentiles.

3.3.3 Caffeine's effect on IR and FMD

To ascertain the effect of caffeine alone on FMD and the IR injury model, the first five participants underwent the protocol outlined above but without a remote preconditioning stimulus.

Caffeine had no significant effect on FMD or IR. Baseline FMD in this group was 4.9+/-0.85 and post caffeine infusion was 5.1+/-0.57 ($p>0.05$ - repeated measures ANOVA).

After IR, FMD reduced to 0.8+/-0.22 again demonstrating a significant reduction from both baseline and post caffeine FMDs (see Fig. 3.8). Again, NMD was not significantly affected by either caffeine or IR injury (data not shown).

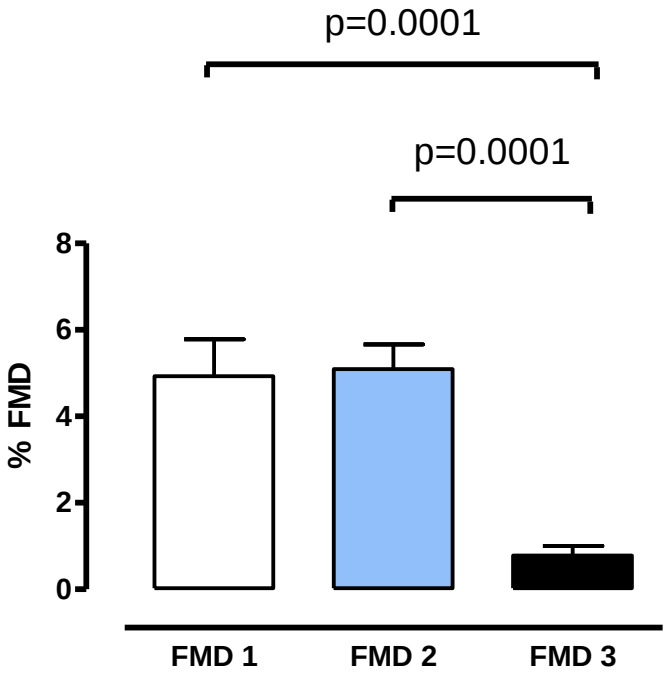


Figure 3.8 Caffeine itself does not significantly affect FMD or the IR injury model. In an initial cohort of participants (n=5) baseline FMD was measured (FMD 1), prior to intravenous caffeine infusion and re-measurement of FMD (FMD 2). Participants did **not** undergo rIPC but proceeded straight to IR injury as described above. Post IR FMD (FMD 3) was significantly reduced, this being consistent with effects previously seen in this model and suggesting caffeine *per se* does not significantly affect it. Columns represent mean + SEM.

3.3.4 Placebo +rIPC and IR

In the main cohort, in the group receiving placebo one study was excluded due to very poor image quality so that n=10. All analyses were by repeated measures ANOVA with post-hoc Bonferroni testing

Baseline FMD was 6.2+/-1.3 and this did not change significantly after placebo infusion to 5.2+/-0.4 ($p>0.05$). After rIPC and IR, there was no significant change in FMD which was 5.4+/-1.2 in comparison with either the baseline or post infusion FMDs demonstrating the protective effect of rIPC against IR injury ($p>0.05$ for both) (see Fig. 3.9).

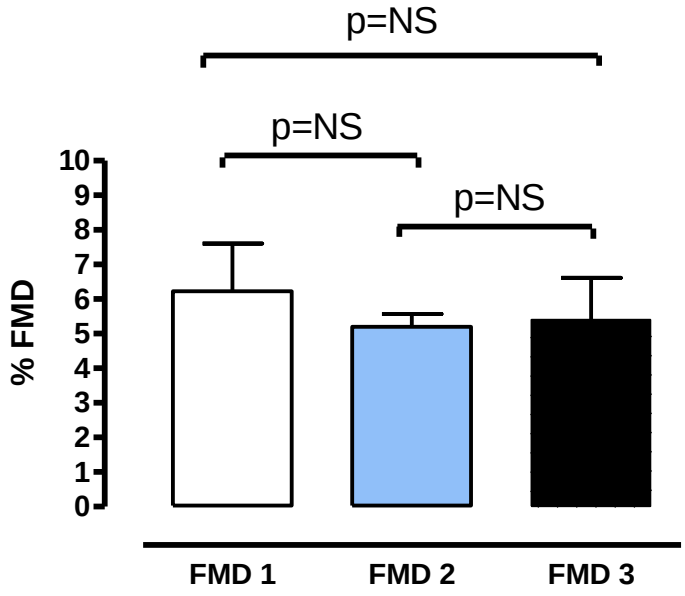


Figure 3.9 rIPC protects against IR injury. FMD was measured at baseline (FMD 1) followed by infusion of placebo and re-measurement of FMD (FMD 2). Following rIPC and IR, FMD was re-measured (FMD 3). FMD 2 was not significantly different to FMD 1 suggesting no change in FMD with placebo and FMD 3 was also not significantly different demonstrating rIPC protected against the effects of IR injury. Columns represent mean + SEM.

3.3.5 Caffeine + rIPC and IR

In the group receiving caffeine (n=10) mean baseline FMD was 7.5+/-1.4 and this did not change significantly with caffeine to 7.9+/-1.0. However, after rIPC and IR, FMD decreased significantly to 3.5+/-1.4 demonstrating an abrogation of the protective effect of rIPC by adenosine receptor blockade (p=0.0009 repeated measures ANOVA) (see Fig. 3.10).

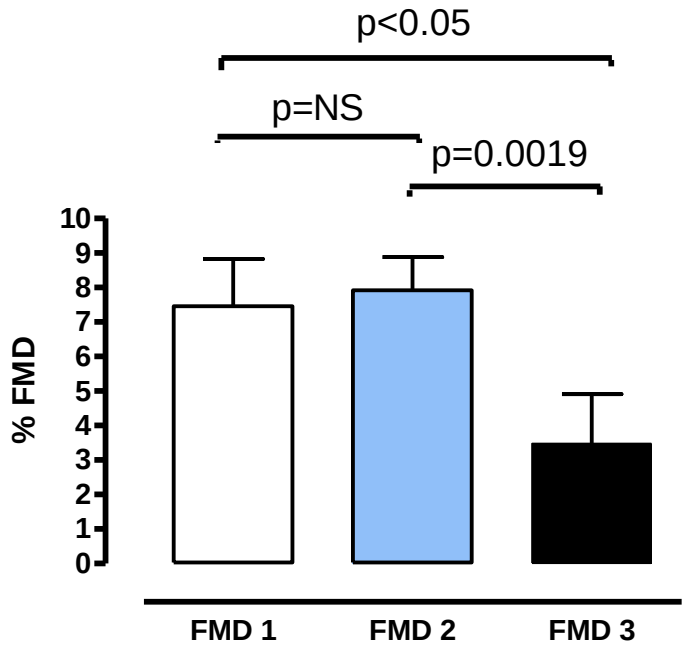


Figure 3.10 Caffeine attenuates the protective effect of rIPC against IR injury. FMD was measured at baseline (FMD 1) followed by infusion of caffeine and re-measurement of FMD (FMD 2). Following rIPC and IR, FMD was re-measured (FMD 3). FMD 2 was not significantly different to FMD 1 suggesting no change in FMD with caffeine but FMD 3 was significantly different demonstrating caffeine attenuates the protective effect of rIPC against IR injury. Columns represent mean + SEM.

3.4 Discussion

This study demonstrates that inhibition of adenosine receptors is able to abrogate the effect of rIPC in humans and implicates adenosine as a key component in the rIPC pathway. While adenosine receptor activation has been implicated as a key step in rIPC pathways in several animal models this is the first demonstration that this is also true in human rIPC and builds on earlier data suggesting adenosine receptor activation in human IPC is important[2].

My initial studies confirmed IR and rIPC were effective in the human forearm model using FMD as the readout. Using this model, I then went on to demonstrate that systemic caffeine infusion had no significant effect on either FMD or on IR injury in the model and then in the final series of experiments demonstrated that caffeine infusion (but not placebo) significantly attenuated the protective effect or rIPC.

The finding of this study, that adenosine receptor activation is important in rIPC, provides important information in describing the pathways of human rIPC. However, the study does have several limitations.

It is not possible to determine from this data whether the blockade of rIPC is absolute or partial. While my studies demonstrate that adenosine is clearly important, other

autocoids including bradykinin and endogenous opioids have also been implicated in rIPC. While these would still be active, the level of GPCR activation by these substances alone may not be enough to trigger intracellular preconditioning signalling cascades and hence switch to a cytoprotected phenotype.

As is common with many human models, there is a good deal of inter-individual variation present between subjects making direct comparisons of cohorts challenging and this is clearly demonstrated here with the degree of variability present in healthy human subjects' baseline FMDs in the various cohorts varying from 5.2+/-0.4 to 9.9+/-2.3. Similar variations are commonly found in the literature with population mean FMDs having a wide range, even in seemingly healthy individuals with one meta-analysis quoting mean values of between 0.2% and 19.2%[192]. While methodological differences may account for some of this discrepancy even in large single study cohorts variations are seen with quoted means from 8.2+/-3.1% [193] and 4.4+/-2.8% [194].

This variation within cohorts is challenging and the final analysis is based on the failure to observe a difference in one cohort, in comparison with a second cohort whom are receiving an intervention. Differences are therefore inferred rather than directly observed and may be influenced by the statistical power inherent in that cohort. However, this is a commonality in many similar interventional studies [76, 176, 195] and with all comparisons within this study being within groups only, the variation seen

between groups is less important as each individual effectively acts as their own control.

Whilst the initial power calculations were used to estimate the size of the cohort required for study from historical data, repeating these calculations with the mean values seen in the caffeine and placebo groups in the main cohort study using the same parameters as previously, suggest that statistical power is preserved at 90% with an α=0.05 with 7 subjects in each group.

Although the human forearm model of preconditioning has been used in many previous translational studies[65, 196-199], there may be important differences in the mechanisms of endothelial rIPC and rIPC in other tissues such as the myocardium, with data from one tissue not necessarily pertinent to other tissues. Despite this limitation, the endothelial model of ischaemia-reperfusion and preconditioning is well described and has been used as a surrogate to attempt to describe human physiological processes *in-vivo[76, 136]*. While models of experimentally induced cardiac ischaemia in humans have been described [184, 185], these studies are inherently technically challenging and not conducive to the simple and rapid testing of physiological hypotheses, being more useful in the testing of potential therapeutic drug candidates. However, despite these provisos it is likely that preconditioning processes at play in the forearm endothelial ischaemia-reperfusion model are similar to those taking place in the cardiomyocyte

triggered by rIPC, and information derived from this model at least provides a starting point from which to investigate other systems.

As previously alluded to, the measurement of endothelial function by brachial artery FMD is prone to error and careful controls were exercised to aim to minimise these. All studies were undertaken in a temperature controlled environment, with care taken to try and perform studies at a similar time of day, after the subject had rested for a minimum of 30 minutes and fasted overnight. All study subjects were non-smokers, not taking any regular medications and were not known to have any chronic diseases. Whilst some studies have also attempted to recruit females in the same phase of their menstrual cycle [200, 201], this was not considered to be a major issue in this study as all scans were performed in each subject on the same day and each individual affectively acted as their own control.

All studies were also performed by a single sonographer using the same ultrasound equipment, performing an average of 300 studies per annum, well above the guideline recommended 100 to maintain competencies [186]. Unfortunately, no local data exists for the coefficient of variation for this sonographer but quoted literature figures suggest an intra-individual coefficient of variation of less than 2% and an inter-individual variation of less than 5% in experienced individuals [187, 202].

A single blinded observer analysed all studies utilising an automated software package to further attempt to minimise any sources of potential bias. Although not undertaken in this particular series of studies due to logistic considerations, the addition of a second blinded observer independently analysing the same data may have further improved the accuracy of FMD measurement and would be a consideration in future work.

A further strength of the method of FMD measurement was the continuous recording technique used in the study. Whilst in other studies a single time point such as 60 seconds post cuff release is taken as the point of maximal increase in diameter and images analysed at this single instance, there is inherent variability in this and the true peak diameter may be missed [188, 189]. This technique also allows the observer to compare the baseline with the vessel diameter in the post FMD peak phase, an important parameter to discount significant movement artefact, and all scans were seen to return to within an acceptable 1.5% of the baseline value.

Finally, whilst other imaging modalities have been trialled in the measurement of FMD, ultrasound remains the pre-eminent method for imaging due to a combination of equipment availability, patient acceptance, low cost and excellent spatial and temporal resolution. Whilst other techniques such as MRI have been well described and are used in studies of endothelial function[203, 204], in particular, the long acquisition times involved do not allow continuous recording of arterial diameter and subtle changes may be lost.

As is common with all pharmacological inhibitors, caffeine is not specific and therefore may have unpredictable off-target effects that could also be conceivably affecting the preconditioning response. Functional studies on human cells *in-vitro* have shown that caffeine effectively blocks the A_1, A_{2A} and A_{2B} receptors at concentrations of between 10-30μM [205]and so effective inhibition would be expected at the 20μM serum levels seen in this and other studies utilising a 4mg/kg dose[2]. As has been previously noted, the A_3 receptor is not effectively inhibited by caffeine with concentrations in excess of 100μM being necessary to cause blockade[205].

Caffeine is known to affect other physiological receptors and previous work has demonstrated its ability to increase free intracellular calcium concentrations in muscle cells through activation of the ryanodine receptor in the endoplasmic reticulum but only in concentrations of more than 1mM[191, 206]. Similarly, caffeine has been shown to inhibit (cAMP) phosphodiesterase, but again, only at high concentrations, with an EC_{50} of 1100μM[207, 208].

Whilst it is acknowledged that caffeine cannot be considered to be a specific inhibitor of adenosine receptors, at the concentrations achieved in this study, it is known to provide effective blockade without having known off-target effects, and so it remains likely that adenosine receptor blockade is at least involved in the loss of preconditioning seen.

Another important point is that this study does not directly demonstrate that adenosine itself is important in rIPC, only that adenosine receptor activation is, but as there are currently no other proven endogenous ligands for the adenosine receptor it would seem likely that adenosine itself is therefore a key molecule in rIPC.

Adding to this point, this study is also unable to demonstrate which adenosine receptor subtypes are important in this response. Caffeine itself only blocks three of the four adenosine receptor sub-types suggesting that the fourth, the A_3, is not important in endothelial preconditioning or is not important in human preconditioning *per se*. In the literature, in animal models, selective agonists of the A_3 receptor have been shown to reduce experimentally induced myocardial infarct size when given prior to ischaemia-reperfusion[209, 210] but the relevance of these models to endogenous preconditioning mechanisms is unclear and it may be that these agonists are triggering pharmacological rather than physiological effects. While caffeine is effective, it unfortunately does not allow us to investigate receptor subtypes beyond ruling out the A_3 receptor.

Unfortunately, other clinically available antagonists such as theophylline are similarly non-specific and other selective antagonists used in laboratory models are not available for human use. There are some clinically available selective adenosine receptor agonists such as regadenoson (a selective A_2A receptor agonist used in myocardial perfusion imaging) but these would not be useful in illuminating the intrinsic

mechanisms of rIPC for the reasons outlined above. Similarly the so-called adenosine regulating agents such as acadesine and GP531 which are now entering phase III recruitment protocols would again fail to be helpful in this regard.

In this cohort, caffeine infusion failed to have any significant effect on flow mediated dilation. The effects of caffeine on endothelial function in the literature are mixed with studies claiming both deleterious [211, 212] and beneficial effects[213]. However, these investigators used disparate techniques and protocols, in some instances utilising caffeinated beverages which may have a variety of effects on endothelial function not related to caffeine *per se*. In this study, at the dosages used, caffeine does not have a confounding effect and allows FMD to be used as a readout for the IR model.

Furthermore, no effect was seen on blood pressure or heart rate from caffeine infusion and is at odds with previous studies using this dose of caffeine [2, 3]. One important difference in this study was that participants remained relaxed and recumbent throughout the three hour study period and this relaxation, without any muscular exercise at all and this may account for the lack of change in haemodynamics, with no effect of caffeine being seen without exercise driven adrenergic stimulation.

Finally, as the adenosine receptor antagonist (caffeine) was given systemically, this study is also unable to differentiate between whether adenosine receptor activation is

important in the' trigger' or 'effector' (or both) phases of rIPC. This will be further explored in subsequent studies.

In conclusion, this study demonstrates that adenosine receptor blockade is able to abrogate the protective effects of rIPC in a human endothelial forearm model of ischaemia-reperfusion injury. This implicates adenosine receptor activation as an important mediator in rIPC.

CHAPTER 4: ADENOSINE RECEPTORS IN THE 'TRIGGER' AND 'EFFECTOR' PHASES OF rIPC

4.1 Introduction

While the study in Chapter 3 demonstrated that systemic blockade of adenosine receptors blocks the protection induced by rIPC, this did not address where in the pathway of rIPC adenosine receptor activation was important. As previously discussed, rIPC may be considered to act in two phases, the 'trigger' phase in which the rIPC stimulus is initiated, and the 'effector' phase in which the cytoprotective stimulus activates a protected phenotype in the target organ which subsequently becomes more tolerant of ischaemia-reperfusion injury. Giving an adenosine receptor antagonist systemically (as in Chapter 3) would block adenosine receptor activation globally providing no information as to where in the pathway adenosine receptors are important. To further investigate this issue the current study was designed and initiated to test the hypotheses:-

i. Adenosine receptor activation in both the 'trigger' and 'effector' phases of rIPC mediates its cytoprotective effects
ii. Adenosine receptor activation in rIPC is important in mediating the release of a circulating humoral protective factor

4.2 'Trigger' and 'Effector' Phase study

The forearm model of IR injury was again used to investigate adenosine receptor activation in the 'trigger' and 'effector' pathways of rIPC. Caffeine has been used at a dose of 90microgmin^{-1} per 100 ml forearm volume, infused directly into the brachial artery to cause local but not systemic blockade of adenosine receptors [214, 215]. This allows the selective inhibition of adenosine receptors in one tissue without systemic spill-over of caffeine at a sufficient concentration to cause widespread adenosine receptor blockade.

Using this model, I selectively infused caffeine into either the 'trigger' arm or the 'effector' arm to ascertain whether it was possible to better characterise adenosine's role in rIPC signalling. In a second aspect of the study, blood was also collected from participants to investigate the production of a circulating preconditioning factor. This study was granted ethical approval (ethics ref. 09/H0604/118) by Oxfordshire Research Ethics Committee A. The results presented below also contain data from the study 08/H0604/169 and the study 09/H1207/99. Both of these studies were conducted by myself during my DPhil period on separate aspects of remote preconditioning, not directly relevant to adenosine's role in rIPC. However, as they both used the forearm model of IR injury, the data from them is usefully presented here to demonstrate the establishment of the IR model with the technique of VOP.

4.2.1 Study Design and Participants

The study was a non-randomised cross-over investigation examining the role of adenosine receptors in the 'trigger' and 'effector' phases of rIPC. Recruits were healthy male volunteers aged 18 or above, not taking regular medications and non-smokers. Only male participants were recruited in this study due to concerns over phlebotomy induced anaemia, and the higher risk of this in females. All participants abstained from caffeine-containing drinks for a minimum of 24 hours, alcohol for 24 hours and from food for at least 4 hours before participation in each study visit. The order of study visits was randomised to avoid the possibility of carry-over effects affecting the results, but for convenience are referred to as study visits 1 ('trigger') and 2('effector') below. All studies were carried out in a dedicated vascular laboratory, a quiet temperature controlled room maintained at 22-24˚C. Studies were of a hybrid design using both FMD and VOP measures of endothelial function as explained below.

4.2.2 Power Calculation and Statistical Analysis

Power calculations were based on the same parameters as for the previous study. By assuming that the difference in response of FMD in sequential studies is Normally distributed with a standard deviation of 5% it was estimated that with eight subjects per group it would be possible to detect a true difference in mean response of 6% with power of 90% and an α=0.05.

For 'visit 1' ('trigger' phase) in which there was a comparison of FMD before and after rIPC and IR I used paired Student t-tests. In 'visit 2' ('effector' phase) in which VOP was used, I used repeated measures ANOVA testing. When a significant difference was observed, post-hoc Bonferroni testing was undertaken to check the validity of multiple comparisons.

4.2.3 Study Visit 1 – 'trigger' phase

In this phase of the study the aim of the protocol was to inhibit adenosine receptor activation in the arm in which the rIPC stimulus was being delivered – the 'trigger' arm - and to achieve this, caffeine was infused directly into the arterial supply of the limb to aim to achieve high tissue concentrations in this tissue only without significant systemic spill-over. Briefly, the brachial artery of attending participants was cannulated using a sterile 27-gauge needle under local anaesthetic (1% lignocaine) and to maintain needle patency a saline infusion commenced at 1ml/min through a fine bore epidural catheter (see brachial needle manufacture in Materials and Methods). Forearm volume was estimated using the formula for a truncated cone and the caffeine infusion dilution adjusted appropriately.

80ml of heparinised venous blood was then taken and a further smaller sample (3ml) taken to measure caffeine levels. The blood was centrifuged and the plasma fraction further processed by dialysis – see materials and methods section for further details. A

baseline FMD was then recorded as described previously, in the non-dominant arm (i.e. the arm not cannulated with the brachial needle). The brachial infusion was then switched to caffeine (infused at 90microgmin^{-1} per 100 ml forearm volume) and a rIPC stimulus delivered in the arm cannulated with the brachial needle.

At the end of this procedure the brachial needle was switched back to a saline infusion. A further 80ml of blood was taken at this stage, a second sample taken for caffeine levels to ascertain whether there had been significant spill-over into the systemic circulation and the rest of the blood heparinised and used for dialysis. IR was then induced in the non-dominant arm as described above. A final FMD was recorded as a readout of IR injury and whether rIPC had been protective or not (see Fig. 4.1).



Figure 4.1 Schematic of procedures during visit 1 ('trigger' phase) of the study. The contralateral arm of participants is cannulated with a 27-gauge sterile needle and during a rIPC stimulus (3x5 minute cycles of inflation of an upper arm cuff) caffeine is infused at a dose of 90microgmin^{-1} per 100 ml forearm volume to block adenosine receptors in this tissue only. Blood was taken both before and after rIPC to generate dialysate and check caffeine levels. FMDs before and after rIPC and IR in the other arm (non-dominant) act as assessment of endothelial function and whether rIPC has been effective in protecting against IR injury.

4.2.4 Study Visit 2 – 'effector' phase

Participants returned a minimum of eight weeks later. This period was chosen to allay fears regarding phlebotomy induced anaemia (as was the stipulation for male volunteers only) and to also allow a sufficiently long period to avoid problems with carry-over effects from the first study. In this study, caffeine was infused into the 'effector' arm to investigate its effects on rIPC (as opposed to the generation of the rIPC signal). As this arm would need to be cannulated with a brachial needle, making ultrasound assessment of the brachial artery extremely challenging, it was decided to use the technique of venous occlusion plethymography to assess endothelial function. Venous occlusion plethysmography is a well-described technique to assess endothelial function and is more fully described in the Methods section.

Briefly, mercury-in-silastic strain-gauges are placed around each forearm at the widest point and pressure cuffs are attached to the wrist and proximal upper arms on both sides. During recordings the wrist cuffs are inflated to 200mmHg to exclude the hand circulation and the upper arm cuffs are inflated for 9 seconds out of every 15, to 40mmHg allowing arterial in-flow but restricting venous return. This in-flow leads to an increase in forearm volume, measured by the forearm strain gauges, which is directly proportional to arterial in-flow. Forearm blood flow responses are measured in response to the infusion of the endothelium-dependent dilator acetylcholine (ACh; 25, 50, and 100 nmol/min; each dose for 3 minutes) or the endothelium-independent dilator glyceryltrinitrate (GTN; 4, 8, and 16 nmol/min; each dose for 3 minutes) infused through the brachial needle. The non-infused arm acts as a contemporaneous control

throughout the study. Typical traces are shown in the Methods section and not repeated here. Traces are analysed by calculating the mean gradient of the slope in the infused arm in comparison with the non-infused arm and values are expressed as a percentage of baseline flow.

Although VOP assesses a different vascular bed to FMD (resistance vs conduit vessels) the two techniques are broadly similar in measuring arterial endothelial function and both these vascular beds have been shown to have similar preconditioning responses and responses to IR injury[76, 176]. The protocol for study visit 2 is outlined below (see Fig. 4.2). Briefly, after a baseline VOP (with caffeine being continuously co-infused directly into the brachial artery at the same dose as previously), rIPC was conducted on the contralateral arm. This was followed by IR injury and then finally a repeat VOP study. Blood was again taken both immediately before and after rIPC to generate blood for dialysate production, caffeine level measurement and serum for storage and future use.

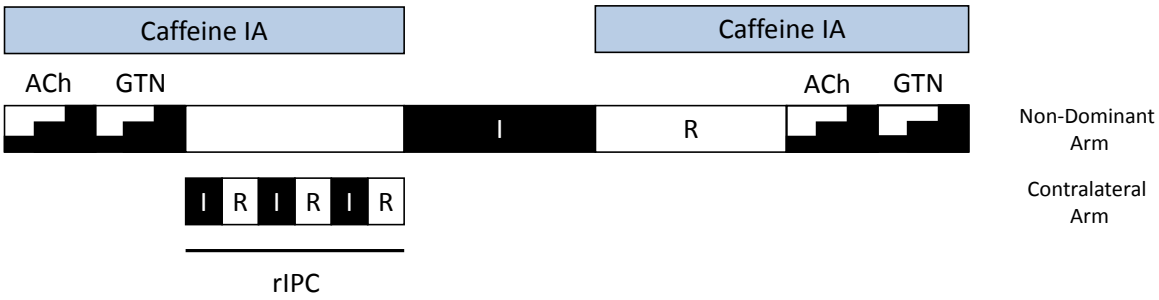


Figure 4.2 Schematic of procedures during visit 2 ('effector' phase) of the study. Caffeine is continuously co-infused into the non-dominant arm and endothelium-dependent and endothelium-independent vasodilations are assessed by the change in forearm blood flow both before and after rIPC and IR injury. Blood was again taken before and after rIPC for the generation of dialysate.

4.3 Results

A total of 11 healthy male volunteers were recruited for this crossover study. In one individual, due to extreme technical difficulties in cannulating the brachial artery on both study visits, no meaningful data was acquired and hence n=10 for all comparisons. Demographic data are displayed below in Table 4.1.

	"Visit 1"	**"Visit 2"**
Mean Age (yrs)	23.8+/-1.3	
Est. Caff. Intake (mg/day)	186+/-54	
Fasting Glc (mmol/l)	5.2+/-0.1	5.2+/-0.2
BMI (kg/m^2)	23.4+/-0.7	23.6+/-0.6
Forearm Vol. (cm^3)	1162+/-18	
Baseline MAP/HR	102+/-3/58+/-3	104+/-2/56+/-2
Post Infusion MAP/HR	96+/-3/65+/-4	96+/-1/54+/-1
Caffeine Pre (µmol/l)	1.25+/-0.31	1.28+/-0.36
Caffeine Post (µmol/l)	2.15+/-0.31	2.61+/-0.31
Haemoglobin (g/dl)	15.8+/-1.1	15.9+/-0.8

Table 4.1 Demographic characteristics of participants in study. No significant changes were seen in the 8 weeks between visits. All values are quoted as the mean+/-SEM.

4.3.1 Confirming the IR model for VOP and the effectiveness of rIPC

I conducted preliminary studies to confirm previously published data in this model. Five volunteers attended for forearm studies as part of the protocol for study 09/H1207/99. During this study volunteers underwent a baseline VOP study, followed by the standard forearm IR protocol (20 mins ischaemia, 15 mins reperfusion) and then a further VOP study to ascertain endothelial function before and after IR. The results are presented below in Fig 4.3 demonstrating a significant difference before and after IR in forearm blood flow responses to Ach.

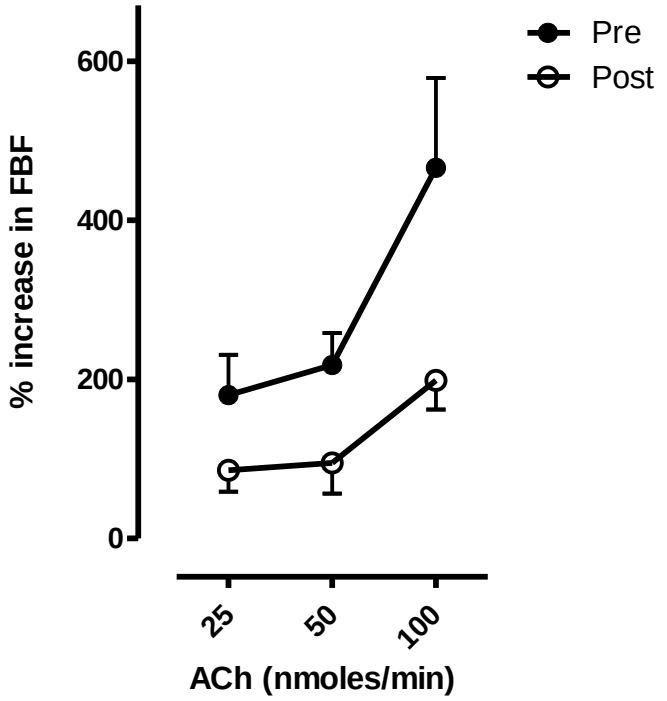


Figure 4.3 Forearm Blood flow responses to incremental doses of Ach are attenuated after IR injury in the human forearm (Repeated measures ANOVA with post-hoc Bonferroni, P=0.03)(n=5). Points represent mean +/- SEM.

As in previous studies there was no significant change in GTN response before and after IR.

Having confirmed IR injury, I subsequently confirmed rIPC was protective in this model. Using data from the study 08/H0604/169 in which a total of 11 healthy volunteers attended for VOP studies as above but in which a standard rIPC protocol (3x5 cycles of 5mins IR of contralateral arm) was carried out prior to IR injury, the effect of rIPC is demonstrated below in Fig 4.4.

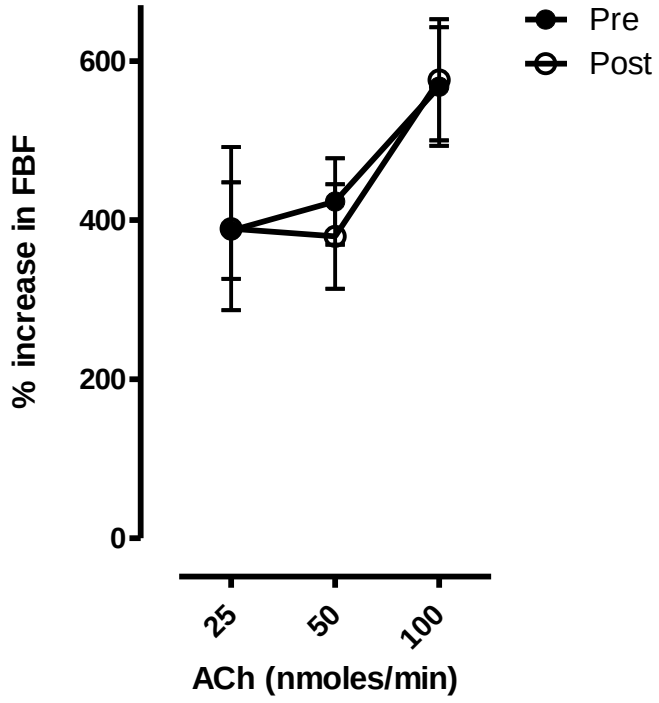


Figure 4.4 Forearm blood flow responses to Ach are maintained when IR is preceded by rIPC (Repeated measures ANOVA with post-hoc Bonferroni, P=0.91)(n=11). Points represent mean +/- SEM.

Data from the study in Chapter 3 had already established rIPC and IR for the FMD model and this was not repeated.

4.3.2 Visit 1 – 'Trigger' phase

The forearm model data for the 'trigger' phase is presented below. Serum caffeine levels at baseline were very low suggesting subjects had observed the protocol for caffeine abstention. Post-infusion of caffeine, there is a statistically significant rise in systemic caffeine levels but the absolute levels are not of biological significance with levels approximately 10-fold lower than those observed in previous studies[2] necessary for systemic blockade and also in the study in Chapter 3 following intravenous caffeine infusion (see Fig. 4.5 and 4.6).

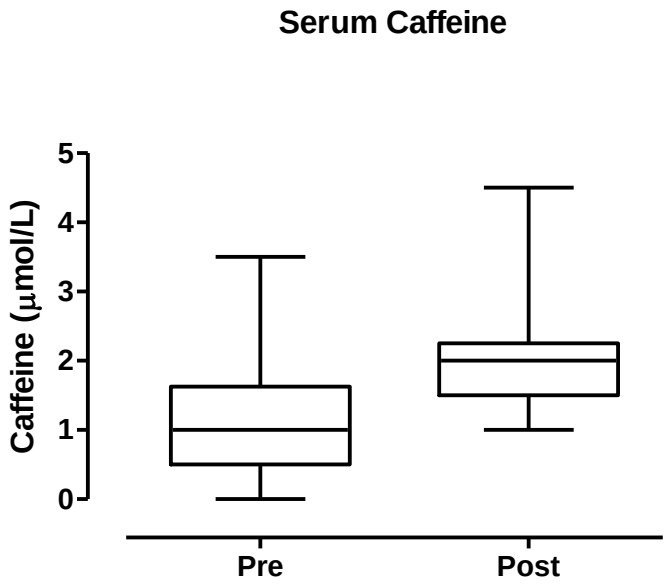


Figure 4.5 Systemic caffeine levels demonstrate a highly statistically significant rise pre- and post-infusion with levels rising from 1.2+/-0.3 to 2.6+/-0.3 (paired t-test P<0.0001). However, this is unlikely to be of biological significance with absolute levels being 10-fold lower than those observed in previous studies required to cause systemic adenosine receptor blockade. Box and whiskers represent median and 5/95 percentiles.

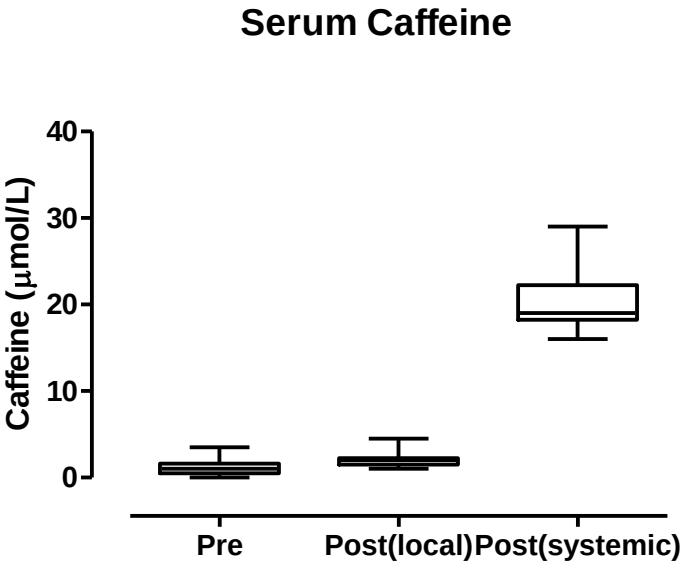


Figure 4.6 Comparison of data from Fig 4.5 with the addition of the rise in caffeine levels seen with systemic infusion (post (systemic)) necessary for global adenosine receptor blockade in previous studies. In this comparison there is a highly significant difference between the post (systemic) levels and the other comparators (p<0.0001 for

both comparisons (1-way ANOVA with post-hoc Bonferroni) with no significant difference demonstrated between the pre and post (local) levels. Box and whiskers represent median and 5/95 percentiles.

There was no significant change in blood pressure or heart rate with the local infusion of caffeine (see Fig 4.7)

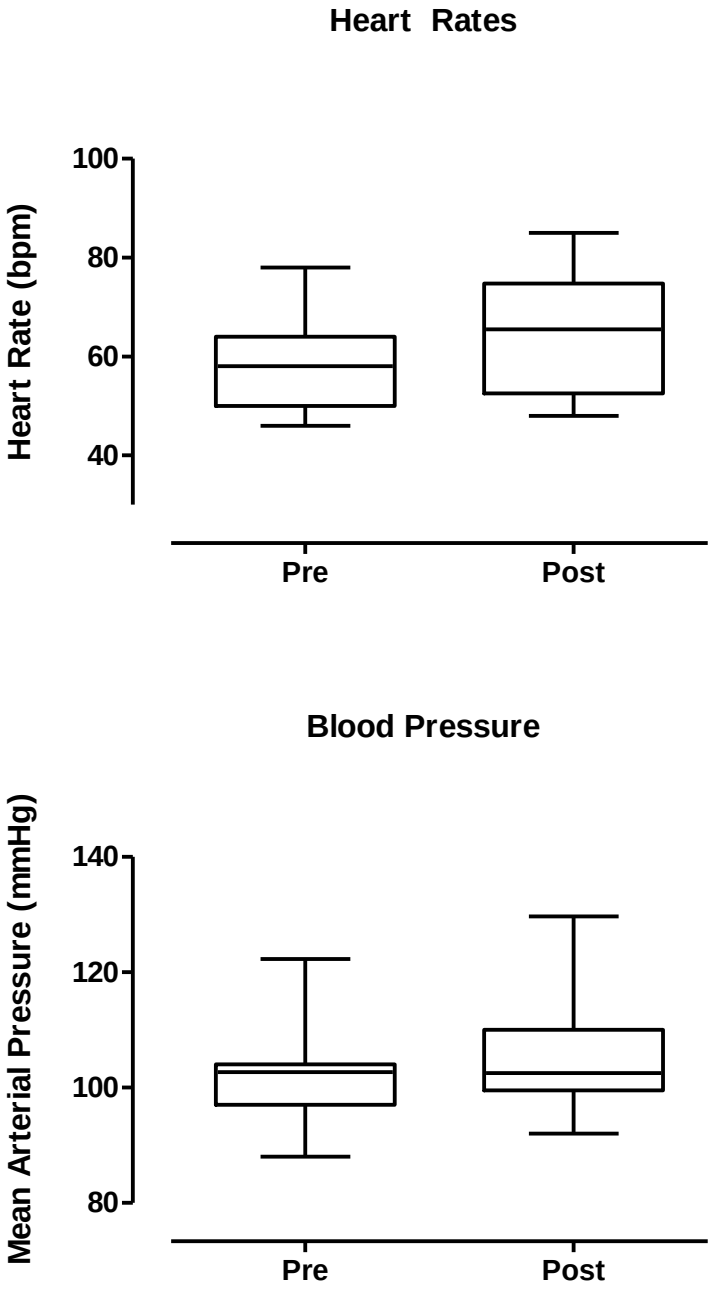


Figure 4.7 Neither blood pressure or heart rate was significantly different before and after caffeine infusion. Heart rate was a mean of 63.6+/-2.7 at the commencement of

infusion and 59.1+/-4.7 at the end (paired t-test, P=0.1). Similarly, MAP was 102+/-2.5 at the beginning and 105.8+/-3.5 at the finish (paired t-test, P=0.4). Box and whiskers represent median and 5/95 percentiles.

Baseline vessel diameters were similar at the start of both FMD studies (See Fig 4.8)

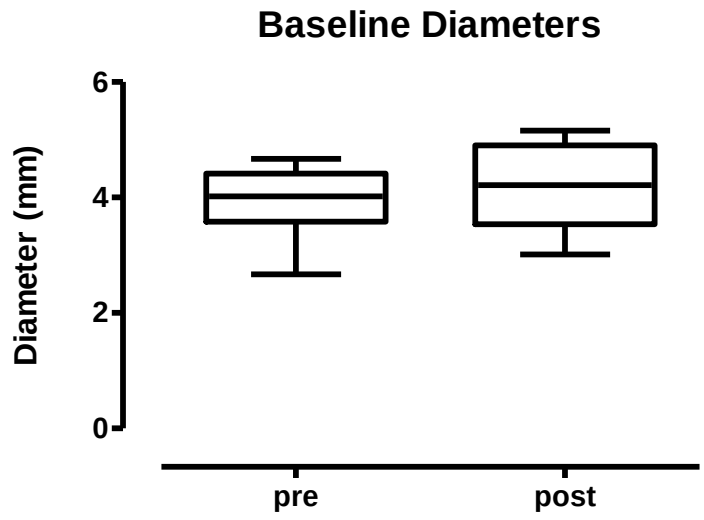


Figure 4.8 Baseline vessel diameters are similar during the first FMD study and during the second (performed post rIPC and IR). Vessel diameters pre- were 3.9+/-0.2 and post were 4.2+/-0.2 (paired t-test, P=0.2). Box and whiskers represent median and 5/95 percentiles.

Flow-mediated dilation post rIPC and IR demonstrated a statistically significant decrease in FMD post rIPC and IR in the presence of an intra-arterial caffeine infusion demonstrating that adenosine receptor blockade in the "trigger" arm of rIPC was able to abrogate the protective effect of rIPC (see Fig 4.9). As previously, there was no significant change in NMD throughout the study.

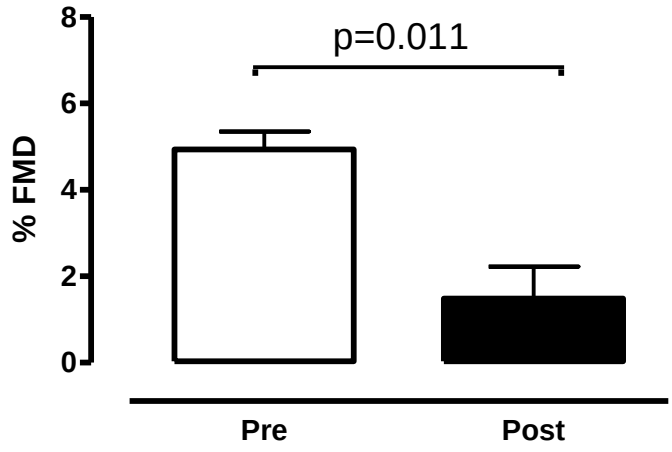
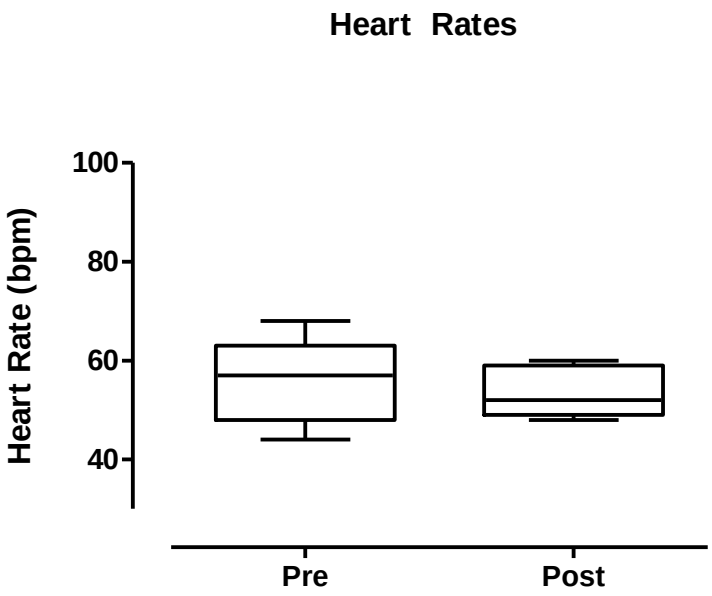
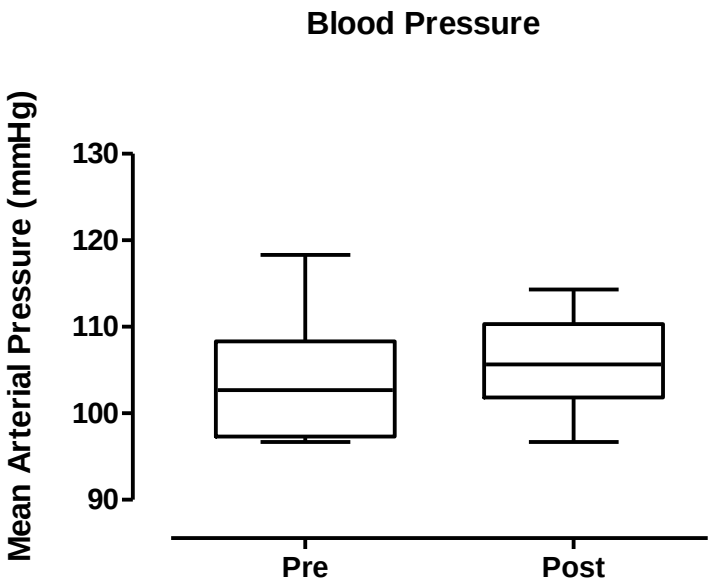


Figure 4.9 Intra-arterial caffeine infusion into the 'trigger' arm abrogates the effect of rIPC. In FMDs performed before and after rIPC and IR there is a statistically significant decrease in FMD from a mean of 4.9+/-0.4 at baseline to 1.5+/-0.7 demonstrating a loss of the protective effect of rIPC in this model (paired t-test, P=0.011). Columns represent mean + SEM.

4.3.3 Visit 2 – 'Effector' phase

As in the 'trigger' phase visit, there were no significant changes in heart rate or blood pressure noted before and after caffeine infusion. Caffeine levels showed a statistically significant rise in systemic levels but in absolute terms (as demonstrated above), remained low (Fig 4.10).


Heart Rates
Heart Rate (bpm)
100
80
60
40
Pre
Post


Blood Pressure
Mean Arterial Pressure (mmHg)
130
120
110
100
90
Pre
Post

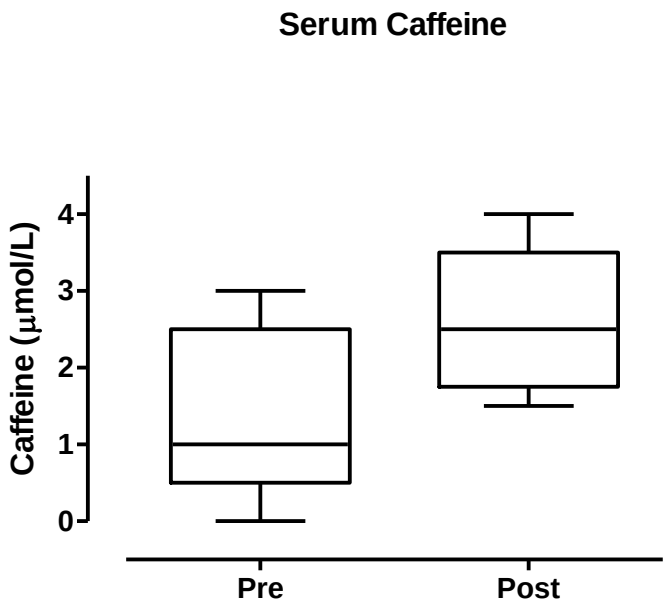


Figure 4.10 Heart rate demonstrated no significant change from 65+/-2.2 to 64.2+/-2.7 (paired t-test, P=0.8) and blood pressure similarly demonstrated no significant change from a mean MAP of 104.2+/-2.3 to 105.8+/-1.6 (paired t-test, P=0.4). Serum caffeine demonstrated a highly significant rise from 1.3+/-0.4 to 2.6+/-0.3 (paired t-test, P<0.0001) but absolute levels remained low. Box and whiskers represent median and 5/95 percentiles.

Forearm blood-flow before and after rIPC and IR is presented below. There was no statistically significant decrease in forearm blood flow response to ACh seen in this model demonstrating that adenosine receptor blockade in the "effector" arm does not effectively abrogate the protective effects of rIPC against IR injury (Fig 4.11).

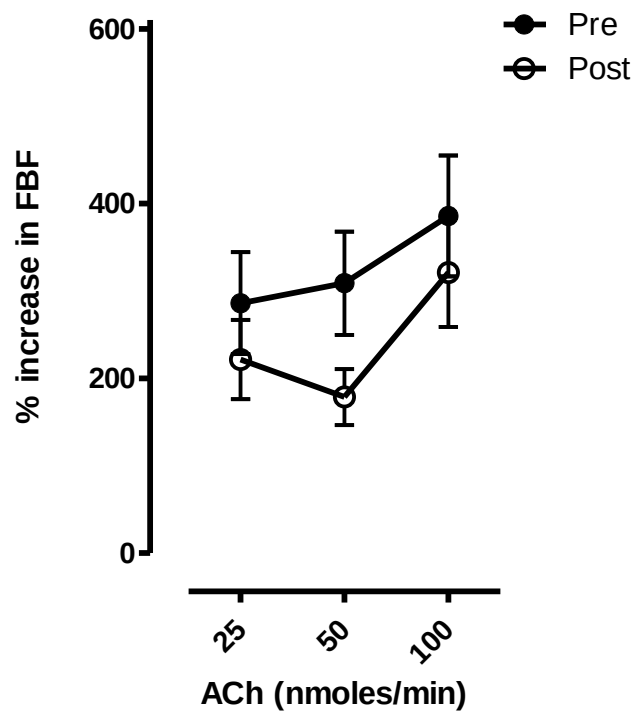


Figure 4.11 There is no significant difference in forearm blood flow response to ACh when caffeine is infused intra-arterially into the 'effector' arm (repeated measures ANOVA, P=0.22). This demonstrates that the protective effect of rIPC remains intact despite adenosine receptor blockade. Points represent mean +/- SEM

Once more there was no significant change in GTN response as has been previously demonstrated (data not shown).

4.4 'Trigger' and 'Effector' phase Dialysate Effects

During both visit 1 ('trigger') and visit 2 ('effector') blood was taken following rIPC, and the blood dialysed against a modified KH solution and the resulting dialysate stored.

The aim of this aspect of the study was to ascertain whether adenosine receptor blockade interfered with the production of the circulating preconditioning factor whose release by a rIPC stimulus has been previously described. The identity of the factor is currently unknown hence its presence or absence can only be inferred through its functional effect, with a mouse Langendorf infarct assay being utilised to test for its presence or absence. By taking blood both before and after rIPC, each individual effectively acted as their own control.

In initial experiments, dialysate was generated from healthy volunteers using a protocol of 3 cycles of 5 minutes of ischaemia and 5 minutes of reperfusion of the upper limb generated by a pressure cuff, blood taken and dialysed as above. In a small pilot group of 8 mouse hearts infused with either control dialysate (i.e. from blood taken prior to rIPC) or rIPC dialysate (blood taken after rIPC) there was a significant difference in infarct size between groups (control IS 37.7+/- 4.2 (n=4)), and rIPC IS 21.32+/-4.7 (n=4), p=0.04) (Fig. 4.12).

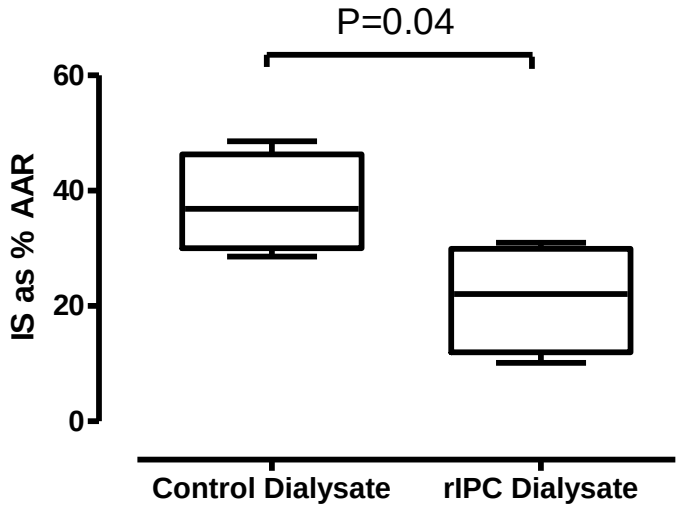


Figure 4.12 Standard rIPC dialysate is protective against IR in a mouse Langendorf model. This proof of concept study validated the technique for manufacture of dialysate. Box and whiskers represent median and 5/95 percentiles.

This pilot study demonstrated the feasibility of the project, establishing the correct manufacture technique for dialysate in this institution, and demonstrating that activity of a circulating cardioprotective substance could be shown with this technique.

4.4.1 'Effector' Phase Dialysate

All Langendorf mouse infarct studies were performed by Dr j Li of the University of Toronto who was blinded to study visit, and analyses of infarct size undertaken by a second blinded observer also unaware of study visit status.

The results for infarct size and haemodynamic recovery for visit 2 ('effector') are demonstrated below. With caffeine being infused in the 'effector' arm, adenosine receptors in the trigger arm should have been unaffected. This part of the study was therefore important in providing evidence for the release of preconditioning factor *per se*. As can be seen in Fig 4.13, there is a highly significant decrease in myocardial infarct size following infusion with dialysate manufactured from blood taken post-rIPC (26.5+/-2.0) as compared with blood from the same individuals pre-rIPC (47.8+/-2.4) (P<0.0001, paired t-test) demonstrating that a highly protective circulating factor is released into the blood stream by an rIPC stimulus (n=9)

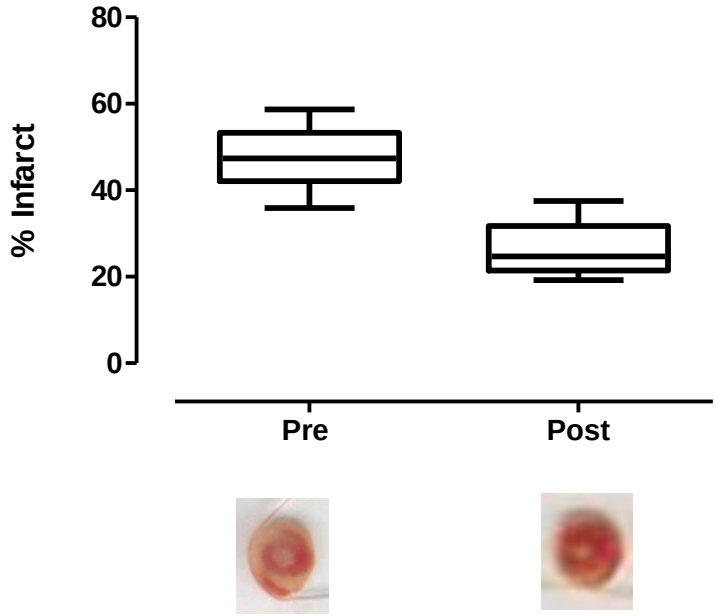


Figure 4.13 There is a highly significant reduction in mean infarct size from 47.8+/-2.4 to 26.5+/-2.0) (P<0.0001, paired t-test) demonstrating that a circulating protective factor is elaborated by rIPC. Representative images of myocardial slices are presented. Box and whiskers represent median and 5/95 percentiles.

Despite this highly significant reduction in infarct size, no statistically significant improvement in post-infarct haemodynamics could be demonstrated (Fig 4.14)

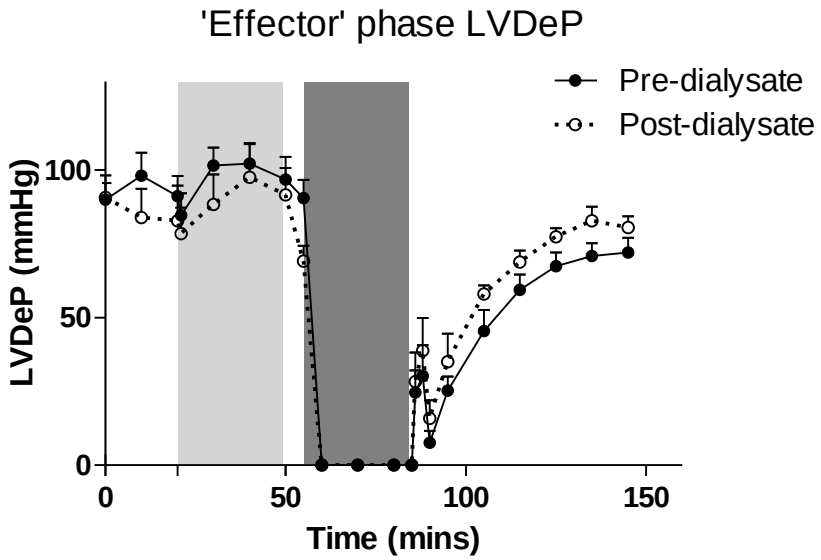


Figure 4.14 Haemodynamic data from mouse Langendorf study for the 'effector' phase. No significant difference in post-infarction haemodynamic recovery could be demonstrated for the post-dialysate. Light grey shaded area, dialysate infusion; dark grey shaded area, global ischaemia. Points represent mean +/- SEM

4.4.2 'Trigger' Phase Dialysate

The data for 'trigger' phase is presented below. In this case, caffeine was infused directly into the 'trigger' arm causing adenosine receptor blockade locally. However while from the above data this clearly appeared to block preconditioning, as the mechanism of rIPC is unclear and both neural and circulating mechanisms have been postulated and demonstrated, it was uncertain as to whether this might have any effect

on the production of the circulating preconditioning factor. Again, this was assayed for by use of the same mouse Langendorf protocol as previously described. In this case there was no significant difference in infarct size which was 37.3+/-1.9 pre-rIPC and 34.25+/-2.0 post-rIPC (p=0.31, paired t-test) (n=10) inferring that adenosine receptor blockade inhibits the release of the circulating preconditioning factor (Fig. 4.15).

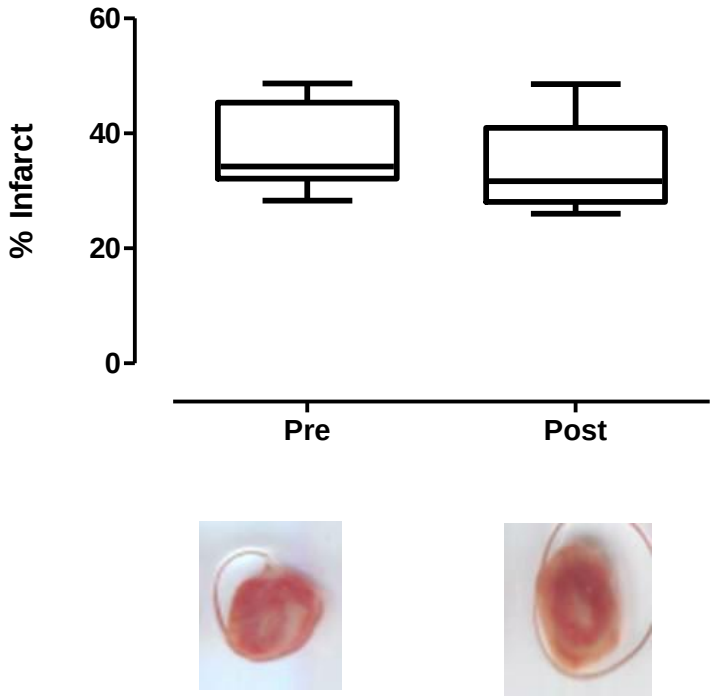


Figure 4.15 With adenosine receptor blockade in the 'trigger' phase there is loss of generation of the circulating preconditioning factor and no significant difference in infarct sizes with dialysate generated pre and post rIPC. Representative images of myocardial slices are presented. Box and whiskers represent median and 5/95 percentiles.

Haemodynamic data from the same study is demonstrated below (Fig. 4.16). Once more, there was no significant difference in post infarct haemodynamic recovery seen.

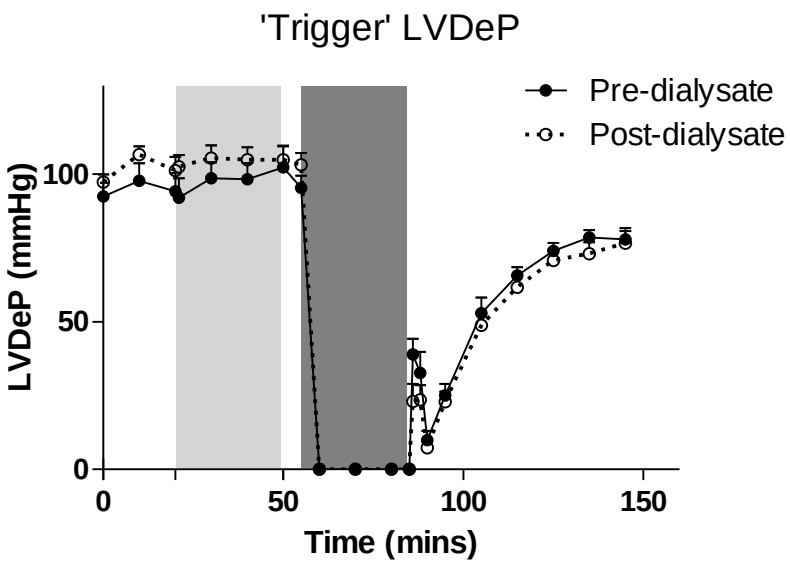


Figure 4.16 Haemodynamic data from mouse Langendorf study for the 'trigger' phase. No significant difference in post-infarction haemodynamic recovery was seen between dialysate groups. Light grey shaded area, dialysate infusion; dark grey shaded area, global ischaemia. Points represent mean +/- SEM

4.5 Discussion

The results of this study, in conjunction with those of the study in Chapter 3, go towards defining the role of adenosine in human rIPC. In the previous study, systemic adenosine blockade lead to abrogation of rIPC with the results of this study demonstrating that adenosine receptor activation appears to be a crucial event in the 'trigger' phase of rIPC. There was no statistically significant evidence of ischaemia-reperfusion injury seen when adenosine receptor blockade was undertaken in the 'effector' arm, demonstrating rIPC is still protective in this scenario.

These results are reinforced by the data from the dialysate study where there is a clear difference demonstrated between dialysate generated during the two arms of the study. Where adenosine receptors were blockaded in the 'trigger' arm there is no evidence in this study that the dialysate generated with blood taken post-rIPC is protective when used in the Langendorf mouse infarct model, suggesting adenosine receptor blockade inhibits the production or release of a circulating preconditioning factor. During the 'effector' phase of the study, dialysate generated from blood taken post-rIPC is highly protective in the mouse Langendorf model, significantly reducing infarct size. This second result is important not only to act as a positive control in showing that post-rIPC dialysate is protective, but also allays fears that the small systemic leak of caffeine measured in serum samples is able to inhibit the action of the

circulating preconditioning factor as caffeine levels were found to be extremely similar after both visits.

Whilst in both phases of the study a highly statistically significant rise in systemic caffeine levels was demonstrated with local intra-arterial infusion, as has been alluded to previously, the absolute levels remained extremely low at 2.6+/-0.3μM and far below the 10-30μM range at which effective inhibition of the A_1, A_{2A} and A_{2B} receptors has been previously demonstrated in human cells [205]. Whilst again, potential off target effects are always possible with any pharmacological inhibitor, as has been discussed in the previous chapter, these are only known to occur at very high concentrations of caffeine and it is likely therefore that the majority of the effect seen in this study is due to adenosine receptor antagonism.

Despite the highly significant decrease in infarct size seen with dialysate, no statistically significant improvement in post-infarction haemodynamic recovery was demonstrated. The reasons for this are speculative, but one possible explanation is that rIPC dialysate alone fails to protect against post-infarction ventricular stunning. The effects of preconditioning stimuli on myocardial stunning remain unclear with conflicting studies in the literature[216, 217] but the largest human study to date suggests that rIPC is not protective against stunning [218].

While there is no statistically significant evidence of IR injury seen during visit 2 ('effector'), it may be argued that there is evidence of at least partial abrogation of the rIPC effect with the pre and post rIPC and IR forearm blood flows not seen to be overlapping, as was demonstrated when establishing the rIPC and IR model for VOP. VOP, despite being regarded as the most sensitive technique to measure *in-vivo* endothelial function, is not precise enough to demonstrate such a partial blockade and with the large inter-individual variations seen with human subjects this seeming partial abrogation may simply be secondary to chance. Adding to this, there is no difference seen at peak dose when the vessel is maximally dilated suggesting that no true difference exists.

Again, as discussed in the previous chapter, the final analysis is based on the failure to observe a difference in one cohort, in comparison with a second cohort. It is again acknowledged that differences are therefore inferred rather than directly observed and may be influenced by the statistical power inherent in that cohort. However, as was previously mentioned all comparisons within this study are within groups only, with each individual effectively acting as their own control.

Once again, if the sample size calculations are repeated with the values seen in the actual cohorts then statistical power is once again preserved at 90% with an $\alpha=0.05$ with 6 subjects in each group.

These results stand against previous work in animal models of rIPC [120] in which blockade of adenosine receptors in the target 'effector' organ was able to effectively inhibit rIPC. Clearly, there are many differences between human and animal models and the purpose of the translational approach of this thesis is to ascertain which data is pertinent in humans. In this regard it would seem that in human rIPC at least, there are differences from animal models with adenosine receptor activation in the 'effector' arm of less importance than in the 'trigger' arm, although tissue effects (human endothelium vs. animal myocardium) should also be considered.

In the 'effector' phase of preconditioning, a variety of G-protein coupled receptor subtypes have been implicated including adenosine, bradykinin and opioid receptors. With this multiplicity of potential receptors, it may be that inhibition of a single receptor sub-type is insufficient to entirely block the effects of preconditioning whilst in the 'trigger' phase, adenosine receptor activation alone is far more important and inhibition is able to effectively block the entire arc. While the direct administration of adenosine at pharmaceutical doses to the 'effector' tissue has been repeatedly demonstrated to act as a preconditioning-like stimulus [170, 219, 220], this may be a very different process to that which occurs with the intrinsic mechanisms of rIPC.

Adenosine itself would not appear to be the humoral transferrable factor associated with rIPC with levels previously measured in dialysate samples seemingly far too low to activate preconditioning (personal communication, Dr M Tropak, University of Toronto).

Instead it would appear that there are either several factors acting in concert, akin to a stress signal, or there is a single as yet unidentified moiety capable of stimulating multiple different receptors to activate a preconditioning response. Work remains on-going to attempt to more firmly establish which of these possibilities is likely to be correct, but remains very much in progress.

CHAPTER 5: PERI-INFARCT ADENOSINE ADMINISTRATION AND MYOCARDIAL INFARCT SIZE

5.1 Introduction

Whilst this thesis concentrates mainly on the mechanisms of remote ischaemic preconditioning, the majority of clinically relevant episodes of myocardial ischaemia-reperfusion injury occur on an unpredictable basis and so would not necessarily be suitable for intervention with a preconditioning stimulus. The development of remote ischaemic perconditioning with which a cardioprotective stimulus could be given at the time of ischaemia has successfully dealt with this issue and heralds an important progression in the treatment of acute myocardial infarction. The mechanistic inter-play of pre-, per- and post-conditioning remain to be fully elucidated but while there are clearly important differences in these stimuli, there are also striking similarities. With growing evidence that adenosine played an important role in triggering preconditioning, in this proof of concept study, I decided to ascertain whether peripheral adenosine, given at the time of ischaemia, could reduce myocardial infarct size in a large mammal model of myocardial infarction.

5.2 Study Design and Goals

This study was designed as a proof of concept study to ascertain whether adenosine infused peripherally at the time of myocardial ischaemia was able to effectively reduce myocardial ischaemia-reperfusion injury. As a precursor to potential future human clinical trials, a large mammal model was chosen as being the most clinically applicable to human myocardial injury.

In a second aspect of the study I elected to use microdialysis catheters to measure interstitial intermediary metabolites and adenosine levels to ascertain tissue metabolic changes and whether there was evidence of central adenosine release in response to peripheral adenosine infusion as has been demonstrated in previous models and has been thought to be an important constituent in the effector phase of preconditioning [120]. This necessitated using an open chest model rather than the more physiologically elegant percutaneous angioplasty model, but this compromise was necessary to obtain information on interstitial metabolites.

With little previous data in similar models to guide adenosine dosing, this was derived from a study in patients undergoing aortic valve replacement where a large dose of adenosine was injected intra-aortically (1.5 mg/kg) while patients were on bypass, as a post-conditioning stimulus[221]. Finally, as there was no suitable large animal facility

locally, this work was carried out in the facility in Skejby University Hospital, Aarhus, Denmark with which our group has on-going collaborative links.

5.2.1 Methods

These methods are extensively described in the Methods chapter but are re-iterated here. Briefly, healthy 30kg mixed Danish Landrace and Yorkshire pigs were preanesthetized with midazolam (0.2mg/kg) and azaperone (7mg/kg) by subcutaneous injection. An ear vein was cannulated and animals induced with intravenous etomidate (0.4mg/kg), the trachea intubated and subsequently ventilated at 4.5 L/min with a 50/50 mixture of atmospheric air and oxygen maintaining a PEEP of 3.5cmH_2O and a tidal volume of 400ml. Anaesthesia was maintained with intravenous pentobarbital (6mg/kg/hr). By means of a cut-down procedure the jugular vein and carotid arteries were identified and cannulated with 6Fr introducer sheaths. A median sternotomy was performed, the pericardial sack divided and the heart exposed. A snare was placed around the LAD just distal to the first diagonal artery but not tightened at this stage. CMA 20 microdialysis catheters with a molecular cut off of 20000 Da were placed in 5 areas – the right atrium, the left ventricular free wall in the non-risk area, 2 probes were placed in the area at risk of the LV and a fifth probe placed in the muscle of the hind limb.

A 5000u loading bolus of heparin was administered following completion of surgical procedures and a further 2000u of heparin were given hourly during the study. Core temperature was carefully maintained between 36.5 and 38˚C with the use of heating blankets. Hydration was maintained by saline infusion at 200ml/hr. Hourly blood gas analysis allowed alterations in ventilation and electrolytes to maintain parameters within physiological norms. After a 60 minute stabilisation period the LAD snare was tightened and a 40 minute ischaemia period commenced. Ischaemia was confirmed by visual inspection of the LV wall which rapidly appeared cyanosed. Five minutes prior to the end of ischaemia a standard 6Fr pigtail catheter was passed into the distal descending aorta and a bolus of 1.5mg/kg of adenosine or 10ml of saline (placebo) was administered. At the completion of 40 minutes of ischaemia the snare was loosened and reperfusion commenced. During ischaemia and reperfusion episodes of VF or haemodynamically unstable VT were treated with DC cardioversion but no antiarrhythmic drugs or inotropes were utilised with only fluid supplementation allowed to maintain blood pressure if necessary. Reperfusion was for 120 minutes after which the snare was retightened around the LAD and the heart perfusion stained with 10% Evans Blue. The animal was euthanized and the heart excised. The right atrium and ventricles were removed and the heart placed in the freezer for approximately thirty minutes (see Fig. 5.1).

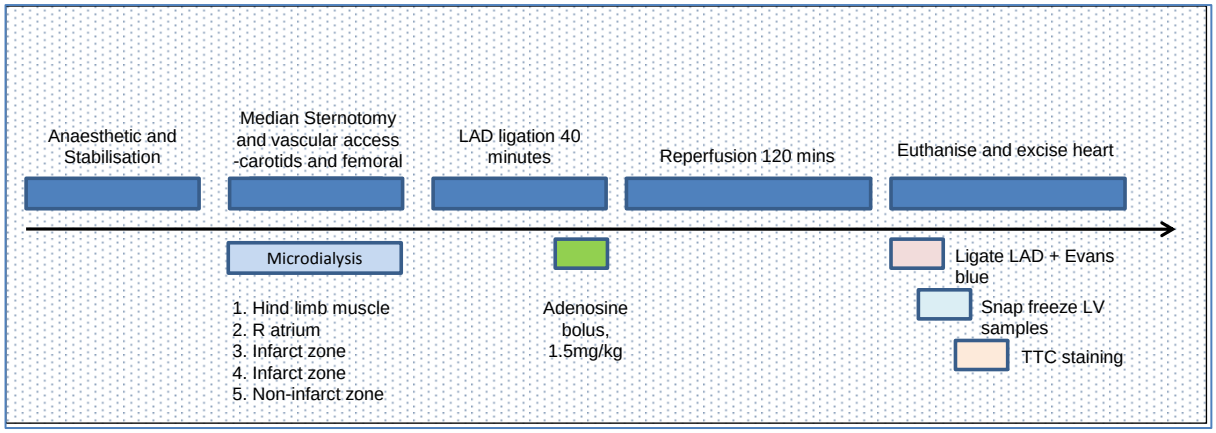


Figure 5.1 Schematic of protocol for peri-infarct adenosine studies. Adenosine (or placebo) was delivered peripherally through a catheter passed into the distal descending aorta five minutes prior to reperfusion.

The left ventricle was then sliced into 8 mm slices from the apex to the base perpendicular to the septum and slices incubated in 1% 2,3,5-triphenyltetrazolium chloride (pH 7.4) at 37°C for 15 minutes. All slices were then scanned on a flat-bed digital scanner and weighed. Computerised planimetry used to assess area at risk, area not at risk and infarct size allowing calculation of final infarct size as a percentage of mass of area at risk.

Microdialysis sample analysis was achieved using a novel mass spectrometry protocol developed by Dr R Birkler at the Department of Forensic Medicine, Aarhus University. This is fully described in the methods section and is not repeated here.

5.2.2 Power Calculation and Statistical Analysis

Power calculations were based on historical data from our group and others using the Aarhus facility. By assuming that the difference in infarct size between studies is Normally distributed with a standard deviation of 11% it was estimated that with nine subjects per group it would be possible to detect a true difference in mean response of 15% with power of 90% and an α=0.05.

The difference in means between the adenosine and control groups was tested using unpaired t-tests.

5.3 Results

A total of 32 studies were performed with animals randomly allocated in a 1:1 fashion to either adenosine or placebo. Of these only 22 studies survived to protocol end and were suitable for dye staining and infarct size estimation leaving n=11 in each group. One animal died peri-anaesthesia, three had a fibrinous pericarditis demonstrated on opening of the chest making them unsuitable for further study and the others did not survive due to intractable arrhythmias or the haemodynamic consequences of their infarcts. Of these animals, 3 had adenosine and 2 received placebo with one dying prior to the administration of either. Four animals had episodes of atrial fibrillation prior to induction of ischaemia but were electrically cardioverted back into sinus rhythm prior to further study procedures.

In baseline characteristics, there was no significant difference found between the two groups for either area at risk or for LV mass. The area at risk as a percentage of the left ventricle in the control group was 43.1+/-1.4% and in the adenosine group was 40.1+/-1.9% (p=0.22 – unpaired t-test) (see fig 5.2) and similarly left ventricular mass in the control group was 103.6+/-3.3g and in the adenosine group was 109.4+/-4.3g (p=0.29 – unpaired t-test) (see Fig 5.2).

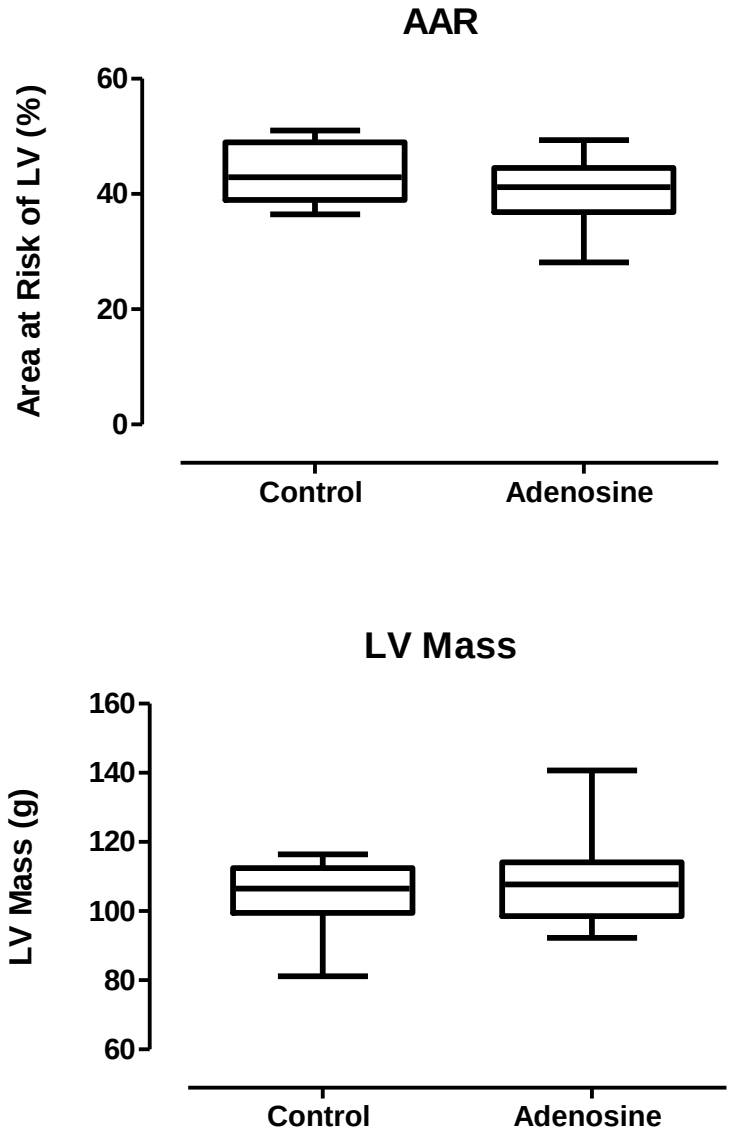


Figure 5.2 Area at risk and left ventricular mass. There were no significant differences seen between groups in either parameter. Box and whiskers represent median and 5/95 percentiles.

Adenosine infusion just prior to reperfusion reduced infarct size from a mean of 62.9+/-3.1 % of LV area at risk to 46.4+/-3.6% (p=0.003 – unpaired t-test) (Fig. 5.3). No significant difference was seen in the number of malignant arrhythmias requiring DC cardioversion between the two groups with similar numbers of episodes of VT and VF occurring.

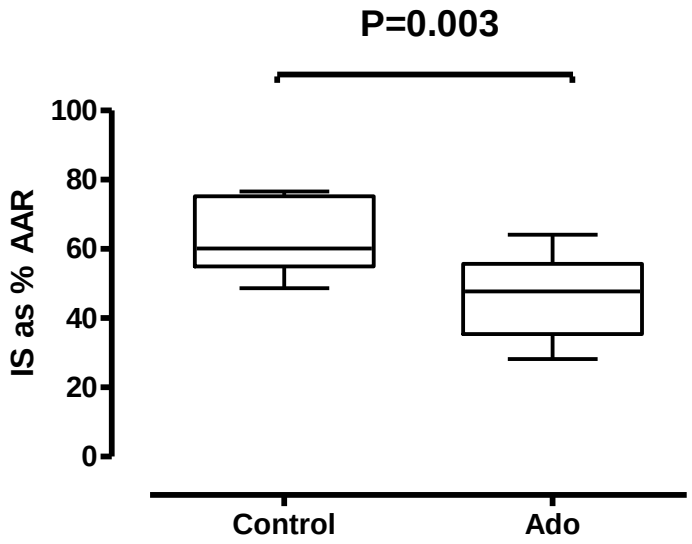


Figure 5.3 Peri-infarct peripheral adenosine infusion reduces infarct size. Adenosine infused peripherally five minutes before reperfusion reduced mean infarct size from 62.9+/-3.1 % to 46.4+/-3.6% (P=0.003, unpaired t-test). Box and whiskers represent median and 5/95 percentiles.

The data for mean arterial pressure and heart rate are presented below, derived from logged ventilator data with mean arterial pressure directly transduced through a carotid arterial line and heart rate derived from the ECG (Fig 5.4). There was no significant difference apparent between the adenosine and control groups for either parameter (2-way ANOVA). No significant episodes of AV block were seen in any study on adenosine administration.

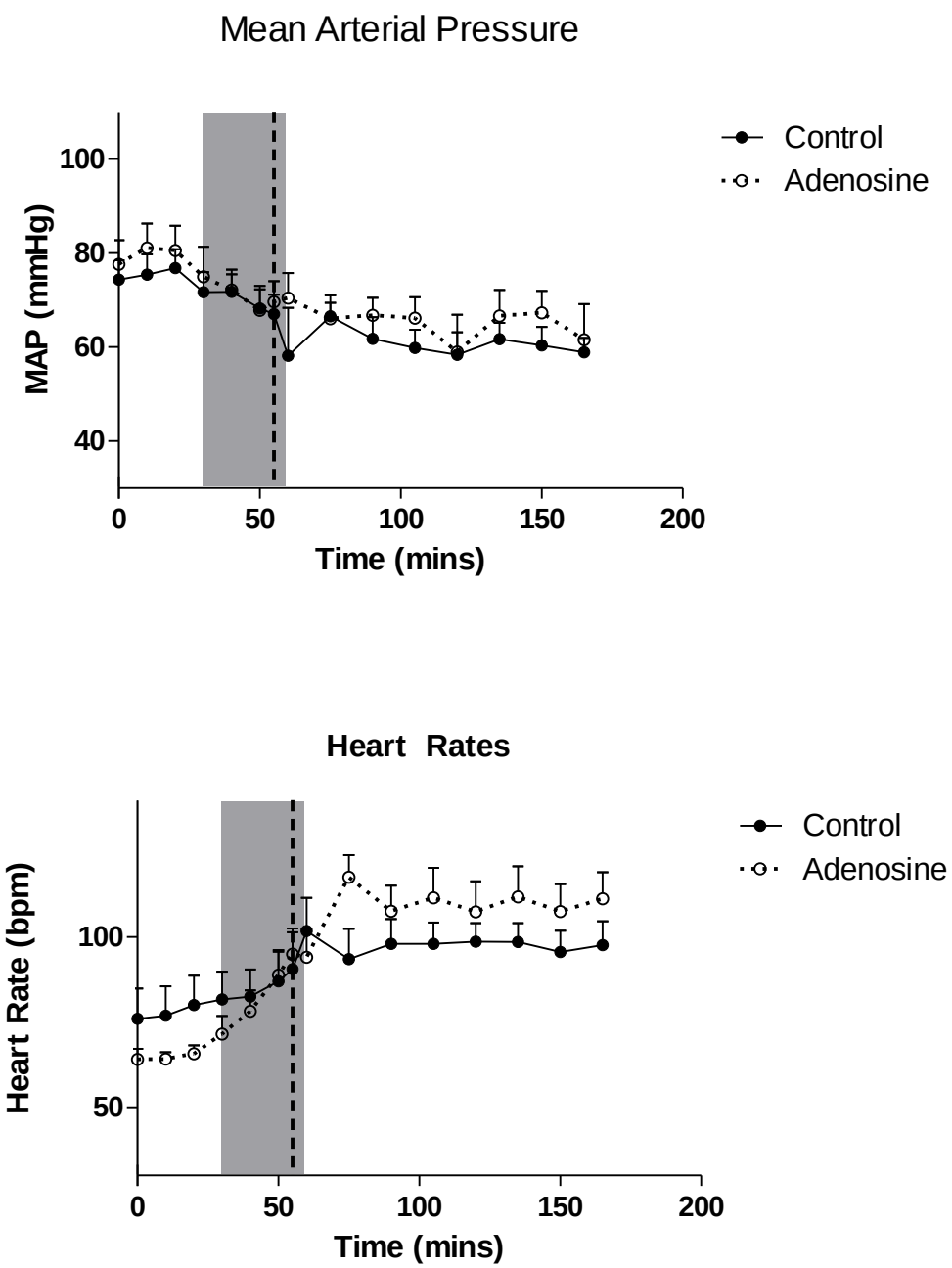


Figure 5.4 Mean arterial pressure and heart rates for adenosine and control groups. There were no significant differences seen between groups for either parameter and no evidence of improved haemodynamic recovery in the adenosine group. Grey shaded area, ischaemic period; Dashed line, time of peripheral adenosine infusion. Points represent mean +/- SEM.

5.3.1 Microdialysis Results

Due to a series of technical problems and the novel institutional use of microdialysis in a large animal model, a meaningful analysis of the microdialysis data is challenging with many samples lost during processing with consequent analysed sample numbers ranging from only 2-6 in many cases. This being the case, no meaningful analysis was possible from the results of the data.

5.4 Discussion

This study examines whether peripheral arterial adenosine delivery just prior to reperfusion is able to confer cardioprotection. In a second aspect of the study novel microdialysis techniques were used to try and examine whether such an infusion triggers a reflex arc, with subsequent central adenosine release important in the stimulus as has been previously described [120].

The findings from the study demonstrate that peripheral adenosine delivery does reduce experimental infarct size, presumably by triggering a cytoprotective stimulus that modulates the effects of reperfusion injury. While highly statistically significant, the absolute effect size was not large with only a 15% reduction in absolute infarct size although this equates to a 25% relative reduction in size. In terms of preconditioning

effects therefore this modulation is modest with reductions of greater than 50% being reported when ischaemic conditioning stimuli are used.

Several factors may be important in this regard. Most reductions in infarct size of this magnitude are reported in experimental models where the stimulus is delivered prior to the onset of ischaemia, which may modulate both ischaemic injury and reperfusion injury. With the aim being to investigate a clinically relevant model of myocardial infarction, the stimulus in this case was delivered after the completion of more than 85% of the ischaemic period by which time point a substantial amount of cardiac tissue may have been irretrievably injured.

Secondly, while preconditioning is clearly a phenomenon with multiple redundant pathways and multiple potential stimuli, it may be that when activated by delivery of a single autocoid stimulus, with only one cell surface receptor type being activated, this may lead to a sub-maximal conditioning stimulus.

Thirdly, there is little available literature describing adenosine receptor distribution in pigs. The dose of 1.5mg/kg was derived from a study of human subjects undergoing aortic valve replacement where delivery of adenosine at this dose into the aorta while under the carefully controlled conditions of cardiopulmonary bypass was shown to be effective in reducing tissue injury. Due to the lack of a large animal facility locally, this work was conducted in the facility based at Skejby Hospital in Aarhus, Denmark. Due to

the complex logistics and high expense associated with this work, little time was available for model development and pilot experiments reducing the ability to ascertain the effects of other doses in this model. This was undoubtedly a high dose and whilst not captured in the ventilator haemodynamic data, significant transient hypotension was seen in a number of experiments potentially limiting the use of such a dose in standard clinical practice. However, the study did provide valuable proof of principle data.

The microdialysis data, whilst initially seen as a very important aspect of the study, unfortunately proved disappointing. The use of microdialysis in an intact large mammal model was not well-established at the time of the study, nor was the use of MS technology to characterise an array of metabolites in microL quantity samples, in that centre. Both endeavours provided a steep learning curve with multiple technical problems encountered with sample collection equipment, catheter placement and displacement and catheter fracture due to "grind" from the contracting myocardial tissue. With further technical problems besetting the MS analysis, it has taken the majority of samples over two years to be analysed, leaving no scope to revisit studies where samples were inadequate or to repeat work to generate further substrate for analysis. With the experience gained from my studies and others, the majority of these issues have now been dealt with and microdialysis now forms a routine part of many studies conducted in similar models in Aarhus.

The use of microdialysis necessitated the switch to an open chest model of myocardial infarction (as opposed to an angioplasty balloon model). The open chest model, whilst widely used, clearly adds a number of physiological compromises that may affect myocardial infarct size. The initial surgical procedure of median sternotomy will inevitably activate systemic inflammatory responses, clouding normal physiological responses. Similarly, the open chest leads to the loss of normal intrathoracic pressure as does the loss of the surrounding pericardial sack which may subtly change myocardial contractility and function and, perhaps more importantly, greatly effect thermoregulation. This is extremely important and great care was taken to keep core temperature within tight prescribed limits, but it is difficult to definitively measure and regulate myocardial temperature in such a model. Finally, the use of a snare to externally occlude the epicardial coronary artery is entirely different to the intraluminal obstruction seen with a conventional myocardial infarction and due to the venous anatomy of the heart, cardiac veins are inevitably encompassed in the same snare, likely significantly increasing myocardial tissue oedema. Whilst all of these limitations apply equally to the intervention and control group, the model as a whole should be considered in the light of these.

A further criticism of similar models is the possibility of collateral circulation affecting results. The incidence of a significant collateral circulation in porcine hearts is thought to occur in less than 10% of animals (personal communication, J Vinten-Johansen, Emory University). Some investigators utilise injectable microspheres to test for collateralisation but this technique was not available at the experimental centre where

the work was conducted. However, with a relatively low incidence and a randomisation of group allocation, it is unlikely that collateral arterial circulation will have greatly affected the results of this study.

Once more no significant difference in post infarction haemodynamics was seen despite the reduction in infarct size. As mentioned previously preconditioning may not affect stunning, and this would seem the most likely explanation for this.

Future experiments with a closed chest model using angioplasty to induce myocardial infarction may be preferable to attempt to minimise many of the above issues. Indeed, in this respect, a more appropriate model may be in an atherosclerotic pig, with some mini-pig models being bred to display atherosclerotic features[222] although studies in these animals remain expensive. However, as a whole, large mammal models provide an excellent translational stepping-stone in testing interventions in a clinically relevant fashion, and as a whole, this study fits well with the goals of this thesis.

In summary, adenosine was effective at reducing infarct size when delivered into the peripheral arterial circulation prior to reperfusion. This proof of concept study lays the basis for further experimental and future clinical studies in this field.

CHAPTER 6: ADENOSINE IN THE RELEASE OF A CIRCULATING PRECONDITIONING FACTOR

6.1 Introduction

Previous experimental work presented as part of this thesis and work by other groups demonstrates that adenosine receptor activation is important in 'triggering' a rIPC response and that rIPC itself releases a blood borne circulating factor that is transferrable to other mammalian species and is able to induce cytoprotection against the effects of ischaemia-reperfusion injury. In conjunction with these findings, my data also demonstrates that peripheral adenosine infusion can trigger conditioning-like mechanisms and reduce myocardial infarct size in a large mammal model.

Taken together, the logical next step was to ascertain whether peripheral adenosine infusion could trigger the release of a circulating cardioprotective humoral factor in humans, particularly humans with atherosclerotic coronary disease, in whom such protection is likely to be of the greatest benefit.

The aims of this study were therefore to ascertain the translational relevance of my previous findings by administering intra-arterial adenosine to human patients in a range of doses which might elicit a cardioprotective factor, to examine whether there was

any evidence of a dose response relationship, and finally to observe the side-effect and tolerability profile of these doses. The study protocol received ethical approval from Oxfordshire Research Ethics Committee A (ref 10/H0604/28).

6.2 Study Design and Participants

The study was a randomised dose ranging study of the effectiveness of adenosine in elaborating a humoral preconditioning factor. Study participants were male or female patients aged 18 years or over with known or suspected coronary artery disease, undergoing clinically mandated coronary angiography. Study participants were supplied with study information a minimum of 7 days prior to attending for coronary angiography and were asked to abstain from caffeine containing substances for a minimum of 24 hours prior to their procedure and be fasted for a minimum of 4 hours on the morning of their study.

Exclusion criteria included a significant angina episode within the previous 4 hours of the study or a myocardial infarction or admission with unstable angina within 4 weeks of the study, a history of diabetes mellitus or impaired glucose tolerance and/or recent use of sulfonylureas, known asthmatic or history of bronchospasm or currently taking theophyllines or dipyridamole, left main stem disease or severe triple vessel disease requiring CABG and severe (ejection fraction < 30%) impairment of left-ventricular function.

6.3 Methods

The study was conducted in the coronary catheter laboratory at the John Radcliffe Hospital. After obtaining written informed consent, patients were draped in the usual sterile fashion and a 6Fr femoral arterial sheath inserted for angiography (all angiograms were conducted from the femoral route). A 4Fr sheath was inserted into the femoral vein to allow rapid vascular access in-case of urgent clinical necessity and also acted as a convenient route from which to take blood samples.

Standard diagnostic coronary angiography was performed and if the operator was satisfied that the participant's coronary anatomy was suitable, the study proceeded with the withdrawal of 75ml of blood from the femoral venous sheath into heparinised containers. Patients were randomised to one of 4 doses of adenosine (0.25mg/kg, 0.5mg/kg, 0.75mg/kg or 1.0mg/kg). An adenosine solution (total volume 30ml) was then infused through the arterial sheath over 1 minute with central pressure monitoring and continuous ECG recording.

If the operator developed concerns regarding patient symptoms, haemodynamics or heart rhythm disturbances the infusion could be terminated or the rate slowed, with residual adenosine likely to be cleared within a few seconds. Five minutes after the completion of infusion a second blood sample was taken of the same volume as the first and the study concluded (see Fig. 6.1).

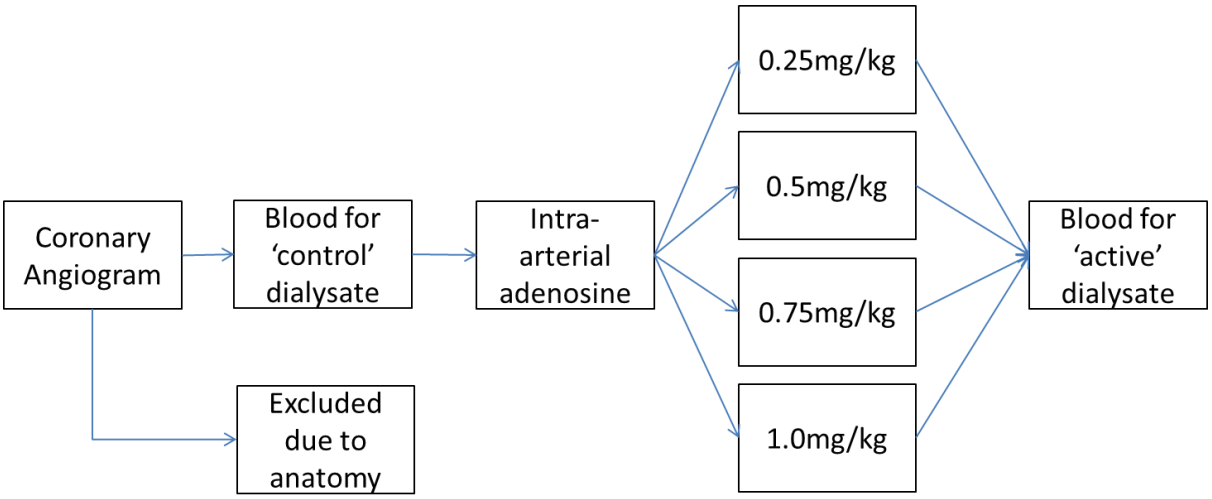


Figure 6.1 Schematic of study protocol. Participants are patients attending for clinically mandated elective coronary angiography.

6.3.1 Manufacture of Dialysate.

Heparinised blood was transported immediately on ice and centrifuged at 4˚C for 10 minutes at 4640g. The plasma was separated off and used to prepare dialysate, whilst the cellular fraction was discarded. The plasma was placed into a 15kDA cut-off membrane and dialysed against a modified Krebs-Henseleit buffer for 48 hours at 4˚C. The plasma was subsequently discarded and the dialysate decanted and stored. By taking blood both before and after infusion, both pre and post infusion dialysate was generated from each study subject. By perfusing this dialysate into an isolated perfused heart model prior to inducing no-flow ischaemia and reperfusion, it has been previously demonstrated that dialysate containing the preconditioning factor is able to reduce infarct size in comparison to control dialysate.

6.3.2 Mouse Langendorf Model of Ischaemia-Reperfusion

Mouse Langendorf ischaemia-reperfusion studies were carried out by Dr J Li at the Division of Cardiology, Hospital for Sick Children, Toronto. All Langendorf studies were performed blinded to group allocation and analyses undertaken by a second blinded observer also unaware of group allocation. Briefly, 25g C57BL/6 mice were euthanized using an IP injection of pentobarbital (300mg/kg) also containing heparin (150 IU). A midline sternotomy was performed and the heart rapidly excised and placed into cold cardioplegic solution (4˚C). The heart was transferred to the Langendorf apparatus, the aorta cannulated and the heart retrogradely perfused at a constant pressure of 80mmHg under non-recirculating conditions with a modified Krebs-Henseleit (KH) buffer (see methods section). Hearts were immersed in a water-jacketed chamber containing KH buffer maintained at 37^{O}C. The left atrium was excised and left ventricular (LV) pressure transduced through a fluid-filled balloon inserted into the LV cavity and left ventricular end-diastolic pressure (LVEDP) was adjusted to between 5 and 10 mmHg.

6.3.3 Power Calculation and Statistical Analysis

Power calculations were based on historical data from previous mouse Langendorf studies. By assuming that the difference in infarct size between studies is Normally distributed with a standard deviation of 10.8% it was estimated that with nine subjects

per group it would be possible to detect a true difference in mean infarct size of 15% with power of 90% and an α=0.05.

The difference in means between the pre- and post-adenosine dialysate groups was tested using unpaired t-tests.

6.4 Results

Results are presented below and are divided into haemodynamic and arrhythmia data which represents the primary safety and tolerability aspect of the study and infarct data from the mouse Langendorf studies representing the primary efficacy outcome. Data is further sub-divided by adenosine dose. A total of 33 patients were eligible and agreed to participate in the study out of a cohort of 219 patients whom were invited over the 12 month study period. Patients were invited simply from routine elective angiography lists with no prior knowledge of their clinical status so many were excluded on the day due to ineligibility. Four studies did not proceed to adenosine infusion due to high risk characteristics found at angiography or unrelated procedural issues. Demographics for the participants receiving adenosine are detailed below (Table 6.1).

	0.25mg/kg	0.5mg/kg	0.75mg/kg	1.0mg/kg
N (M/F)	10 (9/1)	5 (3/2)	10 (9/1)	4 (3/1)
Age (years)	60.4+/-4.1	64.8+/-4.4	61.9+/-2.4	62.8+/-4.7
Smoking status n (%)				
Current or recent	3 (30)	0 (0)	1 (10)	0 (0)
Ex-smoker	3 (30)	2 (40)	4 (40)	1 (25)
Never	4 (40)	3 (60)	5 (50)	3 (75)
Resting MAP (mmHg)	96.8+/-6.5	93.6+/-6.3	92.9+/-3.0	99.2+/-13.3
BMI (kg/m^2)	28+/-1.1	27.3+/-1.4	25.9+/-1.1	25.1+/-0.7
Hypertension n (%)	3 (30)	2 (40)	4 (40)	0 (0)
Family History n (%)	2 (20)	3 (60)	4 (40)	1 (25)
Previous MI n (%)	1 (10)	3 (60)	4(40)	0 (0)
Statin use n (%)	6 (60)	4 (80)	9 (90)	4 (100)
ACE Inh use n (%)	4 (40)	4 (80)	4 (40)	2 (50)
Aspirin use n (%)	9 (90)	5 (100)	10 (100)	4 (100)
Coronary Disease				
No sig (%)	4 (40)	0 (0)	1 (10)	2 (50)
1-vessel (%)	3 (30)	4 (80)	3 (30)	1 (25)
2-vessel (%)	2 (20)	0 (0)	4 (40)	1 (25)
3-vessel (%)	1 (10)	1 (20)	2 (20)	0 (0)

Table 6.1 Demographic characteristics of patients participating in the study. Patients are divided by dose of adenosine infused. ACE Inh, angiotensin converting enzyme inhibitor.

6.4.1 Patient Haemodynamic and Arrhythmia Data

Patient data for haemodynamic response and arrhythmias is presented below by dose.

a) 1 mg/kg

	Occurrence n (%)
1st Degree	0 (0)
2nd Degree	2 (50) (all 2:1)
3rd Degree	0 (0)
Other	1 (25) – paroxysmal AF

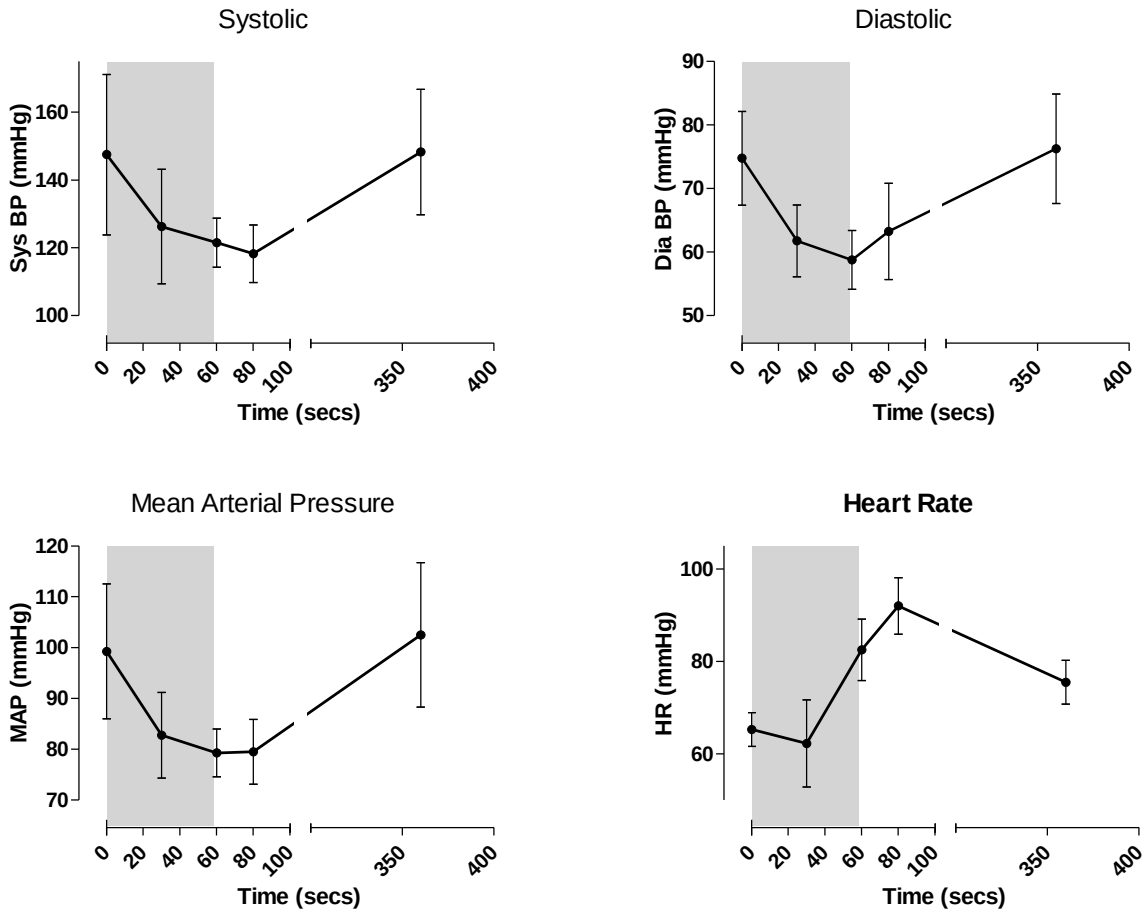


Table 6.2 and Figure 6.2 Arrhythmia and haemodynamic data for participants receiving 1mg/kg adenosine. Episodes of 2:1 block were transient but one individual required the infusion to be slowed due to symptoms. The episode of PAF was in a patient with prior history of PAF and self-reverted within 2 hours. Mean arterial pressure dropped from a mean of 99.2+/-13.3 to 79.2+/-4.7 at 60 seconds but did not recover rapidly, with a MAP of 79.5+/-6.4 recorded at 80 seconds (p=0.33, 1-way ANOVA). MAP had returned to baseline by 5 mins. Similarly heart rate increased from 65.3+/-3.7 to 82.5+/-6.6 at 60 seconds (p=028, 1-way ANOVA) and continued to increase to 92 +/-6.1 at 80 seconds before returning towards baseline at 5 mins. Grey shaded area, adenosine infusion. Points represent mean +/- SEM.

b) 0.75mg/kg

	Occurrence n (%)
1st Degree	1 (10)
2nd Degree	4 (40) (all 2:1)
3rd Degree	0 (0)
Other	0 (0)

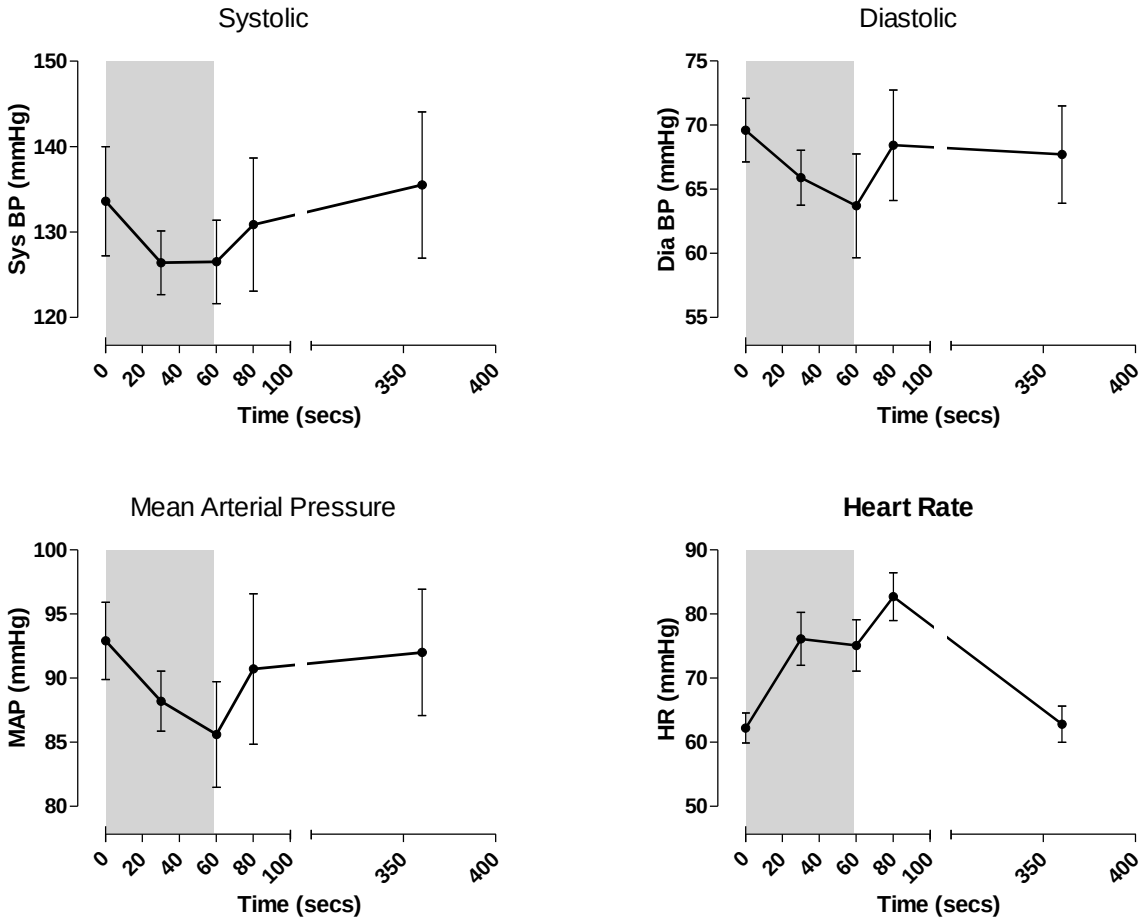


Table 6.3 and Figure 6.3 Arrhythmia and haemodynamic data for participants receiving 0.75mg/kg adenosine. The first degree heart block was pre-existing but became significantly prolonged (without symptoms) prior to returning to baseline. Episodes of 2:1 block were transient and required no intervention. Mean arterial pressure dropped from a mean of 92.9+/-3.0 to 85.6 at 60 seconds (p=0.69, 1-way ANOVA) but recovered rapidly, with a MAP of 90.7+/-5.9 recorded at 80 seconds. Heart rate increased from 62.2+/-2.3 to 75.1+/-4.0 at 60 seconds (p=0.006, 1-way ANOVA with post hoc Bonferroni) and continued to increase to 82.7+/-3.7 at 80 seconds before returning to baseline at 5 mins. Grey shaded area, adenosine infusion. Points represent mean +/- SEM.

c) 0.5mg/kg

	Occurrence n (%)
1st Degree	0 (0)
2nd Degree	1 (20) (all 2:1)
3rd Degree	0 (0)
Other	0 (0)

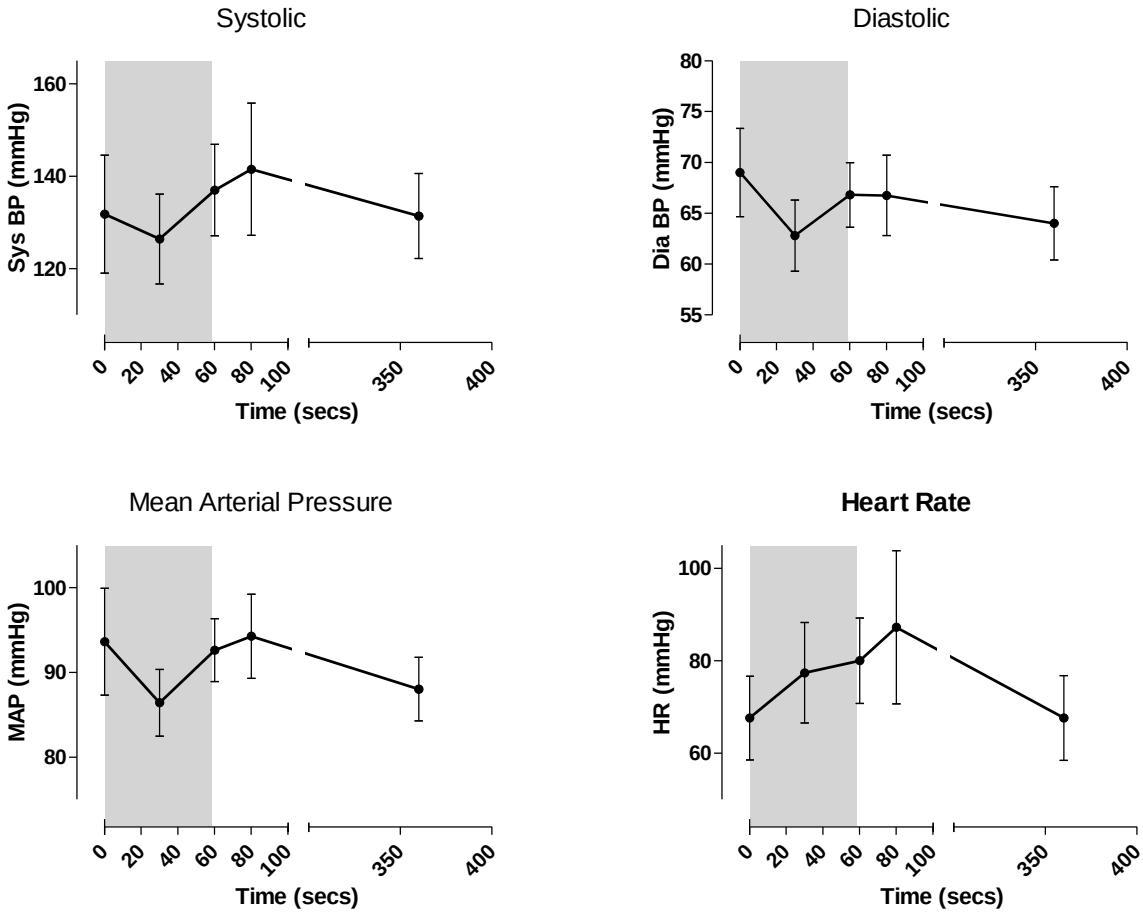


Table 6.4 and Figure 6.4 Arrhythmia and haemodynamic data for participants receiving 0.5mg/kg adenosine. Episodes of 2:1 block were transient and required no intervention. Mean arterial pressure dropped from a mean of 93.6+/-6.3 to 86.4 at 30 seconds but recovered very rapidly, with a MAP of 92.6+/-3.7 recorded at 60 seconds (p=0.68, 1-way ANOVA). Heart rate increased from 67.6+/-9.0 to 80+/-9.2 at 60 seconds (p=0.69, 1-way ANOVA) and continued to increase to 87..2+/-16.6 at 80 seconds before returning to baseline at 5 mins. Grey shaded area, adenosine infusion.

d) 0.25mg/kg

	Occurrence n (%)
1st Degree	0 (0)
2nd Degree	0 (0)
3rd Degree	0 (0)
Other	0 (0)

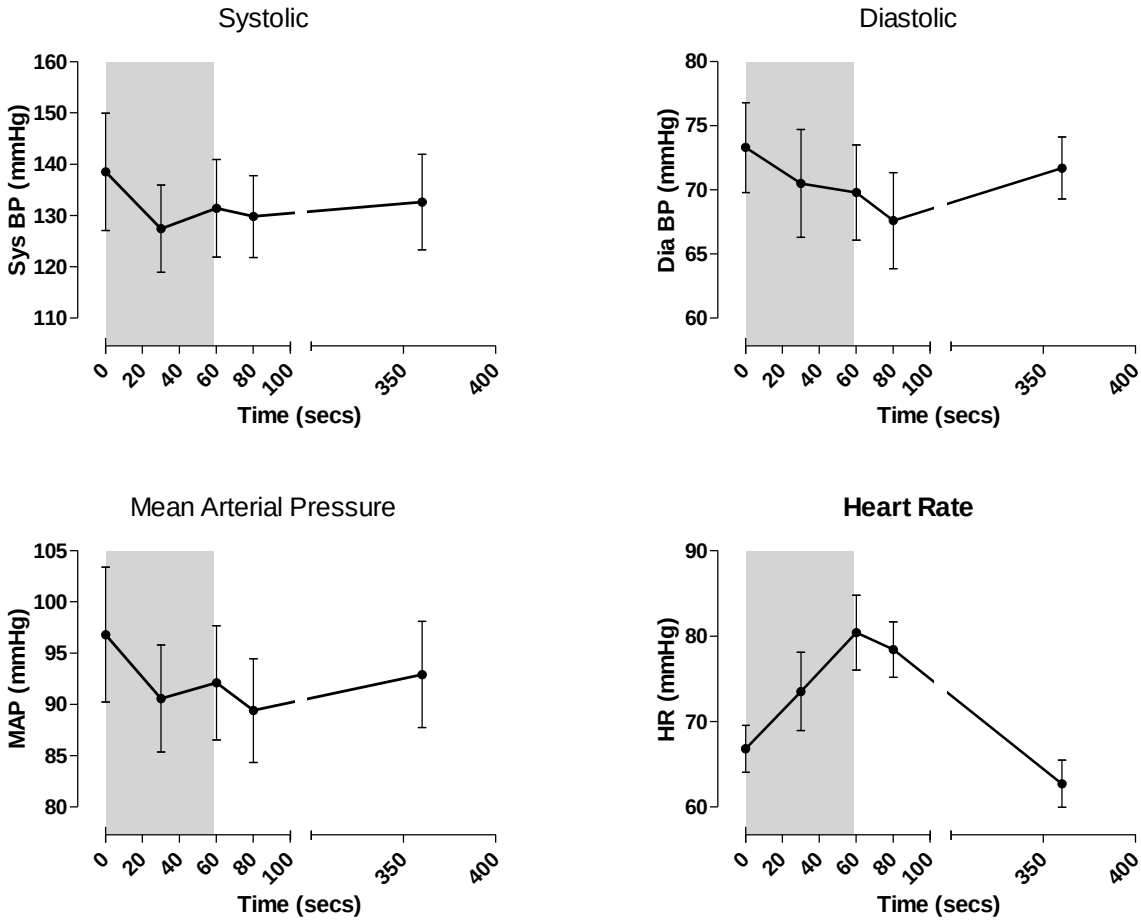


Table 6.5 and Figure 6.5 Arrhythmia and haemodynamic data for participants receiving 0.25mg/kg adenosine. There were no episodes of arrhythmia noted. Mean arterial pressure dropped from a mean of 96.8+/-6.6 to 90.6+/-5.2 at 30 seconds and recovered rapidly (p=0.9, 1-way ANOVA at 60 seconds). Heart rate increased from 66.8+/-2.8 to 80.4+/-4.4 at 60 seconds (p=0.005, 1-way ANOVA with post hoc Bonferroni) but decreased to 78.4+/-3.2 at 80 seconds and had returned to baseline when recorded at 5 mins. Grey shaded area, adenosine infusion. Points represent mean +/- SEM.

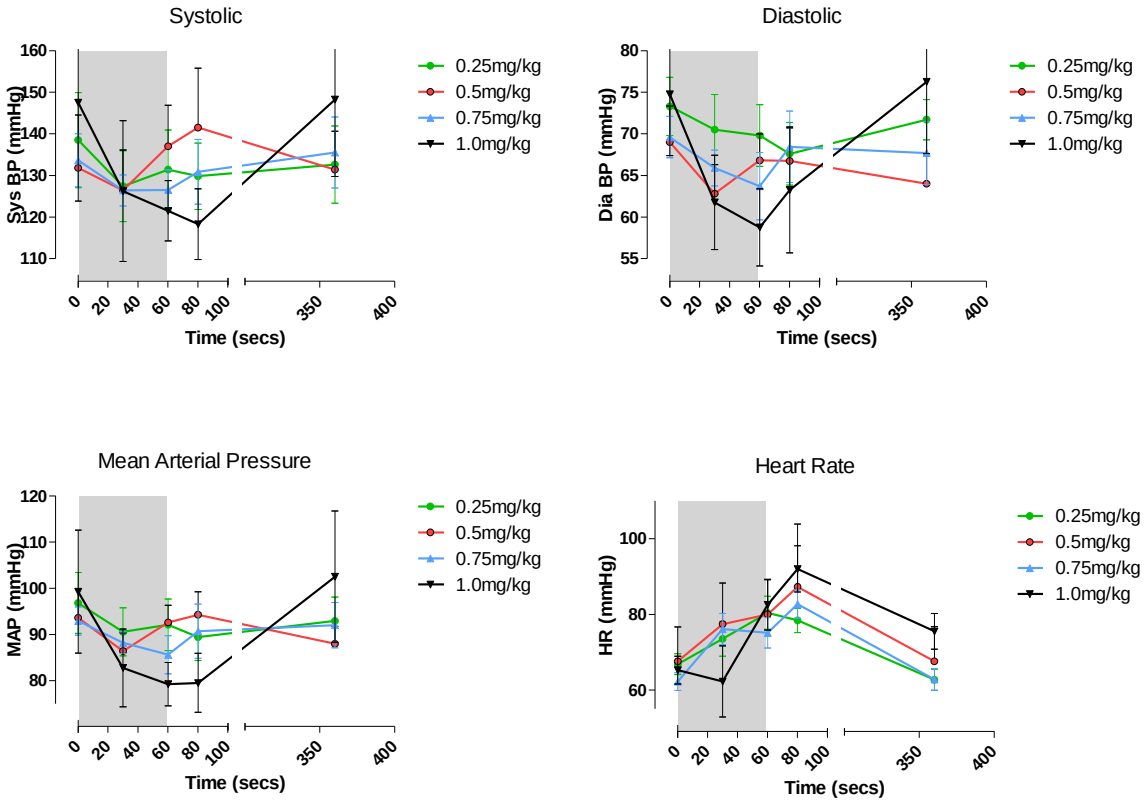


Figure 6.6 Amalgamated haemodynamic data for participants receiving adenosine. Grey shaded area, adenosine infusion. Points represent mean +/- SEM.

As can be seen from the above descriptive figures and is particularly illustrated in Fig. 6.6, not unexpectedly, the higher dose adenosine infusions caused greater degrees of haemodynamic compromise, which took longer to recover and also had a higher incidence of transient degrees of AV block and arrhythmias. While no emergency corrective therapy was required for any patient to reverse these effects, the degree of patient compromise seen with the highest dose of 1.0mg/kg was such that it was rapidly apparent that whilst not unsafe *per se*, this dose was not well tolerated and was not likely to be clinically viable.

Further randomisation to this dose was therefore discontinued and with the not inconsiderable difficulties surrounding recruitment (only 15% of potentially eligible candidates were recruited) it was decided to concentrate further efforts on the highest and lowest remaining doses in the study with further data presented here being for the 0.25mg/kg and 0.75mg/kg doses only.

6.4.2 Langendorf Infarct size data

For dialysate generated from the 0.75mg/kg cohort, mean infarct size was 47.1+/-1.9% with pre-infusion dialysate which significantly reduced to 35.7+/-2.1% when hearts were perfused with post-infusion dialysate (n=12 hearts, p=0.001 – paired t-test) (Fig. 6.7). There was no significant difference seen in post-infarct haemodynamic recovery (Fig.6.8).

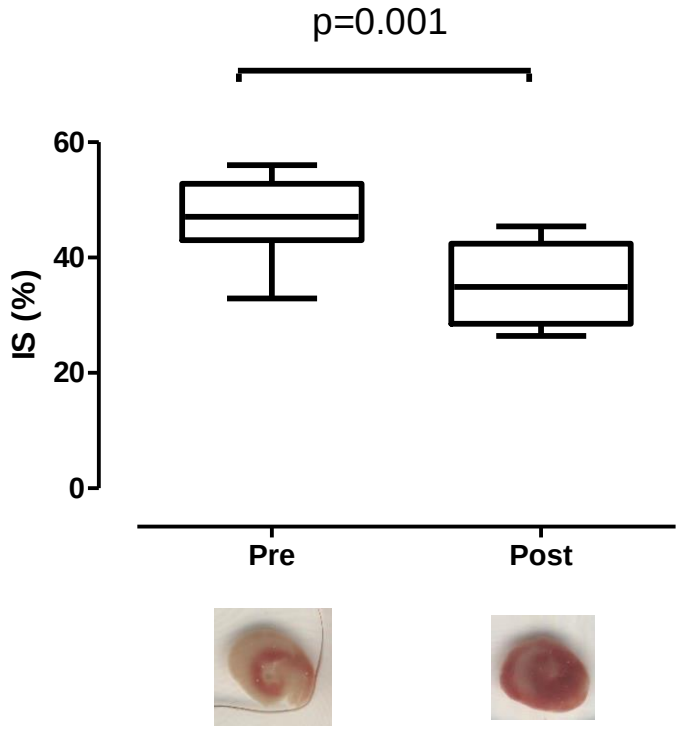


Figure 6.7 Dialysate post 0.75mg/kg peripheral adenosine infusion significantly reduces infarct size in a mouse Langendorf model. Representative heart slices shown. Box and whiskers represent median and 5/95 percentiles.

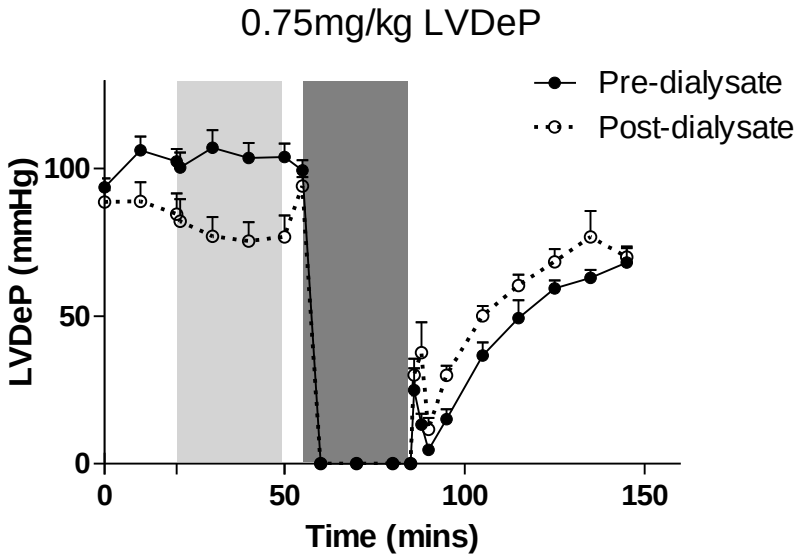


Figure 6.8 Dialysate post 0.75mg/kg peripheral adenosine infusion does not significantly improve post infarction haemodynamic recovery. Points represent mean +/- SEM.

For dialysate taken from the 0.25mg/kg cohort, mean infarct size was 48.3+/-2.9% with pre-infusion dialysate which significantly reduced to 34.0+/-2.6% when hearts were perfused with post-infusion dialysate (n=10 hearts, p=0.006 – paired t-test (Fig 6.9). Again, no significant difference was seen in post-infarct haemodynamic recovery (Fig.6.10).

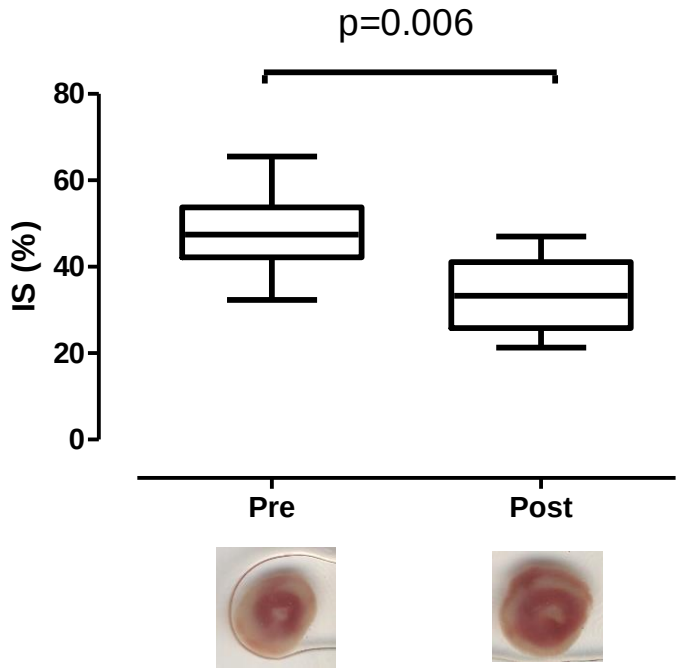


Figure 6.9 Dialysate post 0.25mg/kg peripheral adenosine infusion significantly reduces infarct size in a mouse Langendorf model. Representative heart slices shown. Box and whiskers represent median and 5/95 percentiles.

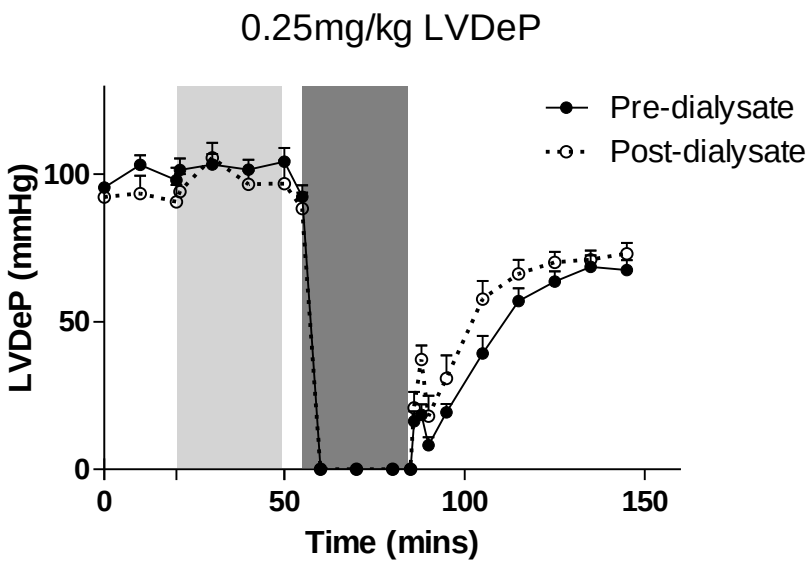


Figure 6.10 Dialysate post 0.25mg/kg peripheral adenosine infusion does not significantly improve post infarction haemodynamic recovery. Points represent mean +/- SEM.

6.5 Discussion

This study demonstrates for the first time that peripheral intra-arterial adenosine infusion in humans elaborates a transferrable blood borne cytoprotective factor that is able to reduce myocardial infarct size. At the doses tested, haemodynamic responses were acceptable and any side effects were transient and did not require corrective intervention, laying the basis for future clinical studies.

Both the 0.75 and 0.25mg/kg doses significantly reduce myocardial infarct size in the mouse Langendorf model and both were well tolerated by patients. Clearly however, the 0.75mg/kg dose caused more profound haemodynamic and conduction system disturbance than the 0.25mg/kg dose hence this would be the preferred dose for future translational studies.

This study has concentrated only on ascertaining the effects of intra-arterial adenosine and it remains unclear whether in humans intravenous adenosine may produce similar effects. It was the original intention of the study to examine intravenous infusion of adenosine also in a similar patient cohort and ethical approval was sought and gained for this aspect of the study. However, certainly at the higher doses, and even at the lower dose of 0.25mg/kg, intravenous infusion is likely to lead to much greater haemodynamic disturbance and occurrence of AV block, with adenosine travelling rapidly to the myocardium. Due to this, and the aforementioned recruitment difficulties, no intravenous infusions were undertaken. Certainly, the recurrent

inference from pre-clinical models in the literature is that intra-arterial and not intravenous adenosine infusion is key in elaborating cardioprotection. With the success of adenosine in achieving this in humans it is doubtful whether a great deal will be achieved by further study of the intravenous route, as adenosine is unlikely to be delivered in a pre-hospital environment currently.

A second issue regarding delivery route also pertains to the increasing use of radial access in interventional coronary procedures. All infusions undertaken in this study were performed from the femoral route as a pre-mandated part of the study design. This decision was taken in light of a human conditioning study in which two cycles of IR were sufficient to elicit cytoprotection when done on the leg but insufficient when done on the arm where three cycles were required[136], with the inference that tissue mass was an important determinant of preconditioning response. This choice of route may limit future translatability and further work will be required on this aspect of delivery.

With the identity of the cardioprotective factor(s) remaining elusive, no direct measurement of elaboration is possible with its presence inferred from its biological activity. The mouse Langendorf bioassay with which the dialysate was tested is a long established means of testing candidate cardioprotective drugs in a controlled environment. The assay however does have several shortcomings, being time and resource expensive, requiring a high degree of technical expertise to run and also not providing evidence for direct protection of human tissue. It is also unclear as to

whether the Langendorf assay is able to differentiate as to the degree of cytoprotection of a particular sample, and indeed it remains to be proven whether preconditioning stimuli are a simple binary on-off phenomenon or whether a true dose response is present, with higher degrees of protection capable with increased stimuli. Future work is needed to develop new model systems which provide a high degree of throughput and automation and preferably use human derived cells or tissues to examine cytoprotective stimuli, preferably with the ability to grade responses. Until these assays are developed, such work will remain laborious and challenging.

Finally, as intimated above, we have not directly demonstrated that peripheral adenosine infusion is able to protect humans against the effects of ischaemia-reperfusion injury. The use of a surrogate marker of protection in this study provides good evidence for future work in models that may provide more evidence of true human cytoprotection.

In summary, this study demonstrates that peripheral adenosine infusion is able to elaborate a cytoprotective humoral factor in humans that is able to reduce myocardial infarct size.

CHAPTER 7: DISCUSSION AND FUTURE WORK

7.1 Discussion

As each chapter has a discussion section, many of the issues regarding individual studies have been covered in the relevant chapters and will not be repeated here. In summary the principal findings of my work are as follows.

1. Systemic adenosine receptor antagonism in humans is able to inhibit remote ischaemic preconditioning *in-vivo.*
2. Local adenosine receptor antagonism in humans inhibits cytoprotection only in the 'trigger' phase, with no significant effect seen in the 'effector' phase. In addition, adenosine receptor antagonism in the 'trigger' phase also inhibits the elaboration of a humoral cardioprotective factor.
3. Peripheral intra-arterial adenosine infusion in humans is able to elaborate a humorally borne cardioprotective factor
4. Peripheral intra-arterial adenosine infusion during the late stages of ischaemia, prior to reperfusion, in an experimental large mammal model is able to significantly reduce myocardial infarct size

7.2 Future Directions

While in these studies I have demonstrated that peripheral intra-arterial adenosine infusion is able to elaborate a circulating cytoprotective factor(s), I have not as such demonstrated a direct protective effect of this factor(s) on human tissue. This would be an important step going forward and both *ex-vivo* and *in-vivo* approaches may be utilised. *Ex-vivo*, a potential model is that of the human atrial trabeculae model which has been previously used to test cytoprotective stimuli. Having already spent some time in developing this model in this centre, it would seem relatively straight-forward to translate the dialysate assay to this model. Unfortunately, while the model is advantageous in utilising human myocardium, and therefore demonstrating direct protection of this tissue, it remains laborious and is not entirely suitable as a high throughput, automated assay as previously discussed.

In-vivo, two approaches to demonstrating cytoprotection seem feasible. An initial approach would be to utilise the human forearm model with peripheral adenosine infusion acting as the intervention prior to IR injury and either before and after FMD or VOP as the readout. Whilst this study has been considered, it is limited by several practical issues including the need for an in-dwelling arterial line in a non-acute clinical environment.

Perhaps the more ambitious but fruitful approach therefore is to pursue an in-vivo human myocardial conditioning study with peripheral adenosine infusion. Pioneered by Prof Bolli and colleagues, an angioplasty model of repeat balloon inflations with measurement of ST-segment elevation and cardiac vein sampling of metabolites has been accepted as demonstrating evidence of myocardial conditioning responses [184, 185, 219]. With the advantage of directly examining *in-vivo* human myocardial responses, such a study has already gained ethical approval and is beginning recruitment of study subjects.

Clearly, the long-term direction of such studies is to ascertain the use of adenosine in human IR syndromes. Adenosine would be one of many potential candidates that are entering clinical trials in this light, including cyclosporine [35], exenatide[223, 224] and remote ischaemic perconditioning[60]. To fundamentally change clinical practice, such a study would need to be powered for hard clinical end-points such as mortality. With the current low mortality levels seen with modern treatment strategies, such trials would require very large numbers of patients with prolonged follow up periods, necessitating enormous resource allocation. Whether the characteristics of adenosine are sufficiently attractive to warrant such allocation in comparison to other candidate therapies remains to be seen.

7.3 Conclusions

In conclusion I have demonstrated that adenosine receptor activation is important in the 'trigger' phase of the intrinsic physiological phenomenon of human remote ischaemic preconditioning. Inhibition of adenosine receptor activation blocks the cytoprotective effect of rIPC and inhibits the elaboration of a humoral cardioprotective factor into the circulation. This elaboration of a cardioprotective factor is reproducible by the intra-arterial delivery of adenosine and in a large mammal model, similar adenosine delivery is able to reduce myocardial infarct size. This evidence suggests that adenosine is intrinsically involved in human remote preconditioning and its use as a pharmacological triggering agent may lead to reductions in ischaemia-reperfusion injury.

CHAPTER 8: APPENDICES

8.1 Timing and Venue of Research

The experiments detailed in this thesis were conducted between June 2008 and December 2011. Healthy volunteers were recruited by advertising at the John Radcliffe Hospital and within the University of Oxford. Patients with coronary artery disease were recruited from the Department of Cardiovascular Medicine at the John Radcliffe Hospital, Oxford. Flow-mediated dilation and venous occlusion plethysmography studies were performed in the Cardiovascular Clinical Research Facility at the John Radcliffe Hospital. Porcine infarct studies were performed at Skejby University Hospital, Aarhus, Denmark. Mouse Langendorf studies were performed at The Hospital for Sick Children, Toronto, Canada.

8.2 Sources of Funding

All work was supported by an unrestricted grant from Fondation Leducq (grant no. 06 CVD).

8.3 Attributions

I am extremely grateful for the contributions made to this research by colleagues and collaborators. Apart from the exceptions described below, the author is directly responsible for the planning, execution and analysis of all the experiments and the preparation of this thesis.

Flow-mediated dilation images were acquired by Mr Jonathan Diesch. Microdialysis samples were analysed by Dr Rune Birkler in the Department of Forensic Medicine, Aarhus University. Langendorf perfusion experiments were performed by Dr Jing Li of the Department of Cardiovascular Medicine, University of Toronto. I was aided in my work in the porcine infarct model by both Dr Rasmus Haarup-Li and Dr Nicolaj Støttrup.

8.4 Acknowledgements

I would first like to thank my supervisor, Dr Rajesh Kharbanda, for giving me the opportunity to undertake this research, and for his generous support, advice, guidance and encouragement during my time in Oxford.

My research would not have been possible without the contributions of many other individuals. In CCRF, I am grateful to Dr Paul Leeson, Dr Merzaka Lazdam and Mr Jonathan Diesch for their help and support with my forearm studies.

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In Skejby University Hospital, I would like to extend my thanks to Dr Rasmus Haarup-Lie, Dr Nicolaj Støttrup, Dr Michael Schmidt, Prof Hans-Erik Bøtker and the late Prof Torsten-Toftegaard Nielsen, for their invaluable support in my studies utilising the porcine infarct model and their enduring friendships and camaraderie.

In Toronto, I would like to thank Dr Jing Li for her help with the mouse Langendorf model, as well as Dr Mike Tropak and Prof Andrew Redington for their insightful advice on all aspects of rIPC.

Throughout my time in Oxford, Dr Houman Ashrafian has been a constant source of advice and assistance in many different aspects of my work. I am very grateful for his enthusiastic support.

I would like to say a big thank you to my family for their love and support. In particular, to my wife Belinda – whom I married during this period – and who has endured me throughout my studies in order to support my dreams; for this I will always be grateful. And a word of thanks to Raife, our son, who was born during my studies and to whom this thesis is dedicated.

Finally, I would like to place on record my thanks to all the patients and volunteers who gave up considerable amounts of time to participate in my studies, without whom this work would certainly not have been possible.

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Contractor H, *Ashrafian H, Rahman I, Juul-Dam K, Redington A, Mascaro CJ, Bonser RS, Kharbanda RK* Beta-Catenin Pathway Expression In Human Myocardium Is Increased By A Remote Ischaemic Preconditioning Stimulus **–** Hypertension 2009;54(5):1173- 1174.

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Botker HE, Schmidt MR, Kharbanda RK. Local Ischaemic Preconditioning Protects Against Endothelial Dysfunction In A Novel Ex-Vivo Model Of Human Vascular Ischaemia-Reperfusion Injury. Hypertension 2009;54(5):1174.

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