

**Cardioprotective actions of bradykinin in the normal and hypertrophied myocardium**

Thesis submitted by

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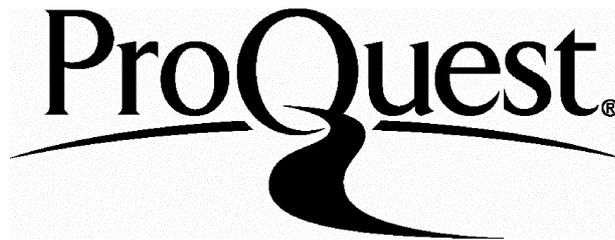
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## Abstract

Very few therapeutic modalities are beneficial in the treatment of acute myocardial infarction. However, the phenomenon of ischaemic preconditioning (IPC) reduces cell necrosis and therefore, may offer protection against ischaemia-reperfusion injury. Bradykinin has been implicated in IPC as a trigger of this protective phenomenon. The protective effects of both IPC and bradykinin are largely under-investigated in models of chronic myocardial hypertrophy. Furthermore, the kallikrein-kinin system is thought to be implicated in hypertension, indeed studies have demonstrated that levels of bradykinin are attenuated in hypertension. Therefore, the aim of this thesis was to further elucidate the cardioprotective actions of bradykinin in both the normal and hypertrophied myocardium.

In preliminary experiments, the deoxycorticosterone acetate (DOCA)-salt rat was used to represent a mild model of left ventricular hypertrophy (LVH) associated with short term hypertension. Although IPC was found to reduce infarct size in the DOCA-salt rat hearts subjected to ischaemic-reperfusion injury, bradykinin induced cardioprotection was impaired in these hearts.

Drugs that inhibit bradykinin degradation, namely, angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) inhibitors can be used therapeutically to augment bradykinin levels. Previous studies have demonstrated that ACE inhibitors can potentiate a subthreshold preconditioning stimulus, however, it is not known whether dual ACE and NEP inhibition also potentiates IPC. It was found that the dual ACE and NEP inhibitor, omapatrilat, analogous to captopril, augmented a subthreshold IPC stimulus via activation of the bradykinin B<sub>2</sub> receptor. In contrast to captopril, omapatrilat also evoked protection when administered directly (ie, in the absence of preconditioning ischaemia), an effect also dependent upon B<sub>2</sub> receptor activation.

The effects of IPC in chronic myocardial hypertrophy associated with long term hypertension were investigated. IPC evoked protection in hearts isolated from young and middle aged SHR and normotensive age matched, WKY rats. However, IPC did not protect the ageing SHR/WKY rat hearts. Therefore, the combination of ageing and long standing hypertrophy interfere with the occurrence of IPC. In an attempt to raise bradykinin levels, captopril was used in conjunction with the IPC protocol. However, no protection was observed in hearts isolated from the ageing SHR. In contrast, modest protection was seen in age matched WKY rat hearts.

In the normal myocardium, it was found that bradykinin administered just prior to reperfusion also induced cardioprotection possibly via activation of the PI3 kinase pathway. However, the protective effect of bradykinin at reperfusion could not be duplicated in the ageing SHR myocardium.

Even though numerous studies have demonstrated that bradykinin elicits classical preconditioning, its role in delayed preconditioning remains elusive. The final set of experiments in this thesis investigated whether bradykinin triggers delayed preconditioning. It was found that a bradykinin bolus given 24 hours prior to infarction triggered protection, an effect dependent upon the generation of nitric oxide.

The work contained in this thesis confirms the cardioprotective potential of bradykinin and bradykinin modifying drugs in normal myocardium. However, the impairment of cardioprotective pathways in hypertensive myocardium was a consistent finding of these studies and therefore requires further investigation.

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## List of Abbreviations

The following abbreviations are used in this thesis;

ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
Akt	Cellular Akt / protein kinase B
AIRE	Acute infarction ramipril efficacy
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ANP	Atrial or type A natriuretic peptide
aSHR	ageing SHR
ATP	Adenosine triphosphate
AT II	Angiotensin II
AT <sub>1</sub>	Angiotensin type 1 receptor
B <sub>1</sub>	Bradykinin type 1 receptor
B <sub>2</sub>	Bradykinin type 2 receptor
B <sub>3</sub>	Bradykinin type 3 receptor
Bad	Bcl-2 <sub>XL</sub> / Bcl-2 -associated death promoter
Bax	Bcl - associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2 gene
BNP	Brain or B type natriuretic peptide
BSA	Bovine serum albumin
cAMP	cyclic adenosine-5-monophosphate
Caspase	Cystein aspartate specific proteases
CCPA	2-chloro N6 cyclopentyl adenosine

CFR	Coronary flow rate
CNP	C type natriuretic peptide
COX-2	Cyclooxygenase -2
CPM	Counts per minute
CT	Cardiotrophin
DAG	Diacylglycerol
DHBA	3,4-dihydroxybenzylamine
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DTT	Dithiothreitol
ECE	Endothelin converting enzyme
EDTA	Ethylene diamine tetraacetic acid
eNOS	Endothelial nitric oxide
ERK	Extracellular signal related kinase
FGF	Fibroblast growth factor
G-protein	GTP binding regulatory proteins
5-HD	5 Hydroxy decanoate
HMWK	High molecular weight kininogen
HOPE	Heart outcomes prevention evaluation
HPLC	High performance liquid chromatography
HSP	Heat shock protein
IGF	Insulin like growth factor
iNOS	inducible nitric oxide synthase
I/R (%)	Infarct-risk ratio (%)
IP <sub>3</sub>	Inositol 3,4,5 triphosphate
IPC	Ischaemic preconditioning

JNK	c-jun N-terminal kinase
K <sub>ATP</sub>	ATP sensitive mitochondrial channel
kDa	kilodaltons
LMWK	Low molecular weight kininogen
L-NA	N ω -nitro-L-arginine
L-NAME	N ω -nitro-L-arginine methyl ester
LV	Left ventricle
LVH	Left ventricular hypertrophy
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinase
MAPKAPK2	Mitogen activated protein kinase activating protein kinase 2
MEK	MAPK/ERK kinase
MLA	Monophosphoryl lipid A
MPG	Mercaptopropionylglycine
mRNA	messenger ribonucleic acid
mSHR	middle aged SHR
mt-K <sub>ATP</sub>	mitochondrial K <sub>ATP</sub> channel
NADH	Nicotinamide adenine dinucleotide phosphate
NEP	Neutral endopeptidase
NF κB	Nuclear factor κB
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
p70 S6 kinase	70 kDa ribosomal S6 kinase
PD98059	2'-amino-3'-methylflavone
PEP	Prolyl endopeptidase
PGI <sub>2</sub>	Prostacyclin

PLSD	Protected least significant difference
PMSF	Phenyl methyl sulphonyl flouride
PTCA	Percutaneous transluminal coronary angioplasty
PI3 kinase	Phosphatidylinositol-3'-OH kinase
PIP	Phosphatidyl inositol phosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
RACK	Receptor for activated C kinase
Raf	MAP kinase kinase kinase
RISK	Reperfusion injury salvage kinases
ROS	Reactive oxygen species
r-PIA	r-N <sup>6</sup> -(2-phenylisopropyl)adenosine
RPM	Rotations per minute
RPP	Rate pressure product
RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Right ventricle
RVH	Right ventricular hypertrophy
SAPK	Stress activated protein kinase
SAVE	Survival and ventricular enlargement
SB203580	4-(4-fluorophenyl)-2-(4-methylsulphonylphenyl)-5-(4-pyridyl)1H-imidazole
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHR	Spontaneously hypertensive rat
SNAP	S-nitro N-acetyl pencillamine

SOD	Superoxide dismutase
SOLVD	Studies of left ventricular dysfunction
8-SPT	8-sulphophenyltheophylline
SR	Sarcoplasmic reticulum
SWOP	Second window of protection
TGF	Transforming growth factor
TGR	Transgenic
TIMI	Thrombolysis in myocardial infarction
TK	Protein tyrosine kinase
TRACE	Trandolapril cardiac evaluation
WKY	Wistar kyoto rat
ySHR	young SHR

## **Publications arising from this thesis**

### **Abstracts**

1. Ebrahim Z, Yellon DM, Baxter GF. Bradykinin induced cardioprotection is attenuated in the hypertrophied rat heart. *Br J Pharmacol.* 2000; (Suppl) 191P.
2. Baxter GF, Ebrahim Z, Yellon DM. AMP 579 an A1/A2 agonist, limits infarct size at reperfusion via a p42/p44 MAPK-dependent pathway. *Circulation.* 2000; 102 (Suppl), I-1028.
3. Ebrahim Z, Yellon DM, Baxter GF. Bradykinin elicits delayed myocardial protection in rat heart via a nitric oxide dependent mechanism. *Br J Pharmacol.* 2001; 133 (Suppl), 90P.
4. Baxter GF, Ebrahim Z, Yellon DM. AMP579, an adenosine A<sub>1</sub> and A<sub>2A</sub> receptor agonist, attenuates lethal reperfusion injury in rat heart via the p42/p44 MAPK pathway. *Br J Pharmacol.* 2001; 133 (Suppl), 7P.
5. Ebrahim Z, Yellon DM, Baxter GF. Omapatrilat lowers the threshold for induction of ischaemic preconditioning via activation of the bradykinin B<sub>2</sub> receptor. *Br J Pharmacol.* 2001; 133 (Suppl), 10P.

### **Manuscripts**

1. Ebrahim Z, Yellon DM, Baxter GF. Bradykinin elicits "second window" myocardial protection in rat heart through an NO-dependent mechanism. *Am J Physiol Heart Circ Physiol.* 2001; 281, H1458-H1464.

2. Ebrahim Z, Yellon DM, Baxter GF. Omapatrilat lowers the threshold for induction of myocardial preconditioning through a bradykinin receptor-mediated mechanism. Paper submitted to J Am Coll Cardiol.
  
3. Baxter GF & Ebrahim Z. Role of bradykinin in preconditioning and protection of the ischaemic myocardium. Review, submitted to Br J Pharmacol.
  
4. Ebrahim Z, Yellon DM, Baxter GF. Characterisation of the myocardial preconditioning response in progressive hypertension. Paper in preparation.

**PART ONE**

**GENERAL INTRODUCTION**

# CHAPTER ONE

## Part I: General introduction to ischaemic preconditioning

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# Chapter One

## Part I: General introduction to ischaemic preconditioning

### 1.1. Mortality and coronary heart disease

Coronary heart disease is the leading cause of mortality in the western world. In 1998, myocardial infarction and angina pectoris accounted for 600,000 deaths in the USA (American Heart Association; 2001 Heart and Stroke statistical update). In the UK, coronary heart disease claimed over 135,000 lives in 1998 making it the most common cause of death (British Heart Foundation; coronary heart disease statistics; 2000 edition). For many years, cardiologists have been searching for therapeutic modalities that can reduce the detrimental effects of myocardial ischaemia. To date, only thrombolytics have proven to reduce damage caused by myocardial ischaemia, however, it has been proposed that the phenomenon of ischaemic preconditioning may confer further protection against myocardial ischaemic injury.

### 1.2. Discovery of ischaemic preconditioning

In 1986, Murry et al reported that four brief periods of ischaemia prior to a sustained ischaemic event paradoxically limited infarct size in the *in vivo* dog model of coronary artery occlusion. In fact, a 75% reduction of infarct size was noted. They named this phenomenon "preconditioning with ischaemia" (Murry *et al*, 1986). This protective effect could not be explained by changes in collateral blood flow, which was similar in both groups, implying alterations in the cellular response to ischaemia. Hence, ischaemic preconditioning (IPC) can be described as a phenomenon whereby short periods of sublethal ischaemia enhance primary myocardial tolerance to a longer, sustained period of ischaemia.

The impact of this study has been tremendous. No therapeutic modality has been shown to reduce cell death by 75% and it now appears that the heart has the innate ability to protect itself from the consequences of ischaemia-reperfusion injury. Following this initial report of IPC, more than 1500 studies have been performed in this field (Yellon & Dana, 2000).

### 1.3. Characteristics of IPC

Following its initial report in dogs, IPC has been demonstrated in a variety of animal models including, mice (Sumeray & Yellon, 1998), rats (Bugge & Ytrehus, 1996a), rabbits (Goto *et al*, 1995), guinea pigs (Chlopicki *et al*, 1999), pigs (Schulz *et al*, 1998), goats (Gattullo *et al*, 1999), sheep (Uematsu *et al*, 1998) and most importantly in humans (Yellon *et al*, 1993).

Several characteristic traits of IPC have been demonstrated. Firstly it is important to note that IPC *delays* cell death, it does not *prevent* cell death. Additionally, the protection is short lived. Investigators have generally shown that if the reperfusion period between the preconditioning ischaemia and the sustained ischaemia is extended beyond one-two hours then protection is lost. Murry *et al* showed that in dogs, protection is lost following a two hour reperfusion period prior to the sustained ischaemic insult (Murry *et al*, 1991). In most models, protection is reduced following a one hour reperfusion period (Miura *et al*, 1992; van Winkle *et al*, 1991). The effects of IPC can however be reinstated once they are lost (Yang *et al*, 1993; Li & Kloner, 1994). Conversely, if the reperfusion period is too short, then IPC does not elicit protection either. Alkhulaifi *et al* showed in isolated rat hearts that a 30 second reperfusion period following the preconditioning ischaemia did not limit infarct size. However when this reperfusion period was extended to one minute, protection was observed (Alkhulaifi *et al*, 1993). A minimum period of reperfusion is required for IPC to protect as it may allow the washout of catabolites from the ischaemic zone. Alkhulaifi *et al*

also found that lactate levels were much lower in groups subjected to one and ten minutes of reperfusion, compared with 30 seconds of reperfusion (Alkhulaifi *et al*, 1993).

The protection induced by IPC is finite. Murry *et al* demonstrated that when the sustained ischaemic episode lasted for three hours, there was no significant difference in infarct size between the control and preconditioned group (Murry *et al*, 1991). Finally, there is general consensus that IPC is an "all or none" phenomenon (Miura *et al*, 1998). An IPC protocol consisting of at least one, two minute cycle of ischaemia is required to initiate the adaptive response. A five minute period of ischaemia is sufficient to elicit maximal protection, additional cycles of ischaemia do not result in enhanced protection (Miura *et al*, 1998; Miura *et al*, 1992; Li *et al*, 1990). Some investigators, however, disagree with this and report that, multiple cycles of IPC induce greater protection than a single cycle. Tanno *et al* reported that whilst IPC with one five minute cycle of ischaemia reduced infarct size, IPC with two five minute cycles of ischaemia produced a further reduction in infarct size in rat (Tanno *et al*, 2000). Importantly, myocardial protection re-appears 24 hours following the preconditioning ischaemia, a phenomenon known as second window of protection or delayed preconditioning, this is discussed in more detail in section 1.4.

### **1.3.1. End points of protection**

Many end points have been used to assess the protective effects of IPC. IPC can attenuate infarct size, occurrence of arrhythmias, post-ischaemic contractile dysfunction, ST segment elevation, creatine kinase / lactate dehydrogenase release and ATP depletion, described overleaf;

#### 1.3.1.1. *Infarct size*

Cell necrosis can be quantified by measuring infarct size. IPC has been shown to reduce infarct size in many species both in the *in vivo* and *in vitro* situations (Bugge & Ytrehus, 1996a; Goto *et al*, 1995; Tanno *et al*, 2000). Infarct size is usually illustrated using quantitative morphological techniques, for example triphenyltetrazolium staining which has been shown to accurately determine infarct size providing a sufficient period of reperfusion is used. Propidium iodide is also used to assess cell death in flow cytometry studies. Propidium iodide is only able to penetrate damaged cell membranes. Once it enters the cell nucleus, it intercalates with DNA, producing a bright red fluorescence (O'Brien & Bolton, 1995; Bhakdi *et al*, 1989). Indeed, the use of propidium iodide to measure infarct size is attractive because it does not require lengthy reperfusion periods like triphenyltetrazolium staining (see chapter two for further details). Recently, Wolff *et al* demonstrated that propidium iodide produced a similar infarct size to that observed with triphenyltetrazolium staining (Wolff *et al*, 2000)

Infarct size however is not always used as an experimental end point in IPC studies. Cell death can be quantified by assaying levels of creatine kinase and lactate dehydrogenase release. Many experimental studies that cannot use infarct size as an end point, measure marker protein leakage instead. In this regard, IPC has been shown to clearly protect by reducing creatine kinase, lactate dehydrogenase and troponin release (Sharma & Singh, 2000; Jenkins *et al*, 1997). In certain experimental models it is not feasible or, investigators may prefer to employ an alternative end point. In isolated myocytes for instance, since infarct size cannot be measured, myocyte viability using trypan blue exclusion, propidium iodide, nitroblue or enzyme release can be used as surrogate markers instead.

#### 1.3.1.2. *Other end points of protection*

Arrhythmia prevalence, usually in the form of ventricular tachycardia or ventricular fibrillation are used to assess the protective effects of IPC, which may correlate well with the clinical problems of sudden cardiac death and severe arrhythmias seen during myocardial ischaemia. Protection against arrhythmias can be demonstrated in the rat (Shiki & Hearse, 1987), dog (Vegh *et al*, 1992) and human (Airaksinen & Huikuri, 1997). In contrast, protection is not observed in the pig myocardium. In fact, Ovize *et al* reported that even though IPC attenuated infarct size, it accelerated the onset of ventricular fibrillation during prolonged ischaemia (Ovize *et al*, 1995). Thus, the anti-arrhythmic effects of IPC may be species dependent or modified by factors such as anaesthesia.

Protection against post-ischaemic contractile dysfunction ("stunning") is debatable. While IPC has been shown to enhance left ventricular contractility by some investigators in the rat heart (Cave & Hearse, 1992; Asimaki *et al*, 1992), other investigators report no reduction in myocardial stunning (Jenkins *et al*, 1995). Studies assessing myocardial stunning are difficult to interpret as improvement of functional parameters may be as a result of reduced necrosis rather than an actual reduction in myocardial stunning. Jenkins *et al* demonstrated that improvement of post-ischaemic contractile function was secondary to reduced infarction, thus, IPC does not appear to protect against myocardial stunning (Jenkins *et al*, 1995).

#### 1.3.2. **IPC in human heart**

There is clear evidence demonstrating the protective effects of IPC in many animal models. However, can this powerful phenomenon be reproduced in humans? The most direct evidence for preconditioning in man comes from a study performed by Yellon *et al*. In a setting of coronary artery bypass grafting, intermittent application of an aortic cross clamp was used to produce global ischaemia and hence provided a stimulus for IPC. Assessment

of ATP levels in biopsy specimens was used as an experimental end point. Patients subjected to the IPC protocol had better preservation of ATP levels during a subsequent ten minute ischaemic episode (Yellon *et al*, 1993). Subsequently, in the same experimental setting, Jenkins *et al* showed that IPC reduced troponin-T release (Jenkins *et al*, 1997) indicating that IPC had a clear ability to directly protect the myocardium against cell necrosis.

Percutaneous transluminal coronary angioplasty (PTCA) involves repeated coronary balloon inflations, separated by periods of balloon deflations. This creates the opportunity to study effects of short, controlled periods of ischaemia-reperfusion in human myocardium. Periods of ischaemia caused by balloon inflations may be sufficient to induce IPC. In fact, studies have demonstrated that if the first balloon inflation is longer than 60-90 seconds then, a IPC like effect is observed (Yellon & Dana, 2000). A reduction in chest pain, ST segment elevation, QT dispersion, and lactate release is observed during subsequent balloon inflations. This indicates that the first balloon inflation was sufficient to act as a trigger of IPC. A major drawback in this experimental setting is that recruitment of collateral vessels may occur during balloon inflations (Billinger *et al*, 1999).

A number of experiments using human myocardial tissue have also demonstrated that IPC can induce protection. Isolated atrial trabeculae suspended in an organ bath and subjected to simulated ischaemia have shown an enhancement of functional recovery following IPC (Walker *et al*, 1995; Bell *et al*, 2000; Speechly-Dick *et al*, 1995; Morris & Yellon, 1997). Similarly, isolated human ventricular myocytes also demonstrate the preconditioning phenomenon (Arstall *et al*, 1998).

If short periods of ischaemia are beneficial prior to a longer ischaemic insult, then theoretically, pre-infarct angina should be beneficial. Indeed, several studies have shown

that patients with angina prior to myocardial infarction have a more beneficial outcome than those without preceding angina. The thrombolysis in myocardial infarction (TIMI) four investigators showed that patients with previous angina had lower in hospital mortality, a lower incidence of heart failure and smaller infarcts (determined by creatine kinase release) compared to patients without previous angina (Kloner *et al*, 1995). Similarly, Andreotti *et al* demonstrated that thrombolytic therapy given to patients with acute myocardial infarction preceded by unstable angina resulted in a more rapid reperfusion and smaller infarcts compared to patients without preinfarction angina (Andreotti *et al*, 1996). Ishihara *et al* also reported that prodromal angina 24 hours before the onset of infarction resulted in a lower in hospital mortality rate and better five year survival (Ishihara *et al*, 1997).

### **1.3.3. Mechanisms of IPC**

The exact molecular mechanisms by which IPC leads to protection remain to be fully established. Nevertheless, our understanding of signalling pathways has dramatically increased since the initial description of IPC in 1986. The IPC signalling cascade can be divided into three parts. (1) The molecular substances generated during the brief ischaemic period which are thought to initiate the protection ("*triggers or activators*"). (2) The molecular substances that are activated by the trigger; their activity is enhanced during the prolonged ischaemic period ("*mediators*" or cell signalling pathway). The mediators or cell signalling pathway determines the activity of the distal effectors which culminate in myocardial protection. (3) The final species or target involved in protection ("*distal effectors*").

### **1.3.4. Triggers of IPC**

The major breakthrough came about when it was revealed that the protection seen with IPC was receptor mediated. During the brief period of ischaemia, many triggers are released by the myocardium (described below) which are thought to initiate the protective signalling

cascade. It should be stressed that the triggers of IPC have to be present in the myocardium prior to the index ischaemic episode in order to elicit protection, their presence during sustained ischaemia alone will not elicit protection.

#### 1.3.4.1. Adenosine

Adenosine released from myocytes as a consequence of ATP degradation has been shown to play a role in IPC. Liu *et al* showed that adenosine receptor blockade with 8-sulphophenyltheophylline (8-SPT) prior to IPC abrogated the infarct-limiting effect in an *in vivo* rabbit model of coronary artery occlusion (Liu *et al*, 1991). Additionally, direct intracoronary infusion of either adenosine or the selective A<sub>1</sub> adenosine analogue r-N<sup>6</sup>-(2-phenylisopropyl)adenosine (r-PIA) mimicked the preconditioning effect (Liu *et al*, 1991). These findings implied that adenosine acting through the A<sub>1</sub> receptor was the trigger of IPC. Subsequently, further evidence supporting a role for adenosine in IPC has been obtained in the pig (Schulz *et al*, 1995), dog (Vander *et al*, 1993) and human studies (Ikonomidis *et al*, 1997). Interestingly, a role for the adenosine A<sub>3</sub> receptor has also been implicated. Liu *et al* showed that a potent A<sub>3</sub> receptor antagonist N<sup>6</sup>-[2-(4-aminophenyl)ethyl]adenosine also abrogated the infarct limiting effect of IPC (Liu *et al*, 1994). The involvement of adenosine in rat remains unclear. Liu and Downey demonstrated that IPC was not abolished with an adenosine receptor antagonist in the rat heart (Ganote *et al*, 1993; Liu & Downey, 1992). During ischaemia, the rat heart has been shown to release approximately three times the concentration of adenosine compared to the rabbit (Headrick, 1996). Thus, higher concentrations of adenosine receptor antagonists may be required to block IPC in rat, explaining why the role of adenosine remains equivocal in this species. However, Wainwright *et al* have demonstrated that the A<sub>1</sub> adenosine agonist (r-PIA) reduced the occurrence of arrhythmias in the rat following coronary artery occlusion (Wainwright *et al*, 1997).

#### 1.3.4.2. Catecholamines

The release of catecholamines following ischaemia has been demonstrated (Lameris *et al*, 2000). Hu *et al* demonstrated that either depletion of noradrenaline stores with reserpine, or blockade of  $\alpha_1$  receptor with prazosin prevented the effects of IPC in rat heart (Hu & Nattel, 1995). Whereas, the  $\alpha_{1B}$  receptor antagonist chloroethylclonidine abolished IPC,  $\alpha_{1A}$  receptor blockade with 5-methylurapidil had no effect. Cohen *et al* showed that in rabbit isolated myocytes, hypoxia caused the release of noradrenaline and adenosine which were *both* required to induce IPC (Cohen *et al*, 1995). Sharma and Singh reported that prazosin attenuated the protective effects of IPC in the dog (Sharma & Singh, 1997).

The  $\beta$  adrenoceptor has also been implicated in IPC. Lochner *et al* demonstrated that alprenolol attenuated the protective effects of IPC in rat heart. Conversely, exogenous isoproterenol mimicked the preconditioning effect (Lochner *et al*, 1999)

#### 1.3.4.3. Bradykinin

The release of bradykinin from the ischaemic myocardium has been shown to occur in a number of studies (Linz *et al*, 1996; Schulz *et al*, 1998; Pan *et al*, 2000; Campbell, 2000). Wall *et al* reported that Hoe 140 (icatibant) (a selective B<sub>2</sub> receptor antagonist) abolished the protective effects of IPC against infarction in an *in vivo* rabbit model of coronary artery occlusion (Wall *et al*, 1994). Goto *et al* also investigated the involvement of bradykinin in IPC. Open chest rabbits, were subjected to ischaemia-reperfusion and infarct size was used as an experimental end point (Goto *et al*, 1995). If three cycles of ischaemia were used to elicit preconditioning then Hoe 140 did not abolish protection. However, if one five minute cycle of ischaemia was used to precondition then protection was abrogated with Hoe 140 (Goto *et al*, 1995). The authors therefore proposed that a "threshold" must be reached in order for the full protective response of preconditioning to occur. It was suggested that

when only one cycle of ischaemia was used, then bradykinin plays a primary role in inducing protection, such that B<sub>2</sub> receptor blockade abolished the effect. However, if three cycles were employed, other triggers are generated in sufficient quantity, such that the “threshold” can be reached even in the presence of the B<sub>2</sub> receptor antagonist. Figure 1.1 illustrates the threshold hypothesis of IPC. The role of bradykinin in IPC is fully discussed in part two of this chapter.

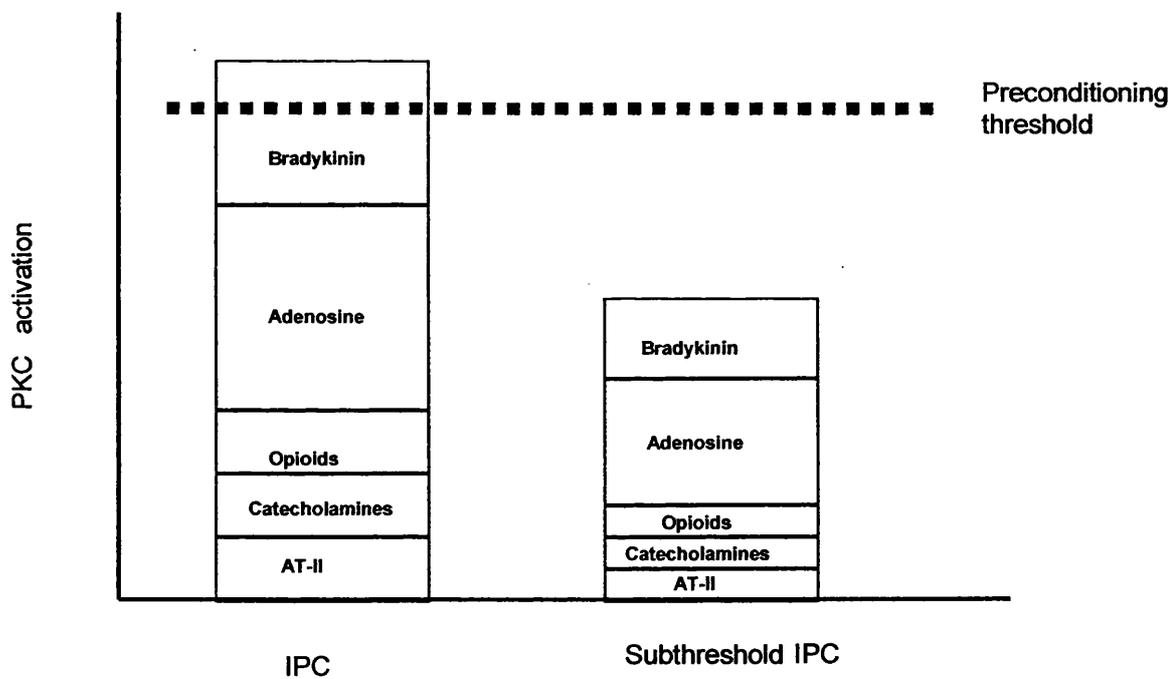


Figure 1.1. The threshold hypothesis of IPC. A standard IPC protocol leads to the liberation of adequate amounts of triggers, so that the threshold for preconditioning is attained (ie, PKC activation is sufficient). During subthreshold IPC sufficient triggers are not released during the preconditioning ischaemia, so that PKC activation is not sufficient to elicit a preconditioning effect.

#### 1.3.4.4. Other triggers of IPC

Studies from Gross’s laboratory have demonstrated the involvement of opioid receptors in IPC in the *in vivo* rat model of coronary artery occlusion. They showed that IPC and morphine induced protection were blocked using naltrindole, a selective  $\delta$  opioid receptor

(Schultz *et al*, 1997). This implied a role for endogenous opioids in IPC and demonstrated that opioids could mimic preconditioning. Aitchison *et al* demonstrated a role for the  $\delta_1$  receptor subtype in isolated rat heart (Aitchison *et al*, 2000). Naloxone (opioid receptor antagonist) was subsequently shown to block IPC in isolated rabbit hearts, suggesting a cardiac and not central nervous system involvement (Chien & Van Winkle, 1996). Indeed, Bell *et al* recently demonstrated that naltrindole abrogated the protective effects of IPC in isolated human heart muscle (Bell *et al*, 2000).

A role for AT II in IPC has also been found. In isolated rabbit hearts, Diaz and Wilson found that blockade of AT<sub>1</sub> receptor abolished the protective effect of IPC (Diaz & Wilson, 1997). Conversely, AT II treatment mimicked the effects of IPC. Nakano *et al* found that AT<sub>1</sub> receptor blockade partially attenuated the effects of IPC in rabbit heart (Nakano *et al*, 1997).

Although not examining the involvement of endogenous acetylcholine in IPC, studies have demonstrated that acetylcholine can mimic preconditioning. Yao and Gross demonstrated that exogenous acetylcholine led to a reduction in infarct size in the *in vivo* dog model of coronary artery occlusion (Yao & Gross, 1993).

Release of endothelin -1 has been shown following ischaemia (Velasco *et al*, 1993). Wang *et al* demonstrated that although IPC was not blocked using an endothelin -1 receptor antagonist, application of exogenous endothelin -1 could mimic IPC in rabbit heart (Wang *et al*, 1996). Therefore, endogenous endothelin -1 does not contribute to IPC but exogenous endothelin -1 can mimic IPC. Similarly, Bugge and Ytrehus found that exogenous application of endothelin -1 in rat hearts reduced infarct size (Bugge & Ytrehus, 1996b). Interestingly, Wainwright's group demonstrated that although exogenous endothelin -1 was

antiarrhythmic, endogenous endothelin -1 released during ischaemia was actually pro-arrhythmic (Sharif *et al*, 1998).

Vegh *et al* demonstrated that the anti-arrhythmic effects of IPC were abrogated in the presence of the cyclooxygenase inhibitor sodium meclofenamate in dog (Vegh *et al*, 1990). Likewise, Arad *et al* found that aspirin abolished the anti-arrhythmic effects of IPC in the isolated rat heart (Arad *et al*, 1996). It must be pointed out that both of these studies however used arrhythmia prevalence as an experimental end point and the role of endogenous prostaglandins using alternative end points of IPC is unknown.

It has been proposed that nitric oxide may play a role in IPC. Vegh *et al* demonstrated that a nitric oxide inhibitor partially attenuated the anti-arrhythmic effects of IPC in the dog (Vegh *et al*, 1992). Although, exogenous nitric oxide can induce protection, the majority of studies however, do not support a role for endogenous nitric oxide in IPC (Nakano *et al*, 2000c; Weselcouch *et al*, 1995; Lu *et al*, 1995) (note, that in delayed preconditioning nitric oxide has however been shown to have a very important role, discussed in the latter part of this chapter).

Several experiments have demonstrated that oxygen free radicals also play a role in IPC. Tanaka *et al* demonstrated that the infarct limiting effects of IPC were attenuated using oxyradical scavengers - mercaptopropionylglycine (MPG) and superoxide dismutase (SOD) in rabbit heart (Tanaka *et al*, 1994b). Similarly, Baines *et al* showed that MPG abrogated the infarct limiting effect of IPC (induced using one five minute cycle of ischaemia) in rabbit heart (Baines *et al*, 1997). Free radicals may also trigger IPC in human heart (Wu *et al*, 2001).

A number of the triggers mentioned above are coupled to G-proteins. Indeed Thornton et al have demonstrated that pre-treatment with pertussis toxin blocked the protective effects of IPC in rabbit, rat and dog implicating an imperative role for G-proteins in IPC (Thornton *et al*, 1993). Central to many diverse triggers of IPC is the fact that their receptors are linked to G-proteins. Therefore, adenosine A<sub>1</sub>,  $\alpha$  adrenoceptors, bradykinin B<sub>2</sub>, muscarinic M<sub>2</sub>, AT<sub>1</sub>, endothelin -1,  $\delta$  opioid are all G-protein linked receptors. Some G-proteins couple to phospholipase C, whose activation leads to the formation of diacylglycerol and inositol triphosphate. Diacylglycerol is subsequently believed to activate protein kinase C (PKC).

### **1.3.5. Intracellular signalling pathways of IPC**

Increased activity of several kinases has been shown to occur during IPC, described below;

#### *1.3.5.1. Protein kinase C*

PKC, initially identified by Nishizuka et al in bovine cerebellum, is a ubiquitous, multifunctional kinase that phosphorylates serine and threonine residues on target proteins (Webb *et al*, 2000). Current evidence suggests that PKC is an important mediator of IPC; Ytrehus et al demonstrated that two specific inhibitors of PKC, namely staurosporine and polymyxin B aborted IPC in rabbit heart (Ytrehus *et al*, 1994). Similarly, Mitchell et al showed that IPC was abolished using PKC inhibitors in rat heart (Mitchell *et al*, 1995). More importantly, Ikonomidis et al demonstrated preconditioning was abolished using PKC inhibitors in human myocytes (Ikonomidis *et al*, 1997). Yellon's group also demonstrated that a PKC inhibitor, chelerythrine abrogated IPC in human atrial muscle (Speechly-Dick *et al*, 1995). Conversely, investigators have also reported that direct activators of PKC namely, phorbol esters or diacylglycerols can mimic preconditioning (Ikonomidis *et al*, 1997; Ytrehus *et al*, 1994; Speechly-Dick *et al*, 1995). This led to the development of the "Downey

hypothesis" which postulates that PKC activation is vital in IPC. However, in larger animals, notably in the pig and dog, a role for PKC in IPC is equivocal (Vahlhaus *et al*, 1996; Przyklenk *et al*, 1995). Vahlhaus *et al*, reported that staurosporine did not block IPC in swine (Vahlhaus *et al*, 1996). However, investigators later reported that when a tyrosine kinase inhibitor and staurosporine were used concomitantly, IPC was abolished (Vahlhaus *et al*, 1998). This implies that both PKC and tyrosine kinase signalling cascades are involved in IPC and indeed PKC plays an important role in IPC in pig myocardium. However, controversy still surrounds the role of PKC in dog. Przyklenk *et al* reported that PKC inhibition did not abolish preconditioning's infarct limiting effect in the dog (Przyklenk *et al*, 1995). In contrast, Kitakaze *et al* found that PKC inhibition resulted in the abrogation of IPC in the dog model (Kitakaze *et al*, 1996). Reasons for the incoherent results obtained in dog and pig are not clear. However, it should be pointed out that PKC is a very complex enzyme, 11 isoforms have been identified, of which ten have been detected in the myocardium (Ping *et al*, 1997). Hence, inhibitors of PKC may not selectively block activity of all isoforms or may lead to the inhibition of other kinases. Indeed, high concentrations of staurosporine inhibit cAMP dependent protein kinase and calcium-calmodulin dependent kinase (Brooks & Hearse, 1996). Thus as with all drugs, inhibitors of PKC may have non-specific effects. Nevertheless, a distinct role for PKC has been found in rat (Mitchell *et al*, 1995), rabbit (Ytrehus *et al*, 1994) and human myocardium (Ikonomidis *et al*, 1997; Speechly-Dick *et al*, 1995). PKC activation has also been shown to be important in pharmacologically induced preconditioning.

For IPC to be protective, PKC activation is essential during the sustained ischaemic insult. Yang *et al* demonstrated that infusion of staurosporine during the IPC protocol did not block the protection in isolated rabbit hearts (Yang *et al*, 1997b). However, when staurosporine was administered just prior to and continued into the sustained ischaemic episode, IPC was

completely abrogated (Yang *et al*, 1997b). These findings imply that PKC activity is not required during the period of IPC, but is imperative during sustained ischaemia. This in turn implies that PKC does not trigger IPC and suggests that mechanism(s) upstream of PKC trigger IPC.

PKC activation leads to its translocation from the cytoplasm to membranes and cytoskeletal structures. Each PKC isoform translocates to the membrane, where it binds to a specific anchoring protein, referred to as receptor for activated C kinase (RACK). Ping *et al* showed that IPC caused a significant translocation of PKC  $\epsilon$  and PKC  $\eta$  isoforms from the cytosolic to the particulate fraction in rabbit heart (Ping *et al*, 1997). Interestingly, the particulate fraction of PKC  $\epsilon$  increased with the number of IPC cycles used, whereas maximal translocation of PKC  $\eta$  occurred after just one cycle of IPC. Armstrong and Ganote demonstrated that activation of PKC  $\epsilon/\delta$  pharmacologically using ingenol-3,20-dibenzoate mimicked IPC in isolated rabbit cardiomyocytes. However, activation of PKC  $\alpha$ ,  $\beta$  and  $\gamma$  using thymeleatoxin had no protective effect (Armstrong & Ganote, 1994). The translocation of PKC  $\epsilon$  and  $\eta$  does not however demonstrate that these isoforms are responsible for the protective effects of IPC. Translocation could simply be an epiphenomenon as a result of ischaemia, and not be involved in protection. However, Liu *et al* demonstrated that only PKC  $\epsilon$  inhibition abrogated IPC and PKC  $\beta$ ,  $\delta$  and  $\eta$  inhibitors failed to block IPC (Liu *et al*, 1999). These results provide further conclusive evidence for a role of PKC  $\epsilon$  in IPC. Very recently, PKC  $\delta$  activation has in fact shown to be detrimental (Chen & Mochly-Rosen, 2001). Using sophisticated functional proteomic techniques to analyse PKC  $\epsilon$ , Ping *et al* showed that PKC  $\epsilon$  is physically associated with 36 proteins, some of which have been implicated in cardioprotection (Ping *et al*, 2001). The exact mechanisms by which PKC  $\epsilon$  leads to protection is not known.

#### 1.3.5.2. Tyrosine kinases

Experimental evidence suggests that kinases other than PKC are involved in IPC. Tyrosine kinases as the name implies phosphorylate tyrosine residues on target proteins. Maulik et al demonstrated that genistein, a selective tyrosine kinase inhibitor, blocked the protective effects of IPC in isolated rat heart (Maulik *et al*, 1996). In rabbit hearts, two structurally dissimilar tyrosine kinase inhibitors, genistein and lavendustin A ablated IPC, when present at the onset of the sustained ischaemic event (Baines *et al*, 1998). Tanno et al reported that even though IPC induced with one cycle of ischaemia-reperfusion was blocked with staurosporine, IPC using two cycles of IPC was not. However, when a combination of staurosporine and genistein was used, IPC induced by two cycles of ischaemia-reperfusion was abrogated (Tanno *et al*, 2000). Thus, it appears that both PKC and tyrosine kinase may be implicated in IPC, the role of each kinase may depend upon the species in question and the IPC protocol used. PKC and tyrosine kinase may co-ordinate mutually in IPC and both protective pathways may run in parallel. Experimental work has indicated that tyrosine kinase is downstream of PKC in the IPC signalling cascade (Baines *et al*, 1998).

#### 1.3.5.3. Mitogen Activated Protein Kinases

Three major families of mitogen activated protein kinases (MAPKs) are thought to exist, these include; extracellular signal regulated kinases (p42/p44 ERK), p38 MAP kinase, and the stress activated C-jun N-terminal kinase (JNK/SAPK). MAPK signalling cascades can be induced by tyrosine kinases, phospholipase C, G-protein coupled receptors and stressful stimuli including ischaemia and hypertension.

An inhibitor of p38 MAPK, SB 203580 has been shown to completely abrogate the protective effects of IPC in isolated rabbit hearts (Nakano *et al*, 2000a). Additionally, direct stimulation of p38 MAPK, with anisomycin has demonstrated protective effects (Nakano *et al*, 2000a).

Mocanu et al reported that when SB 203580 was perfused during the IPC protocol, IPC was still protective, however, when SB 203580 was given just prior to and during the prolonged ischaemic event, IPC was abolished in isolated rat heart (Mocanu *et al*, 2000). This implies that activation of p38 MAPK occurs during the sustained ischaemic episode and hence, timing of administration of SB 203580 is critical. Indeed Mocanu et al demonstrated that phosphorylation of p38 MAPK markedly increased following ischaemia in preconditioned versus control groups. This may help to explain divergent results obtained as some studies do not find an abrogation of IPC in the presence of SB 203580 and in fact report that inhibition of p38 MAPK is beneficial (Barancik *et al*, 2000). Indeed, further complications may be raised as at least four isoforms of p38 MAPKs are thought to exist; p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$ , and p38 $\delta$  (Ping & Murphy, 2000).

Some investigators have found that JNK/SAPK may be important in IPC. Barancik et al reported that in pig myocardium, IPC increased JNK rather than p38 MAPK activity (Barancik *et al*, 1997). However, in rabbit Nakano et al did not observe enhanced activity of JNK following IPC. Hence, there may be species differentiation with respect to the JNK activity following IPC (Nakano *et al*, 2000a).

Lastly, the involvement of the ERK pathway in IPC has not been widely investigated. Evidence suggests that this kinase is not involved in IPC (Maulik *et al*, 1996). Additionally, studies conducted by Mocanu et al (unpublished findings) showed that a potent ERK pathway inhibitor (PD 98059) failed to abolish IPC in isolated rat heart. In contrast, Fryer et al very recently reported that IPC and  $\delta$  opioid receptor stimulation induced protection were reduced with PD 98059 (Fryer *et al*, 2001).

It is relevant to note that studies investigating the signalling pathways implicated in IPC have largely relied upon pharmacological tools to activate or block pathways. However, many pharmacological agents are non-specific and exert effects that are not desired, hence, the use of transgenics or over expression studies in the future may generate more reliable results.

### **1.3.6. End effectors of IPC**

The final pathway in the protective cascade remains elusive. However, much evidence to date appears to suggest an imperative role for the  $K_{ATP}$  channel.

#### *1.3.6.1. Sarcolemmal $K_{ATP}$ channel*

Gross and Auchampach first demonstrated a role for the  $K_{ATP}$  channel in IPC in dog myocardium. The authors found that administration of  $K_{ATP}$  channel inhibitors- glibenclamide or 5-hydroxydecanoate (5-HD) abolished the protective effects of IPC. Additionally, they showed that treatment with aprikalim a  $K_{ATP}$  channel opener, mimicked the protective effects of IPC (Gross & Auchampach, 1992). This finding was subsequently found in a number of different species and experimental models including, humans (Gross & Fryer, 1999). The question is, how would the opening of  $K_{ATP}$  channels lead to a protective effect? It was initially suggested by Noma that opening of the sarcolemmal  $K_{ATP}$  channel would lead to the shortening of the cardiac action potential (Noma, 1983), which would lead to a reduction of calcium entry into the myocyte via L-type voltage operated calcium channels. This in turn would lead to cardioprotection by having a cardioplegic effect. However, more recently, investigators showed that  $K_{ATP}$  channel openers were protective but did not lead to action potential shortening. Yao and Gross found that Bimakalim induced cardioprotection was not due to its action potential shortening effects (Yao & Gross, 1994). Additionally, other  $K_{ATP}$  channel openers such as, cromakalim were protective, independent of any action potential

shortening (Grover *et al*, 1995). When dofetilide (class III antiarrhythmic) abolished action potential shortening in preconditioned hearts but did not abolish the protective effects of IPC, investigators began to question if sarcolemmal  $K_{ATP}$  channel opening was really involved in IPC (Grover *et al*, 1996). Furthermore, isolated non-beating cardiac myocytes could be preconditioned and were amenable to protection induced by  $K_{ATP}$  channel openers in the absence of a ventricular action potential (Armstrong *et al*, 1995). These findings strongly imply that protection is not linked with the action potential duration and thus the sarcolemmal  $K_{ATP}$  channel. This confusion was later lessened when it was realised that there are two types of  $K_{ATP}$  channels, some of which are present in the sarcolemma (sarcolemmal  $K_{ATP}$  channel) and some of which are present in the mitochondria (mitochondrial  $K_{ATP}$  channel).

#### 1.3.6.2. Mitochondrial $K_{ATP}$ channel

Garlid *et al* were the first to show that the mitochondrial  $K_{ATP}$  channel was involved in cardioprotection. They showed that diazoxide, a highly selective mitochondrial  $K_{ATP}$  channel opener led to a protective effect, which was blocked with 5-HD, an inhibitor of the mitochondrial  $K_{ATP}$  channel in rat heart (Garlid *et al*, 1997). Subsequently, numerous investigators showed that diazoxide was protective in isolated rabbit cardiac myocytes and in the *in vivo* rabbit model of ischaemia-reperfusion (Liu *et al*, 1998; Baines *et al*, 1999; Gray *et al*, 1997; Miyawaki & Ashraf, 1997). Furthermore, Auchampach *et al* demonstrated that 5-HD completely abrogated IPC in the dog, which further points to the involvement of the mitochondrial  $K_{ATP}$  channel (Auchampach *et al*, 1992). Final confirmatory evidence implicating a role for the mitochondrial  $K_{ATP}$  channel comes from using a selective sarcolemmal  $K_{ATP}$  channel antagonist, HMR 1883. Gogelein *et al* have shown that HMR 1883 does not abolish IPC (Gogelein *et al*, 1998). Pharmacological agents that induce preconditioning have also been shown to induce protection by activating the mitochondrial  $K_{ATP}$  channel.

Sato et al demonstrated activity of mitochondrial  $K_{ATP}$  channels can be regulated by PKC in intact myocardial cells (Sato *et al*, 1998). Further, the protection evoked by the PKC activator dioctanoyl-sn-glycerol is abolished with glibenclamide (mixed mitochondrial and sarcolemmal  $K_{ATP}$  channel inhibitor), in isolated human atrial trabeculae muscle suggesting that the  $K_{ATP}$  channel is downstream of the signalling pathway and thus likely to be the end effector of IPC (Speechly-Dick *et al*, 1995). This has led to the proposal by several investigators that PKC may phosphorylate the mitochondrial  $K_{ATP}$  channel, which leads to channel opening with subsequent protection. This theory has recently been challenged, and interesting experiments performed by Downey's group implicate that opening of the mitochondrial  $K_{ATP}$  channel is not the end effector of IPC. In a complex series of experiments, these authors showed in isolated rabbit hearts, a five minute exposure to diazoxide was able to induce protection even when washed out for a period of up to 30 minutes. This form of protection is analogous to that observed with a trigger of IPC, eg, adenosine or bradykinin. In addition, 5-HD only abrogated IPC when it was administered during the IPC episode and had no effect when given during the index ischaemia. Obviously, if the mitochondrial  $K_{ATP}$  channel was the end effector then protection should have been abolished when 5-HD was given during the index ischaemia as opposed to during the IPC episode. The authors concluded by postulating that opening of the mitochondrial  $K_{ATP}$  channels is not a distal mechanism of IPC but is in fact an upstream event that triggers the IPC mechanism. The opening prior to ischaemia generates free radicals (they found that diazoxide induced protection was abolished in the presence of free radical scavengers) that subsequently activate kinase cascades that ultimately target an unknown end effector (Pain *et al*, 2000). Although this is an attractive hypothesis, the vast majority of studies conducted to date have implied that the mitochondrial  $K_{ATP}$  channel is the end effector of IPC (Gross & Fryer, 2000).

How the opening of the mitochondrial  $K_{ATP}$  channel leads to protection is not understood. Channel opening would cause depolarisation of the intramitochondrial membrane as  $K^+$  enters the mitochondria. Consequently, mitochondrial swelling may result that may lead to increased respiration and enhanced ATP production. Membrane depolarisation may also reduce mitochondrial calcium entry and thus attenuate calcium overload.

#### 1.3.6.3. *Alternative end effectors*

It has been proposed that IPC may preserve cellular cytoskeletal structure (Downey & Cohen, 1997). Indeed, heat shock protein 27 (HSP 27) promotes actin polymerisation and could maintain cytoskeletal integrity of myocytes. As mentioned above, p38 MAPK may be activated as a consequence of IPC. Incidentally, p38 MAPK leads to the phosphorylation and consequent activation of HSP 27. Shattock's group demonstrated that  $\alpha\beta$  crystallin may be implicated in mediating IPC (Eaton *et al*, 2000). These investigators demonstrated that even though transient hypercarbia induced translocation of  $\alpha\beta$  crystallin to the cytoskeleton, no cardioprotection was observed. IPC however, not only led to the translocation of  $\alpha\beta$  crystallin but also enhanced its phosphorylation (Eaton *et al*, 2000). Thus, these results imply that IPC may be mediated by phosphorylation and translocation of  $\alpha\beta$  crystallin.

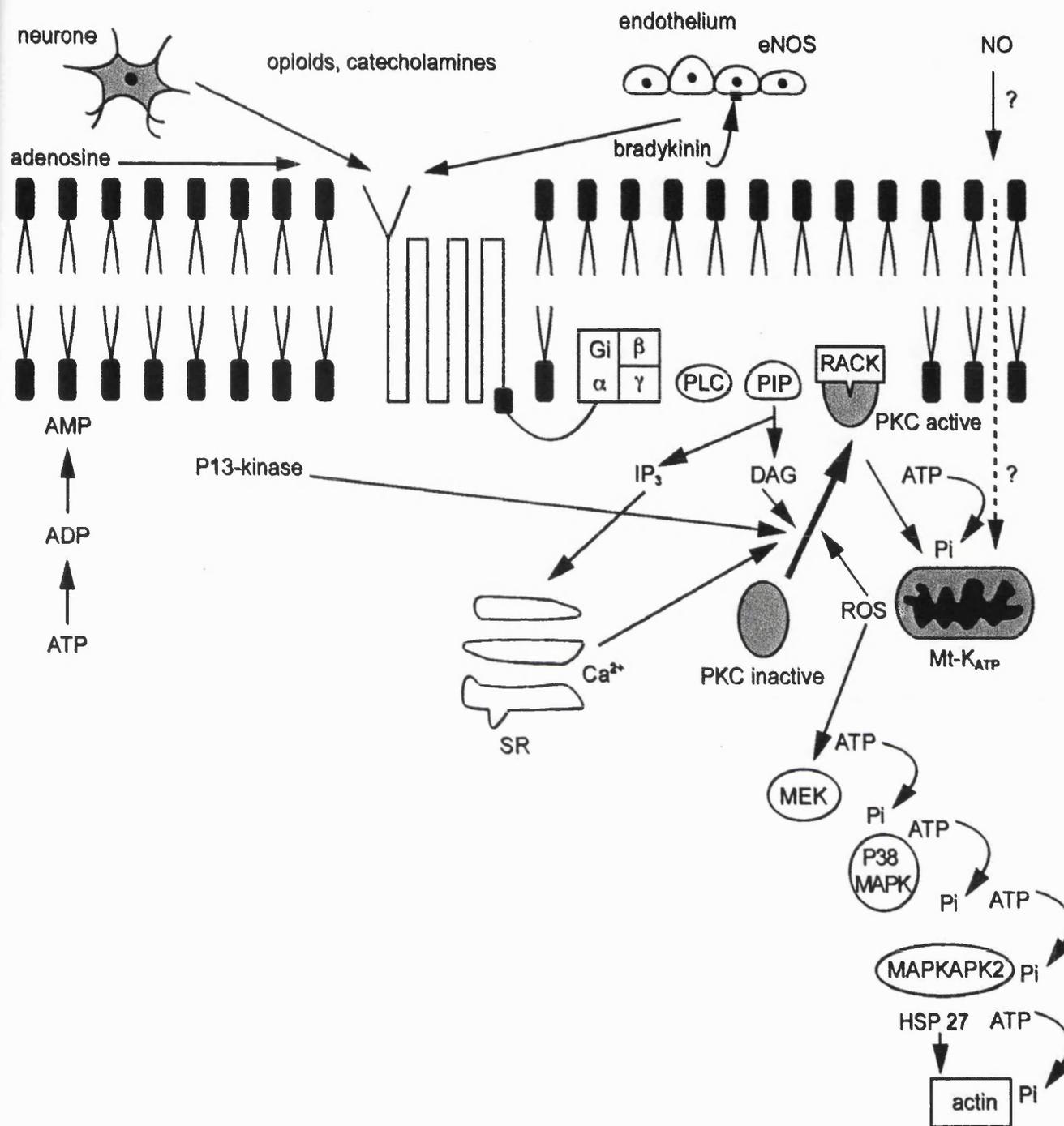


Figure 1.2. Schematic representation of IPC. Activation of G-protein linked receptors leads to the activation of PKC. PKC may phosphorylate the mitochondrial  $K_{ATP}$  channel and thereby induce cardioprotection. Activation of MAPK cascades leads to phosphorylation of HSP 27 and the subsequent polymerisation of actin.

Abbreviations used in figure: eNOS - endothelial nitric oxide synthase; NO - nitric oxide; ATP - adenosine triphosphate; ADP - adenosine diphosphate; AMP - adenosine monophosphate; PLC - phospholipase C; PIP - phosphatidyl inositol phosphate; RACK - receptor for activated C kinase; PKC - protein kinase C; IP<sub>3</sub> - inositol 3,4,5 phosphate; DAG - diacylglycerol; mt- $K_{ATP}$  - mitochondrial  $K_{ATP}$  channel; ROS - reactive oxygen species; MAPK - mitogen activated protein kinase; MEK - MAPK/ERK kinase; MAPKAPK2 - mitogen activated protein kinase activating protein kinase 2; HSP 27 - heat shock protein 27; SR - sarcoplasmic reticulum; P13 kinase - phosphatidylinositol-3'-OH kinase.

#### 1.4. Delayed Preconditioning

IPC described above, is also referred to as “classical preconditioning”. Classical preconditioning begins within minutes of the ischaemic stimulus and protection usually wanes within one-two hours. However, in 1993, two independent groups, Yellon's group and Tada's group demonstrated the existence of another form of preconditioning. They reported that approximately 24 hours following the preconditioning ischaemia, there is the induction of a delayed phase of protection. Yellon's group reported that in the rabbit, four five minute coronary occlusions prior to the prolonged ischaemic event resulted in a reduction in infarct size 24 hours later (Marber *et al*, 1993). Similarly, Tada's group reported that an identical preconditioning protocol produced a similar degree of protection in the dog (Kuzuya *et al*, 1993). This phenomenon termed the *delayed preconditioning* although, not as powerful as classical preconditioning, confers protection for up to three-four days. Thus, this delayed preconditioning, also known as the *second window of protection* (SWOP) or *the late phase of preconditioning*, may be of greater clinical benefit due to its longer duration of action. For the purpose of this thesis, the term delayed preconditioning will be used. The time course of the protection indicates that a molecular adaptation, resulting in altered gene expression and consequently protein synthesis occurs following the preconditioning stimulus. These speculations were confirmed when protein synthesis was blocked using cycloheximide, which also blocked the occurrence of delayed preconditioning (Rizvi *et al*, 1999). Finally, delayed preconditioning confers robust protection against myocardial stunning, infarction and arrhythmias, unlike classical preconditioning in which protection against stunning is equivocal (Bolli, 2000).

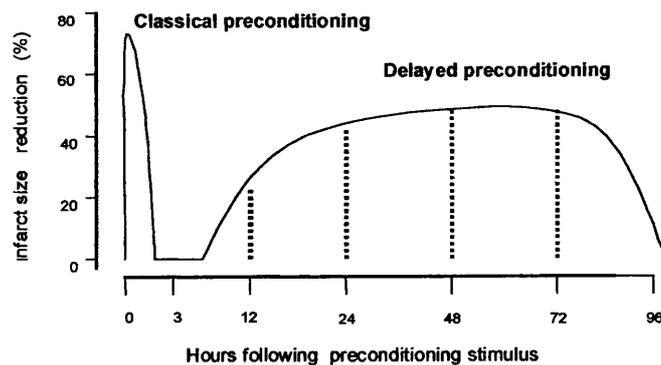


Figure 1.3.  
Time course of classical  
and delayed preconditioning

Like classical preconditioning, delayed preconditioning has been found to occur in many species including rabbit (Marber *et al*, 1993), dog (Kuzuya *et al*, 1993), pig (Sun *et al*, 1995), rat (Yamashita *et al*, 2000) and mouse (Guo *et al*, 1998). Noda *et al* demonstrated the existence of delayed preconditioning in humans (Noda *et al*, 1999). Additionally, Arstall *et al* demonstrated that human foetal cardiac myocytes were amenable to delayed preconditioning (Arstall *et al*, 1998). However, it is important to point out that the physiology of foetal ventricular myocytes is different from that of the adult equivalent (Bustamante *et al*, 1982).

#### **1.4.1. Mechanisms of delayed preconditioning**

The triggers and putative signalling pathways of delayed preconditioning are described below.

##### **1.4.1.1. Triggers of delayed preconditioning**

Agents initially released during the preconditioning ischaemia that initiate the protection can be regarded as the “triggers” of delayed protection. Many triggers (described below) are released during ischaemia, some of which have also been shown to participate in classical preconditioning.

##### **1.4.1.1.1. Adenosine**

Baxter *et al* were the first to report a role for adenosine in delayed preconditioning. These investigators using an *in vivo* rabbit model of coronary artery occlusion found that adenosine receptor blockade using SPT during preconditioning abolished protection against infarction 24 hours later. Conversely, administration of the adenosine A<sub>1</sub> receptor agonist, 2-chloro-N<sup>6</sup>-cyclopentyl-adenosine (CCPA) 24 hours prior to coronary artery occlusion also led to a reduction in infarct size (Baxter *et al*, 1994). It has recently also been demonstrated that

adenosine A<sub>3</sub> receptor activation can trigger protection against infarction (Takano *et al*, 1999). Hence both A<sub>1</sub> and A<sub>3</sub> receptor activation can induce delayed protection against infarction. A role for adenosine in protection against myocardial stunning, however, has not been demonstrated. Bolli's group were unable to abort protection against stunning using SPT and PD (both are A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub> antagonists) in pig and rabbit. Conversely, CCPA administration 24 hours prior to the ischaemic insult did not protect against stunning. Thus, protection against stunning may involve alternative triggers.

#### 1.4.1.1.2 Nitric oxide

An extensive number of studies have examined the involvement of nitric oxide in delayed preconditioning. Bolli *et al* were the first to demonstrate a role of nitric oxide in triggering delayed preconditioning. In conscious rabbits, these authors showed that N<sup>ω</sup>-nitro-L-arginine (L-NA), a non-specific inhibitor of all nitric oxide synthase isoforms (including, neuronal, endothelial and inducible) administered prior to preconditioning ischaemia, abolished protection against stunning (Bolli *et al*, 1997a). These findings suggest the nitric oxide generated during the preconditioning ischaemia can trigger delayed preconditioning. Furthermore, the same authors reported that nitric oxide donors attenuated stunning in conscious rabbits (Shinmura *et al*, 1999). Finally, Bolli's group also demonstrated that L-NA aborted the protection against *infarction* in conscious rabbits (Qui *et al*, 1997). Thus, these investigators have provided robust evidence in favour of endogenous nitric oxide as a trigger of IPC. As protection was blocked using L-NA but not with selective inducible nitric oxide synthase inhibitors (iNOS), aminoguanidine and S-methylisothiurea, endothelial NOS (eNOS) is likely to be the source of nitric oxide during preconditioning ischaemia.

#### 1.4.1.1.3. Reactive oxygen species

Sun et al demonstrated an obligatory role for reactive oxygen species (ROS) in the genesis of delayed preconditioning. In conscious pigs, application of a combination of antioxidants consisting of, superoxide dismutase and MPG during the preconditioning ischaemia abolished the protective effect of late preconditioning against stunning (Sun *et al*, 1996). Similar effects were observed in rabbits when MPG was administered (Tang *et al*, 1997). MPG also abolished delayed preconditioning against infarction and arrhythmias (Yamashita *et al*, 1998b). Conversely, administration of ROS-generating solution prior to sustained coronary artery occlusion led to a protective effect against stunning 24 hours later (Takano *et al*, 1997).

#### 1.4.2. Putative signalling pathways in delayed preconditioning

Analogous to classical preconditioning, kinase cascades appear to be involved in delayed preconditioning as well. A role for PKC, MAPKs, tyrosine kinases and the transcription factor, nuclear factor  $\kappa$ B (NF- $\kappa$ B) have been proposed and described below.

##### 1.4.2.1. PKC

Baxter et al were the first to propose a role for PKC in delayed preconditioning. They demonstrated that delayed preconditioning induced by ischaemia was abrogated with the PKC inhibitor chelerythrine in rabbits (Baxter *et al*, 1995). Conversely, they showed that treatment with diacylglycerol 24 hours prior to infarct induction, significantly attenuated infarct size (Baxter *et al*, 1997a). Subsequently, many studies have reported the involvement of PKC in delayed preconditioning induced by various methods (Dana *et al*, 1997; Joyeux *et al*, 1997; Qian *et al*, 1999; Sharma & Singh, 2000). Ping et al tried to determine which specific PKC isoform is involved in delayed preconditioning. These investigators showed that analogous to classical preconditioning, the initial ischaemic stimulus induced

translocation of PKC  $\epsilon$  and  $\eta$  from cytosolic to particulate fraction. Chelerythrine blocked translocation of PKC  $\epsilon$  and also blocked protective effects of delayed preconditioning against myocardial stunning. Thus, these results implicate a role for PKC  $\epsilon$  in delayed preconditioning (Qui *et al*, 1998). The same authors subsequently demonstrated that administration of L-NA blocked preconditioning ischaemia induced PKC  $\epsilon$  translocation, implying that nitric oxide generation during the preconditioning ischaemia causes the translocation of PKC  $\epsilon$  (Ping *et al*, 1999a). Conversely, these authors showed that direct administration of nitric oxide donors triggered PKC activation in the myocardium. Thus, these results imply that PKC is downstream of nitric oxide in the signalling cascade and nitric oxide released during the preconditioning ischaemia activates PKC  $\epsilon$ .

#### 1.4.2.2. *Mitogen activated protein kinases*

The signalling pathway downstream of PKC is largely unknown. Bolli's group demonstrated that preconditioning ischaemia caused activation of p42/p44 MAPKs, an effect blocked with chelerythrine (Ping *et al*, 1999b). This implies that MAPK signalling is downstream of PKC. The role of MAPKs in delayed ischaemic preconditioning is as yet unknown.

Dana *et al* showed a seven fold increase in p38 MAPK catalytic activity 24 hours following CCPA treatment, an effect abrogated with chelerythrine, indicating that this kinase may also be downstream and in part may be mediated by PKC activation (Dana *et al*, 2000b). Very recently, Kukreja's laboratory showed that CCPA also induced p38 MAPK phosphorylation in mouse hearts. Interestingly, they also reported that CCPA's infarct sparing effect was abrogated using a p38 MAPK inhibitor, SB203580 (Zhao *et al*, 2001). Thus, although a role for MAPK signalling in delayed ischaemic preconditioning is not yet known, there appears to be a role for these signalling intermediates in adenosine A<sub>1</sub> receptor induced delayed preconditioning.

#### 1.4.2.3. *Tyrosine kinases*

Imagawa et al demonstrated that genistein, a tyrosine kinase inhibitor, completely abrogated the protective effects of delayed preconditioning induced by ischaemia in rabbits (Imagawa *et al*, 1997). Dana and et al showed that lavendustin A blocked the cardioprotective effects of CCPA and the CCPA induced increase in p38 MAPK activity, indicating that p38 MAPK signalling is downstream of tyrosine kinase signalling (Dana *et al*, 2000b). Ping et al have also found a role for the Src family of tyrosine kinase (Src and Lck) in delayed ischaemic and nitric oxide donor preconditioning (Bolli, 2000).

#### 1.4.2.4. *Nuclear factor $\kappa$ B*

Xuan et al revealed a role for the transcription factor NF $\kappa$ B in delayed preconditioning. They found that NF $\kappa$ B was briefly activated following preconditioning ischaemia, an effect blocked by agents known to inhibit delayed preconditioning, namely, L-NA, MPG, chelerythrine and lavendustin A (Xuan *et al*, 1999). This suggests that NF $\kappa$ B activation is a common downstream pathway through which nitric oxide, ROS, tyrosine kinase and PKC act to induce gene transcription.

### 1.4.3. **Distal effectors of delayed preconditioning**

Generation of triggers during the preconditioning ischaemia (day 1) subsequently leads to the expression of substances in the myocardium 24-72 hours later (day 2-4). Expression of the distal effectors is observed during the sustained ischaemic event. Numerous distal effectors have been implicated in delayed preconditioning, described below.

#### 1.4.3.1. *Nitric oxide synthases*

Bolli's group have also provided evidence for the involvement of nitric oxide in mediating delayed preconditioning. They showed that administration of L-NA prior to the sustained

ischaemic episode (ie, 24 hours following preconditioning ischaemia) abrogated the protective effects of delayed preconditioning against myocardial infarction (Takano *et al*, 1998) and stunning in rabbits (Bolli *et al*, 1997b). Additionally, the selective iNOS inhibitors namely, aminoguanidine and S-methylisothiourea, given just before index ischaemia, abolished delayed preconditioning (Bolli *et al*, 1997b; Takano *et al*, 1998). These data strongly imply a role for nitric oxide synthesised by iNOS in mediating delayed preconditioning. Final conclusive evidence arises from transgenic mice studies. Mice with a targeted disruption of the iNOS gene are unresponsive to delayed preconditioning (Guo *et al*, 1999). This led Bolli to formulate the “Nitric oxide hypothesis of late preconditioning”, which proposes that nitric oxide is not only the trigger but also the mediator of delayed preconditioning. Several studies have also demonstrated that pharmacologically induced delayed preconditioning is also dependent upon iNOS activity (Zhao *et al*, 1997; Ockaili *et al*, 1999), although some investigators disagree with this (Bell *et al*, 1999; Dana *et al*, 2001).

#### 1.4.3.2. *Manganese superoxide dismutase*

The temporal activity of manganese superoxide dismutase is biphasic after a preconditioning stimulus, with a time course similar to classical and delayed preconditioning (Hoshida *et al*, 1993). Furthermore, in cultured rat myocytes, Yamashita *et al* demonstrated that oligonucleotide anti-sense to manganese superoxide dismutase abrogated the protective effect of delayed preconditioning (Yamashita *et al*, 1994). Dana *et al* also reported the involvement of manganese superoxide dismutase in delayed preconditioning induced pharmacologically with the adenosine A<sub>1</sub> agonist CCPA. Investigators found that CCPA administered 24 hours prior to infarct induction led to a significant limitation of infarct size in rat heart (Dana *et al*, 2000a). Simultaneously, they also noted that activity and content of manganese superoxide dismutase was increased following CCPA treatment, and interestingly, pre-treatment with antisense oligonucleotides to manganese superoxide

dismutase completely abrogated the cardioprotective effects of CCPA (Dana *et al*, 2000a). Similarly, studies have found that heat stress induced (Yamashita *et al*, 1998a) and exercise (Yamashita *et al*, 1999) induced delayed protection involve manganese superoxide dismutase.

#### 1.4.3.3. Cyclooxygenase-2

Shinmura *et al* have shown an imperative role for cyclooxygenase-2 (COX-2) in delayed preconditioning. In conscious rabbit, these investigators found that two unrelated COX-2 inhibitors (NS-398 and celecoxib) administered 24 hours following preconditioning ischaemia abolished cardioprotective effects of delayed preconditioning against both myocardial stunning and infarction (Shinmura *et al*, 2000). They also demonstrated the increase in COX-2 protein levels that occurred 24 hours following preconditioning ischaemia was abolished with the COX-2 inhibitors. These data strongly imply the involvement of COX-2 in mediating delayed preconditioning.

#### 1.4.3.4. Heat shock proteins

The role of HSPs in delayed preconditioning is unresolved. Studies in mice overexpressing HSP 70 have shown that this protein induces protection against ischaemia-reperfusion injury (Marber *et al*, 1995; Radford *et al*, 1996;). Additionally, studies have shown an increase in HSP 70 content following preconditioning ischaemia and heat shock (elevation of basal body temperature to 42 °C for a period of 15 minutes) induced delayed preconditioning (Marber *et al*, 1993; Joyeux *et al*, 1998). Subsequently, however, studies have also showed that ischaemia, CCPA or MLA induced delayed protection do not cause HSP 70 induction (Baxter & Yellon, 1997b; Yoshida *et al*, 1996). Involvement of HSP 70 in delayed preconditioning is therefore questionable and it is perhaps feasible to postulate that HSP 70 induction *per se* does not confer delayed protection and that increased expression of HSP

70 following preconditioning ischaemia or heat shock may be an epiphenomenon as a consequence of myocardial stress. Recently, Dana et al implied a role for HSP 27 in CCPA induced delayed protection (Dana *et al*, 2000b). These investigators demonstrated an increase in phosphorylated HSP 27 in hearts pre-treated with CCPA compared with control hearts, suggesting that the p38MAPK/HSP 27 pathway may be a distal end effector of adenosine A<sub>1</sub> receptor activation (Dana *et al*, 2000b)

#### 1.4.3.5 *K<sub>ATP</sub> channel*

Studies have also found a role for the K<sub>ATP</sub> channel. Mei et al were the first to show that K<sub>ATP</sub> channels are involved in delayed preconditioning. The authors reported that MLA induced delayed preconditioning was blocked by glibenclamide and 5-HD given 24 hours following MLA treatment in dog (Mei *et al*, 1996). Identical results were obtained by the same group in rabbits. Following these reports, several studies have shown that delayed protection induced by opioids (Fryer *et al*, 1999), ischaemia (Takano *et al*, 2000), heat stress (Pell *et al*, 1997) and adenosine A<sub>1</sub> agonist (Baxter & Yellon, 1999) are dependent on K<sub>ATP</sub> channel opening. As these studies have used the so called selective mitochondrial K<sub>ATP</sub> channel antagonist, 5-HD, a role for the mitochondrial K<sub>ATP</sub> channel in delayed preconditioning may be possible, although a role for the sarcolemmal K<sub>ATP</sub> channel cannot be completely excluded. Furthermore, the selective mitochondrial K<sub>ATP</sub> channel opener, diazoxide has been shown to produce delayed preconditioning in rats, an effect also abrogated with 5-HD (Takashi *et al*, 1999). Interestingly, Takano et al reported that although 5-HD blocked the anti-infarct effect of delayed preconditioning induced by ischaemia, it did not abolish the anti-stunning effect of delayed preconditioning (Takano *et al*, 2000). These data suggest that different mechanisms are involved in these two forms of cardioprotection.

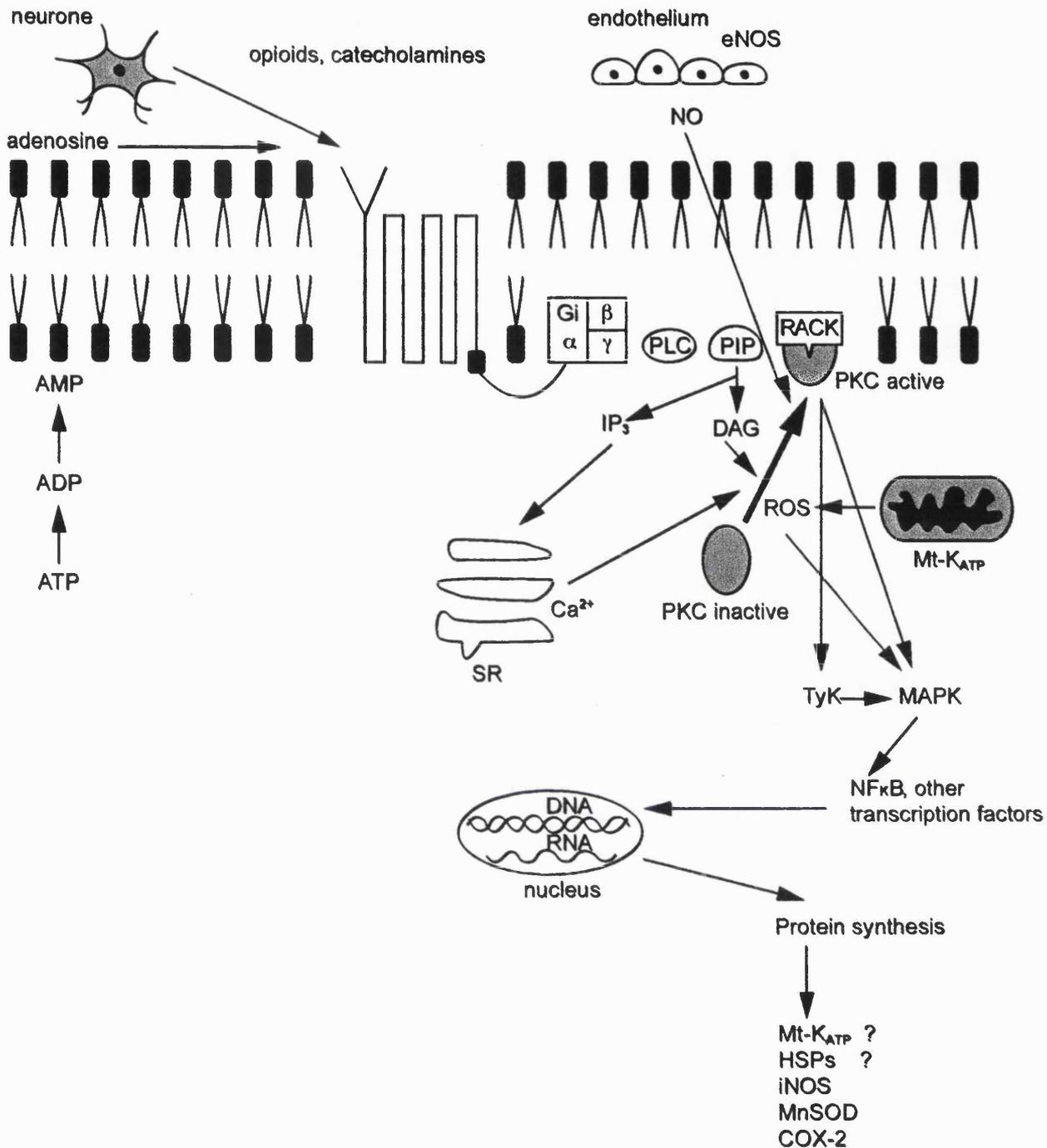


Figure 1.4. Schematic representation of delayed preconditioning. Triggers lead to the activation of kinase cascades which subsequently activate transcription factors eg, NFκB. This promotes the synthesis of various cardioprotective substances.

Abbreviations used in figure: e/iNOS - endothelial/inducible nitric oxide synthase; NO - nitric oxide; ATP - adenosine triphosphate; ADP - adenosine diphosphate; AMP - adenosine monophosphate; PLC - phospholipase C; PIP - phosphatidyl inositol phosphate; RACK - receptor for activated C kinase; IP<sub>3</sub> - inositol 3,4,5 phosphate; PKC - protein kinase C; DAG - diacylglycerol; mt-K<sub>ATP</sub> - mitochondrial K<sub>ATP</sub> channel; ROS - reactive oxygen species; MAPK - mitogen activated protein kinase; HSPs - heat shock proteins; TyK - tyrosine kinase; NFκB - nuclear factor κB; DNA - deoxyribonucleic acid; RNA - ribonucleic acid; MnSOD - manganese superoxide dismutase; COX-2 - cyclo-oxygenase-2; SR - sarcoplasmic reticulum.

## Chapter One

### Part II: The cardioprotective actions of bradykinin

#### 1.5. General properties of bradykinin

Bradykinin is a nonapeptide, belonging to a family of peptides called kinins. The most important physiologically active kinins include kallidin (Lys-bradykinin), bradykinin and des-Arg<sup>9</sup>-bradykinin. Rocha e Silva *et al* in 1949 found that incubation of venom extracts of *Bothrops jararaca* or trypsin with the globulin fraction of dog plasma resulted in the production of a substance that produced a slow, delayed contraction of the isolated guinea pig ileum. They named this substance bradykinin (Greek: 'brady' meaning slow and 'kinin' meaning movement) (Rocha e Silva *et al*, 1949).

Since its discovery, actions of bradykinin in a number of tissues and physiological and pathological responses have been extensively researched (Bhoola *et al*, 1992; Wirth *et al*, 1997). Bradykinin is a potent mediator of tissue pain and tissue inflammation (Calixto *et al*, 2000). Bradykinin causes contraction of smooth muscle of bronchioles, intestine and uterus (Bhoola *et al*, 1992). Bradykinin also promotes glucose and chloride transport and cell proliferation (Bhoola *et al*, 1992). In the cardiovascular system, bradykinin induces vasodilation by potentiating the release of nitric oxide and prostacyclin (PGI<sub>2</sub>) (Hatta *et al*, 1997; Wirth *et al*, 1997). Exogenous bradykinin dilates coronary arteries in isolated hearts and *in vivo*.

##### 1.5.1. Synthesis of bradykinin in the myocardium

Both kallidin and bradykinin are synthesised by catalytic enzymes called kallikreins acting on kininogen precursor molecules (summarised in figure 2.1). Precursors of kallikreins are

found in plasma (pre-kallikreins) and in tissues (pro-kallikreins). They are activated by a variety of stimuli including activated factor XII (Hageman factor). In the rat, both tissue and plasma kallikrein generate bradykinin, however, in man, tissue kallikrein generates kallidin and plasma kallikrein generates bradykinin (Campbell, 2000). The substrates, kininogens are primarily synthesised in the liver. Three forms of kininogens have been found in mammals; the largest type, high molecular weight kininogen (HMWK, 88-115 kDa, depending upon species) and low molecular weight kininogen (LMWK, 50-68 kDa). The third type, T-kininogen (68 kDa) is only found in rat (Blais *et al*, 2000).

Vascular endothelial cells are the primary source of bradykinin (Wirth *et al*, 1997; Linz *et al*, 1997), although, it has recently been proposed that the cardiac myocyte can also synthesise this peptide (Matoba *et al*, 1999). Injured endothelial cells cause the activation of factor XII, which catalyses the conversion of prekallikrein to kallikrein at the endothelial cell surface. Kallikrein subsequently cleaves kininogen to release bradykinin. Pre-kallikrein can also be activated in the absence endothelial cell damage, whereby factor XII is activated by a receptor mediated mechanism. The kininogen has multiprotein receptor complexes which allow the binding of factor XII and subsequent activation. Prekallikrein can also be activated by a factor XII independent mechanism (Blais *et al*, 2000).

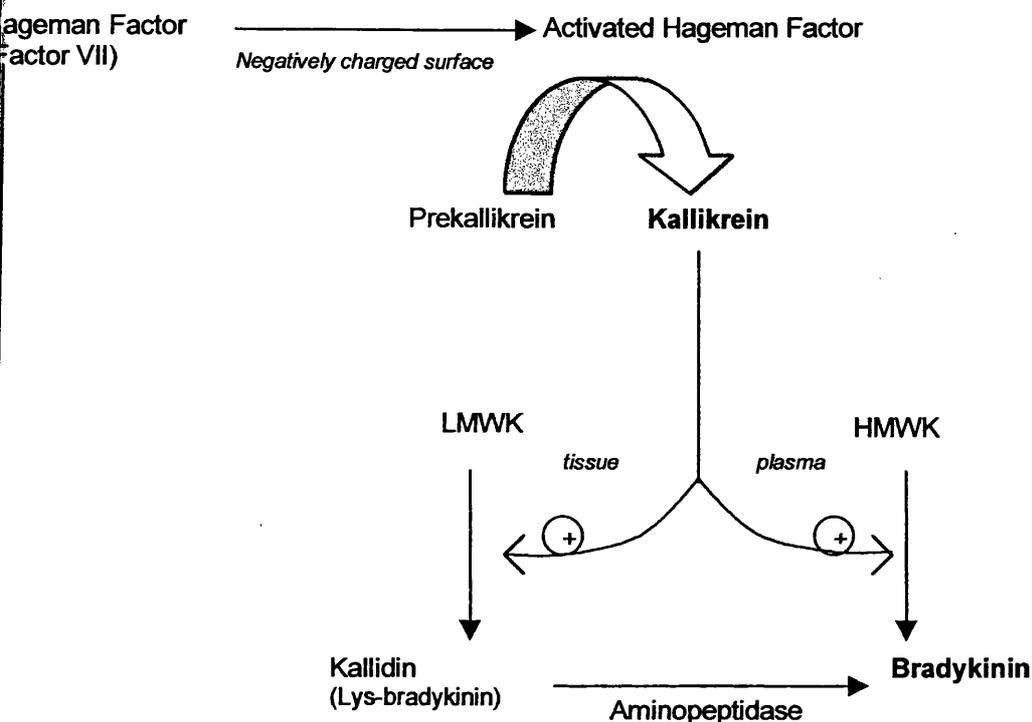


Figure 1.5. Schematic overview of the synthesis of bradykinin.

Activation of Hageman factor leads to the conversion of prekallikrein to kallikrein following which kallikrein converts kininogen to bradykinin.  
 LMWK - low molecular weight kininogen; HMWK - high molecular weight kininogen

A number of studies have provided evidence that even during brief preconditioning periods of ischaemia, tissue and plasma bradykinin levels (assessed by radio-immunoassay) increase markedly (Linz *et al*, 1996; Schulz *et al*, 1998; Pan *et al*, 2000; Campbell, 2000). It is relevant to point out that bradykinin is generated in isolated tissues and endothelial cells in the absence of plasma. Bradykinin released during ischaemia has been shown to primarily originate from endothelial cells (Wirth *et al*, 1997; Linz *et al*, 1997). However, the exact molecular pathological mechanism leading to bradykinin generation during ischaemia is not understood. The reduction in oxygen supply cannot explain the bradykinin release for endothelial cells are more resistant to ischaemia / reperfusion injury than cardiac myocytes

(Silverman *et al*, 1995). However, the plasma kallikrein system is thought to be activated following altered proton release during ischaemia. A fall in pH occurs following IPC which could explain the release of bradykinin (Edery & Lewis, 1962).

### **1.5.2. Catalytic degradation of bradykinin in the myocardium**

Circulating levels of this peptide are usually very low under basal conditions. Enzyme linked and radio-immunoassay of bradykinin has proved to be technically difficult and mean plasma concentrations in human studies range from 6-4200 pmol/L (Blais *et al*, 2000). Such low levels can be attributed to the rapid catabolism of this peptide once it is formed. Once released, bradykinin is rapidly degraded into inactive metabolites within 30 seconds (Bhoola *et al*, 1992). Enzymes that degrade bradykinin are collectively referred to as *kininases* or *kinin peptidases*. The most important of these are kininase I (syn. carboxypeptidase N; EC 3.4.17.3 [enzyme commission number, set by the enzyme committee of the international union of Biochemistry and Molecular Biology]), Kininase II (syn. angiotensin converting enzyme [ACE]; EC 3.4.15.1), neutral endopeptidase (NEP; syn enkephalinase; EC 3.4.24.11), carboxypeptidase M (syn.membrane-bound kininase I; EC 3.4.17.1), and aminopeptidase P (syn. prolyl-aminopeptidase; EC 3.4.11.9). These enzymes are zinc metalloproteases ie, they contain a zinc atom in their active site, and their catalytic activity is dependent on the presence of zinc. Other enzymes may degrade bradykinin but their contribution *in vivo* is probably negligible; they include endopeptidase (EC 3.4.24.15), endothelin converting enzyme (ECE; EC 3.4.24.71) and prolyl endopeptidase (PEP; EC 3.4.21.26) (Mc Dermott *et al*, 1987; Brown & Vaughan, 1998; Piedimonte *et al*, 1994; Ura *et al*, 1987; Ersahin *et al*, 1999; Kuoppala *et al*, 2000). Table 1.1 summarises some of the enzymes involved in the catalytic degradation of bradykinin.

Table 1.1. Some of the enzymes responsible for the degradation of bradykinin

Enzyme	Synonym	EC number
Kininase I	Carboxypeptidase N	EC 3.4.17.3
Kininase II	Angiotensin Converting Enzyme (ACE)	EC 3.4.15.1
Neutral endopeptidase (NEP)	Enkephalinase	EC 3.4.24.1
Carboxypeptidase M	Membrane-bound kininase I	EC 3.4.17.1
Aminopeptidase P	Prolyl-aminopeptidase	EC 3.4.11.9
Endopeptidase	-	EC 3.4.24.1
Endothelin converting enzyme (ECE)	-	EC 3.4.24.7
Prolyl endopeptidase (PEP)	-	EC 3.4.21.2
Aminopeptidase N	-	EC 3.4.11.2
Dipeptidyl peptidases IV	-	EC 3.4.14.5

Among these enzymes, ACE is believed to be one of the most important enzymes involved in degrading bradykinin (Heusch *et al*, 1997; Ersahin & Simmons, 1997; Dumoulin *et al*, 1998; Kuoppala *et al*, 2000). ACE additionally catalyses the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor with growth promoter actions. ACE has a higher affinity for bradykinin than for angiotensin I highlighted by a lower apparent  $K_m$  for bradykinin than for angiotensin I, indicating more favourable kinetics for bradykinin degradation than for angiotensin I (Zisman, 1998). Hence, ACE may be regarded as being primarily a kininase and then an angiotensinase (Blais *et al*, 2000).

Despite the apparent primacy of ACE as a kininase, other enzymes play important roles in the inactivation of bradykinin. Kokkonen *et al* demonstrated using human cardiac membrane

samples that NEP and not ACE is the primary enzyme responsible for the degradation of bradykinin (Kokkonen *et al*, 1999). It has also been demonstrated that a NEP inhibitor prevented isoprenaline induced hypoperfusion in the rat, an effect abolished by Hoe 140, suggesting that the effect was due to reduced degradation of bradykinin as a consequence of NEP inhibition (Piedimonte *et al*, 1994). In contrast to these findings, Dumoulin *et al* reported, using an isolated rat heart model, that ACE is the main enzyme that catalyses the breakdown of bradykinin and that NEP only comes into action when the activity of ACE is impaired (Dumoulin *et al*, 1998). On the other hand, Ura *et al* demonstrated that NEP is the main enzyme contributing to total urinary kininase activity (Ura *et al*, 1987). The reasons for these discrepant findings is not clear. However, it may be feasible that bradykinin degradation mechanisms may differ according to the tissue in question and presence and absence of plasma. Indeed, Kokkonen *et al* have suggested that in the vascular bed of human heart bradykinin is degraded by ACE, and in the interstitium of the human heart, bradykinin is catabolised by NEP (Kokkonen *et al*, 2000).

### **1.5.3. Bradykinin receptors**

Bradykinin is thought to produce its effects by targeting two receptor subtypes, type 1 (B<sub>1</sub>) and type 2 (B<sub>2</sub>) (Hall, 1997) although a B<sub>3</sub> receptor has been found in the trachea. Molecular cloning techniques have identified the gene encoding B<sub>1</sub> receptors in the rabbit, human and mouse, and B<sub>2</sub> receptors in the rat, human and mouse. The B<sub>1</sub> and B<sub>2</sub> receptor show little sequence homology (36%) (Hall, 1997). The bradykinin B<sub>2</sub> receptor usually predominates, with the bradykinin B<sub>1</sub> receptor only being expressed during pathological conditions (Bhoola *et al*, 1992). Highly specific antagonists at the B<sub>2</sub> receptor include the bradykinin-derivative Hoe140 (icatibant) (Wirth *et al*, 1991) and the non-peptides FR173657 and FR167344 (Aramori *et al*, 1997). This is a G-protein coupled receptor, which once occupied by bradykinin, has been shown to release inositol triphosphate and diacyl glycerol,

evoking the release of  $Ca^{2+}$  which can subsequently activate PKC (Minshall *et al*, 1995; Derian & Moskowitz, 1986; Morgan-Boyd *et al*).

#### **1.5.4. Bradykinin and its role in IPC**

Scholkens *et al* were the first to report the cardioprotective effects of exogenously administered bradykinin (Scholkens *et al*, 1988). In the isolated rat heart model of ischaemia / reperfusion, bradykinin increased coronary flow, reduced the occurrence of arrhythmias, improved cardiac function and led to an improvement of metabolic efficiency (Scholkens *et al*, 1988). Following this report, bradykinin was also found to be cardioprotective in the *in vivo* dog and pig models of coronary artery occlusion (Tio *et al*, 1991; Tobe *et al*, 1991; Vegh *et al*, 1991).

Several years following the observation of the cardioprotective properties of bradykinin, a role for this kinin in eliciting IPC was noted. Wall *et al* reported the involvement of bradykinin in IPC (Wall *et al*, 1994). These investigators using an *in vivo* rabbit model of coronary artery occlusion reported that a specific bradykinin B<sub>2</sub> receptor antagonist, Hoe 140, abrogated IPC (Wall *et al*, 1994). They also found that preconditioning could be mimicked by direct administration of exogenous bradykinin (Wall *et al*, 1994). Almost simultaneously, Vegh *et al* (Vegh *et al*, 1994) documented the abrogation of the anti-arrhythmic effects of IPC with Hoe 140 in a canine model of coronary artery occlusion. The ability of bradykinin to mimic IPC has subsequently been confirmed by numerous investigators in a variety of models including, rats (Bugge & Ytrehus, 1996a; Starkopf *et al*, 1997), pigs (Schulz *et al*, 1998) and more importantly also in the human myocardium (Leesar *et al*, 1999).

Bugge and Ytrehus found that application of exogenous bradykinin was able to mimic IPC in an isolated rat heart model of global ischaemia (Bugge & Ytrehus, 1996a). However, IPC

was not abrogated when Hoe140 was used, implying that endogenous bradykinin is not involved in IPC in rat heart. Using a different end point, this group of investigators also demonstrated that IPC was not abrogated with Hoe 140 in rat heart (Starkopf *et al*, 1997). Brew *et al* on the other hand demonstrated that IPC was abolished with the bradykinin B<sub>2</sub> receptor antagonist NPC-349 or B7982 (D-Arg[Hyp<sup>3</sup>-Thi<sup>5,8</sup>-D-Phe<sup>7</sup>]BK) in rat heart (Brew *et al*, 1995). Goto *et al* were unable to demonstrate the involvement of endogenous bradykinin in IPC of isolated buffer perfused rabbit hearts. This was attributed to the lack of blood-borne kininogens (Goto *et al*, 1995). However, they reported that icatibant blocked the infarct limiting effect of preconditioning in rabbit heart *in vivo* when one cycle of ischaemia was used as a preconditioning stimulus (Goto *et al*, 1995). Hence, endogenous bradykinin plays an important role in triggering IPC in rabbit.

Vegh *et al* have demonstrated that IPC is abrogated in the *in vivo* dog model of coronary artery occlusion using Hoe 140 (Vegh *et al*, 1994). In contrast to these findings, Sun and Wainwright demonstrated that endogenous bradykinin did not play a role in IPC and that exogenous bradykinin did not limit the occurrence of arrhythmias in the rat (Sun & Wainwright, 1994). Despite these inconsistencies in the literature examining the participation of endogenous bradykinin in triggering IPC, the ability of bradykinin to mimic IPC has been demonstrated in a variety of models both *in vivo* and *in vitro*, including; the isolated rabbit heart (Goto *et al*, 1995), and the isolated rat heart with infarct size as an end point (Bugge & Ytrehus, 1996a); with arrhythmias (Hassanabad *et al*, 1998) and contractile recovery as end points (Brew *et al*, 1995); and in pigs subjected to infarction (Schulz *et al*, 1998); and in humans undergoing coronary angioplasty with ST segment shift as the end point (Leesar *et al*, 1999).

Table 1.2. The role of bradykinin in IPC

**a. Bradykinin does play a role in IPC**

<i>Study</i>	<i>Species</i>	<i>End point / Model / Finding</i>
Wall et al; 1994	Rabbit	Infarct size/ <i>in vivo</i> coronary artery ligation/ IPC was abrogated with Hoe 140.
Vegh et al; 1994	Dog	Arrhythmias/ <i>in vivo</i> coronary artery ligation/ IPC was abrogated with Hoe 140.
Goto et al; 1995	Rabbit	Infarct size/ <i>in vivo</i> coronary artery ligation/ IPC (induced with one cycle of ischaemia) was abrogated with Hoe 140.
Brew et al; 1995	Rat	Functional recovery/ <i>in vitro</i> coronary artery ligation/ IPC was abrogated with B <sub>2</sub> receptor antagonist NPC-349
Yang et al; 1997	Mouse	Infarct size/ <i>in vivo</i> coronary artery ligation/ IPC was absent in bradykinin B <sub>2</sub> receptor knock-out mice
Yang et al; 1997	Brown Norwegian Katholiek rat strain	Infarct size + reperfusion arrhythmias/ <i>in vivo</i> coronary artery ligation/ IPC was absent in HMWK deficient rats.

**b. Bradykinin does not play a role in IPC**

Sun & Wainwright; 1994	Rat	Arrhythmias/ <i>in vivo</i> coronary artery ligation/ IPC was not abolished with Hoe 140.
Ytrehus & Bugge; 1996	Rat	Infarct size/ <i>in vitro</i> coronary artery ligation/ IPC was not abolished with Hoe 140.
Goto et al; 1995	Rabbit	Infarct size/ <i>in vitro</i> coronary artery ligation/ IPC was not abolished with Hoe 140.
Starkopf et al; 1997	Rat	Functional recovery/ <i>in vitro</i> coronary artery ligation/ IPC was not abolished with Hoe 140.

Further conclusive evidence implying a central role for bradykinin in IPC comes from mice with a targeted disruption of the bradykinin B<sub>2</sub> receptor (Yang *et al*, 1997c). Yang *et al* using a transgenic mouse model discovered that IPC could not protect these mice, using infarct size as an experimental end point. These authors also demonstrated that rats deficient in high molecular weight kininogen, a source of bradykinin could not display the preconditioning response (Yang *et al*, 1997c). Final confirmatory evidence supporting a role of bradykinin in cardioprotection comes from a study by Yoshida *et al* (Yoshida *et al*, 2000). A human tissue kallikrein gene was delivered into rats using adenoviral vector. One week following gene delivery, rats were subjected to ischaemia / reperfusion. It was noted that kallikrein gene delivery caused a significant reduction in infarct to risk ratio from 69.6% to 44.5%, P<0.01. In addition to infarct size limitation, a significant alleviation in the occurrence of ventricular fibrillation was observed. Finally, kallikrein gene delivery also attenuated apoptosis in the ischaemic area compared with the control area as determined using terminal deoxynucleotidyl transferase-mediated nick end labelling assay. All of the above beneficial effects were abolished with Hoe 140, implying a role for the bradykinin B<sub>2</sub> receptor in the protection observed. These authors additionally found the expression of human tissue kallikrein mRNA in the rat heart, kidney, lung, liver and adrenal gland. Cardiac kinin levels were also significantly increased following kallikrein gene delivery (Yoshida *et al*, 2000).

To summarise, a vast number studies have demonstrated the cardioprotective properties of bradykinin. However, a small number of investigators disagree with this (Hatta *et al*, 1997). Indeed, it has been demonstrated that bradykinin can induce some deleterious effects in ischaemia / reperfusion (Hatta *et al*, 1999). Hatta *et al* demonstrated that bradykinin administration enhanced exocytotic and carrier mediated noradrenaline overflow from guinea pig hearts subjected to ischaemia / reperfusion resulting in the exacerbation of arrhythmias an effect prevented by Hoe 140 (Hatta *et al*, 1999; Seyedi *et al*, 1997).

Bradykinin also enhanced carrier mediated noradrenaline release in a human myocardial ischaemia model (Hatta *et al*, 1999). Hence, even though it has been observed that bradykinin may produce unfavourable events in ischaemia / reperfusion, the majority of studies conducted to date have shown that this kinin elicits cardioprotective effects (Wirth *et al*, 1997; Brew *et al*, 1995; Scholkens, 1996; Parratt *et al*, 1997; Bugge & Ytrehus, 1996a).

#### **1.5.5. Molecular mechanisms of bradykinin induced cardioprotection**

The mechanisms underlying the protective actions of bradykinin are not well understood. A number of agents have been proposed to participate in the protection including, nitric oxide, PGI<sub>2</sub>, PKC and tyrosine kinase (Bugge & Ytrehus, 1996a; Goto *et al*, 1995; Feng & Rosenkranz, 1999; Zhu *et al*, 1995).

Despite the confusion in the literature concerning what pathways are involved in bradykinin induced protection, there is general consensus that bradykinin B<sub>2</sub> receptor activation is required for protection, since Hoe 140 in most models abolishes the protection afforded by bradykinin. As mentioned earlier, this is a G-protein coupled receptor whose activation may lead to the subsequent activation of PKC, a kinase shown to be involved in mediating IPC. Brew *et al* (Brew *et al*, 1995), Bugge and Ytrehus (Bugge & Ytrehus, 1996a) and Goto *et al* (Goto *et al*, 1995), have presented evidence that exogenously administered bradykinin protects against ischaemia-reperfusion through a PKC-dependent mechanism in isolated rat and rabbit myocardium respectively.

The role of nitric oxide in mediating the cardioprotective properties of both endogenous and exogenously administered bradykinin has been the subject of some interest. Although there is good evidence that the vasodilator actions of bradykinin in several vascular beds are at least partly mediated by nitric oxide, the protective action of bradykinin against infarction was

not abolished in the presence of nitric oxide synthase inhibitors (Bugge & Ytrehus, 1996a; Goto *et al*, 1995). On the other hand, Feng *et al* and Zhu *et al* showed that bradykinin induced protection involved nitric oxide (Zhu *et al*, 1995; Feng *et al*, 2000). Alternatively, it can be proposed that bradykinin induced nitric oxide release may subsequently evoke the opening of the mitochondrial  $K_{ATP}$  channel which has been proposed as an end effector (O'Rourke, 2000; Sanada *et al*, 2000). In fact Kita *et al* have demonstrated that bradykinin evokes cardioprotection by opening the mitochondrial  $K_{ATP}$  channel (Kita *et al*, 2000). Indeed, various prostaglandins have also shown to activate sarcolemmal  $K_{ATP}$  channels, which have also been suggested to participate in IPC (Bouchard J *et al*, 1994; Sanada *et al*, 2000). Pathways thought to be involved in bradykinin induced cardioprotection are highlighted in figure 1.6.

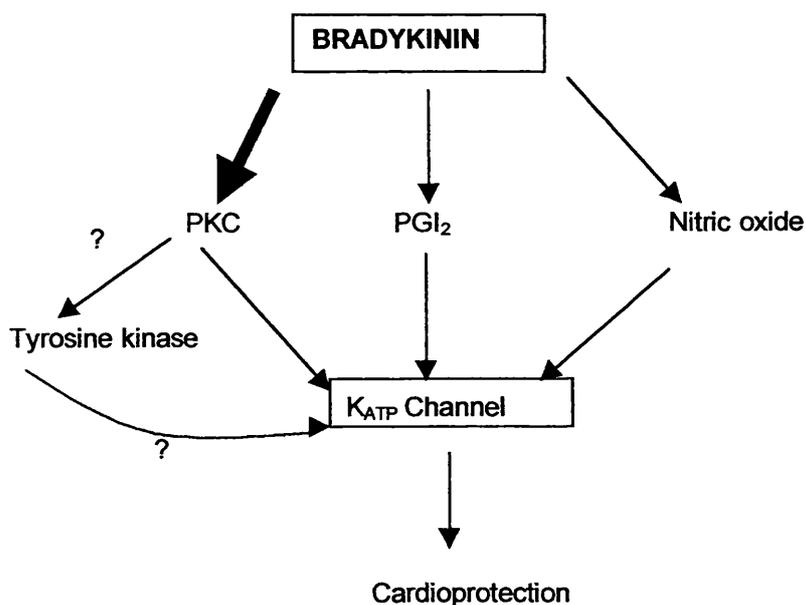


Figure 1.6. Mechanisms involved in bradykinin induced cardioprotection. Bradykinin leads to the activation of PKC, the generation of  $PGI_2$  and nitric oxide. These agents are thought to act on the  $K_{ATP}$  channel to evoke cardioprotection.

The bradykinin  $B_1$  receptor is inducible and only expressed under certain pathological conditions like inflammation and anoxia (Bhoola *et al*, 1992). Its activation has also been

proposed to be involved in vascular protection. Bouchard and colleagues reported that the beneficial effects of IPC on endothelial function was partly mediated by activation of the bradykinin B<sub>1</sub> receptor (Bouchard *et al*, 1998). In addition to this, Chahine *et al* found that bradykinin limited noradrenaline outflow and reduced the occurrence of arrhythmias in the isolated rat heart model (Chahine *et al*, 1993). This protective effect was not abrogated using Hoe140 but with a specific bradykinin B<sub>1</sub> receptor antagonist, Lys [Leu<sup>8</sup>] Des-Arg<sup>9</sup>-bradykinin, implying a role for the bradykinin B<sub>1</sub> receptor as opposed to the bradykinin B<sub>2</sub> receptor (Chahine *et al*, 1993).

#### **1.5.6. Enzymes responsible for the catalytic degradation of bradykinin**

##### **1.5.6.1. ACE**

The importance of ACE as a kininase has been demonstrated by many studies, which have showed that ACE inhibitors can elevate circulating and tissue bradykinin concentrations (Hornig & Drexler, 1997; Baumgarten *et al*, 1993; Pellacani *et al*, 1994). Linz's group in the isolated rat heart demonstrated that ramiprilat caused bradykinin outflow (Baumgarten *et al*, 1993). This study not only hinted that a local kallikrein system exists in the rat heart but additionally suggested that ACE inhibitors were capable of increasing bradykinin levels by inhibiting its breakdown. With this in mind, several investigators have revealed that ACE inhibitors can indeed potentiate a subthreshold preconditioning stimulus by increasing bradykinin levels (Morris & Yellon, 1997; Miki *et al*, 1996). A subthreshold preconditioning stimulus consists of a short ischaemic period which liberates triggers involved in preconditioning (ie, bradykinin) but is not sufficient to trigger the preconditioning response (Morris & Yellon, 1997; Miki *et al*, 1996). Miki *et al* showed that captopril, combined with a subthreshold preconditioning protocol was sufficient to elicit the full preconditioning response in the *in vivo* rabbit model of coronary artery occlusion, which was abrogated with Hoe 140, implying a role for the bradykinin B<sub>2</sub> receptor (Miki *et al*, 1996). Similarly, in our laboratory

using human atrial muscle tissue, Morris and Yellon found that both captopril and lisinopril were able to evoke preconditioning by augmenting bradykinin levels, an effect also abrogated with Hoe 140 (Morris & Yellon, 1997).

The ability of ACE inhibitors to confer protection in the absence of a preconditioning stimulus is more controversial. In the studies described above, administration of either captopril (Miki *et al*, 1996) or lisinopril (Morris & Yellon, 1997) alone, prior to the index ischaemic event, did not result in protection. In contrast to these findings, various other investigators have reported that direct administration of ACE inhibitors induces cardioprotection (Jin & Chen, 2000; Anderson *et al*, 1996; Massoudy *et al*, 1994; Dogan *et al*, 1998b; Matoba *et al*, 1999). Anderson *et al* showed that captopril but not enalapril was protective in the isolated rat heart and attenuated lipid peroxidation (Anderson *et al*, 1996). Indeed, it has been proposed that ACE inhibitors that possess sulfhydryl (SH) moieties are able to act as free radical scavengers and consequently lead to cardioprotection when administered alone (Anderson *et al*, 1996). Indeed captopril does contain a SH moiety and may act as a free radical scavenger, however, ACE inhibitors that do not carry a SH group can also induce cardioprotection (Birincioglu *et al*, 1997; Matoba *et al*, 1999). In 1999, Matoba *et al* communicated the findings of their study. They discovered that the ACE inhibitor, cilazaprilat (non-sulfhydryl containing ACE inhibitor) protected directly against hypoxia / reoxygenation injury in cultured rat myocytes. They were able to demonstrate that cilazaprilat enhanced bradykinin production in the culture media of the myocytes (Matoba *et al*, 1999). The reasons that can explain the discrepancy as to why some investigators found ACE inhibitors to evoke cardioprotection whereas others do not, remain unclear, although, experimental models and end points differ.

Although the question of whether ACE inhibitors are truly “cardioprotective” in experimental acute myocardial ischaemia without preconditioning remains uncertain, clinical trials have confirmed that ACE inhibitors exert beneficial effects on morbidity and mortality (Yusuf & Lonn, 2000). These agents have been shown not only to reduce blood pressure but in addition, have displayed additional beneficial effects, namely cardioprotection by raising levels of bradykinin (Heusch *et al*, 1997; Liu *et al*, 1996; Bachetti, 2000). A recent meta-analysis based on data from multi centre trials, namely: Studies Of Left Ventricular Dysfunction (SOLVD) (The SOLVD investigators, 1992), Survival And Ventricular Enlargement (SAVE) (Rutherford *et al*, 1994), TRAndolapril Cardiac Evaluation (TRACE) (The TRACE investigators, 1995), and Acute Infarction Ramipril Efficacy (AIRE) (The AIRE investigators, 1993), on a total of 12,500 patients over a four-five year follow up, led to a 20% reduction in relative risk reduction in total mortality ( $P < 0.0001$ ) (Yusuf & Lonn, 2000). Furthermore, the recent Heart Outcomes Prevention Evaluation (HOPE) trial demonstrated that ramipril reduced risk of death in patients with coronary artery disease (The HOPE investigators, 2000), an effect that appears to be unrelated to blood pressure reduction alone.

#### 1.5.6.2. *Neutral Endopeptidase*

Other than ACE, several additional enzymes exist which also play a role in the breakdown of bradykinin. After ACE, NEP is probably the most important (Piedimonte *et al*, 1994; Ura *et al*, 1987; Kokkonen *et al*, 1999). The availability of inhibitors of these various enzymes has allowed an examination of their importance in the degradation of bradykinin. NEP, analogous to ACE, is also a cell surface zinc metalloprotease, but unlike ACE its concentration in endothelium is low. NEP is highly concentrated in the epithelial cells of the kidney, it is also found in lung, liver and myocardium (Bhoola *et al*, 1992; Piedimonte *et al*, 1994). Studies with NEP inhibitors have found that these agents can evoke cardioprotection

(Yang *et al*, 1997a; Schriefer *et al*, 1996). Yang *et al* in an *in vivo* rat model of coronary artery occlusion found that the NEP inhibitor CGS24592 was able to induce cardioprotection comparable to that induced by an ACE inhibitor using infarct size as an end point (Yang *et al*, 1997a).

Novel compounds which are dual inhibitors of ACE and NEP have been introduced for the treatment of hypertension and heart failure (Robl *et al*, 1997; Fink *et al*, 1996; Weber, 1999; Kentsch & Otter, 1999; van Veldhuisen & van Gilst, 2000; Asher & Naftilan, 2000). Omapatrilat (BMS 18616) known as a “vasopeptidase inhibitor” is the first in this new class of agents (Asher & Naftilan, 2000).

Rastegar *et al* reported that a dual ACE and NEP inhibitor, Z13752A, produced a protective effect in an *in vivo* dog model of coronary artery occlusion, using arrhythmia prevalence as an end point (Rastegar *et al*, 2000a). They also found Hoe 140 abolished the cardioprotective effect of Z13752A. Additionally, Schrieffer *et al* in the *in vivo* rabbit model of coronary artery occlusion found that dual inhibition of ACE and NEP produced cardioprotective effects over and above treatment with just an ACE or NEP inhibitor alone (Schrieffer *et al*, 1996). As these beneficial effects were blocked with Hoe 140, bradykinin mediated cardioprotection is most likely. As NEP is also responsible for the catalytic degradation of various other vasodilator peptides including ANP (atrial or A type natriuretic peptide), BNP (brain or B type natriuretic peptide), CNP (C-type natriuretic peptide) (although it has a higher affinity for ANP) and substance P (Piedimonte *et al*, 1994; Ozaki *et al*, 1999), it is feasible to hypothesise that ANP or any of these other vasodilator peptides may be involved in the cardioprotective effect observed with NEP inhibitors. Investigators in the past have indeed shown that ANP exerts cardioprotective effects during ischaemia / reperfusion (Takagi *et al*, 2000). However, as Yang *et al* found all protection was abrogated

using Hoe 140, a role only for **bradykinin** and not ANP generated as a consequence of NEP inhibition is likely (Yang *et al*, 1997a). In addition, Yang *et al* used a natriuretic peptide receptor antagonist (HS-142-1) and were not able to completely abrogate the protection afforded by dual ACE and NEP inhibition, although it was slightly attenuated. Indeed, Rastegar *et al* also discovered that all protection was lost using Hoe 140, strongly implicating a role for bradykinin rather than the other peptides (Rastegar *et al*, 2000a).

#### 1.5.6.3. *Aminopeptidase P*

Experiments involving apstatin, an aminopeptidase P inhibitor unveiled that it can also induce cardioprotection by increasing levels of bradykinin in rat (Ersahin *et al*, 1999). Hence, enzymes other than ACE appear to have roles to play in the metabolism of bradykinin. Indeed dual inhibitors like omapatrilat may provide additional benefits in the clinic, where this agent has already shown to display superior properties compared to standard ACE inhibitor therapy (Trippodo *et al*, 1995, 1999; McClean *et al*, 2000; Rouleau *et al*, 2000). However, as bradykinin levels are expected to increase to greater levels then one would expect angio-oedema to be a problem (Coats, 2000; Messerli & Nussberger, 2000). Angio-oedema is a life threatening condition characterised by a non-pitting, non-erythematous oedema of the face, throat and tongue (Agostoni *et al*, 1999). The pathophysiology of angio-oedema is not well understood, however, it is thought that high levels of bradykinin may mediate the capillary leakage (Anderson *et al*, 1996). As omapatrilat inhibits both ACE and NEP, there is a higher risk of angio-oedema compared with ACE inhibitors alone. Indeed, angio-oedema has proved to be a dilemma associated with omapatrilat (Coats, 2000; Messerli & Nussberger, 2000), one also associated with ACE inhibitors (Agostoni *et al*, 1999)

## **1.6. Summary of the cardioprotective properties of bradykinin**

The nonapeptide bradykinin exerts a plethora of cardiovascular actions only some of which have been described in this chapter. Although the role of endogenous bradykinin in mediating IPC remains controversial, bradykinin administered prior to ischaemia exerts a cardioprotective effect and in addition, mimics IPC. Agents that inhibit the breakdown of this substance have displayed cardioprotective effects both experimentally and clinically. We possess a multitude of enzymes capable of degrading bradykinin, inhibition of a single enzyme may not produce maximal protection as metabolism of bradykinin may be switched to an alternative enzyme. Hence, a combination of inhibitors may be required to produce maximal protection. Inhibition of ECE appears to result in a weak reduction of blood pressure, which may be further enhanced using dual or even triple inhibitors of ACE, NEP and ECE to treat severe hypertension / heart failure (Roques BP, 1998) which theoretically should lead to enhanced cardioprotection. It is important to note that even though the acute cardioprotective effects of bradykinin have been extensively investigated, whether this peptide induces delayed preconditioning remains elusive.

## **Chapter One**

### **Part III: The hypertrophied myocardium**

#### **1.7. Hypertension and hypertrophy**

Many therapeutic modalities exist for the management of hypertension. However, prevalence and hypertension associated morbidity still remain high. In England, 41% of men and 33% of women have hypertension and 14% / 12% of cardiovascular deaths are caused by hypertension in men and women respectively (British Heart Foundation; coronary heart disease statistics; 2000 edition). Hypertension claimed the lives of 44,435 individuals in the USA in 1998. In addition to this, hypertension was listed as a primary cause of death or contributing cause of death in 210,000 individuals in a total of 2,000,000 deaths that year (American Heart Association; 2001 heart and Stroke Statistical Update).

Both primary and secondary hypertension initiate a host of cardiovascular abnormalities. The most important initial manifestation of hypertension includes myocardial hypertrophy (Messerli & Aepfelbacher, 1995). While right ventricular hypertrophy (RVH) arises from pulmonary hypertension, left ventricular hypertrophy (LVH) is caused by systemic arterial hypertension. The Framingham study established a strong link between hypertension and LVH. Using electrocardiographic techniques to detect the prevalence of LVH, the Framingham study demonstrated that in normotensive patients, LVH was very rare (1% per year in adults), whereas in individuals with hypertension (blood pressure > 160/95), incidence of LVH increased by roughly tenfold (Levy, 1988). The Framingham study also demonstrated a link between LVH and subsequent mortality (Levy, 1988). Indeed, LVH is associated with high cardiovascular mortality, predicting myocardial infarction, congestive heart failure (Himmelman, 1999; Cleland, 1999) stroke and sudden death (Messerli, 1999).

### **1.7.1. Left ventricular hypertrophy**

When the heart is subjected to an increase in arterial pressure, it can do three things in order to increase cardiac output. It can 1) use the Frank-Starling mechanism, 2) employ neurohormonal mechanisms or 3) increase muscle mass to increase contractility. The first mechanism can be limited and the chronic upregulation of neurohormonal mechanisms can be detrimental (Lorell & Carabello, 2000). Hence, an increase in cardiac mass plays an important part in responding to increased demands in cardiac work. Following birth, myocytes are terminally differentiated, hence do not undergo mitosis. As a consequence, the increase in mass following hypertension, occurs from the enlargement (hypertrophy) of existing myocytes rather than hyperplasia. However, Kajstura *et al* has demonstrated that hypertrophied right ventricle contains more myocytes, indicating that some myocyte mitosis can occur (Kajstura *et al*, 1994). Hypertrophy is initiated by complex cellular alterations and signal transduction cascades which culminate in growth of the myocyte (de Leeuw & Kroon, 1998). The renin angiotensin system (Paradis *et al*, 2000; Stroth & Unger, 1999; Thurmann *et al*, 1998; Akers *et al*, 2000), the sympathetic nervous system (Akers *et al*, 2000; de Champlain *et al*, 1976), cytokines, growth factors (Wollert *et al*, 1996; Isgaard *et al*, 1994; Ito *et al*, 1993; Takahashi *et al*, 1994; Li & Brooks, 1997) and calcineurin (Molkentin *et al*, 1998; Murat *et al*, 2000) (among other agents) have been implicated in initiating hypertrophy.

In eccentric hypertrophy, there is a relative increase in the length rather than the diameter of myocytes (occurs in cardiomyopathy). During concentric hypertrophy, there is a relative increase in the diameter of myocytes as new contractile protein units are constructed in parallel, leading to an increase in wall thickness (Hunter & Chien, 1999). Physiological hypertrophy that occurs in athletes involves proportional increases in both the width and length of myocytes (Hunter & Chien, 1999). Both eccentric and concentric hypertrophy are initially beneficial, enabling the myocardium to meet a higher cardiac output (compensated

hypertrophy). However, chronic hypertrophy ultimately leads to heart failure and death (decompensated hypertrophy) (de Leeuw & Kroon, 1998). It is relevant to note that hypertrophy or "remodelling" also occurs due to myocardial scar following myocardial infarction which can initially maintain cardiac output but subsequently may precipitate premature death (Swynghedauw, 1999).

In summary, LVH occurs when an increased haemodynamic burden is imposed upon the myocardium, a condition which may ultimately lead to heart failure. LVH is not only associated with an increase in size of myocytes, but also with hypertrophy and hyperplasia of non-myocyte cells (eg, fibroblasts), accumulation of collagen and infiltration of lymphocytes and monocytes. Excess collagen is produced by fibroblasts leading to interstitial and perivascular fibrosis. All of these processes initiate changes in the overall structural arrangement of the myocardium. A significant problem is concerned with the increase in interstitial cell mass which may be greater than the increase in myocyte mass due to augmentation of collagen production (de Leeuw & Kroon, 1998)

### **1.7.2. Transition from hypertrophy to heart failure**

Chronic, long-standing hypertension may ultimately progress to pump failure. How does compensated hypertrophy eventually lead to decompensated hypertrophy and heart failure? The answer to this question is not known, although several observations have been reported at the onset of heart failure. A loss of myocardial contractile protein in heart failure may lead to diminished pump function (Boluyt *et al*, 1994). Myocyte loss as a consequence of apoptosis is thought to occur during the transition to heart failure (Olivetti *et al*, 1997). Further accumulation of collagen has been shown to occur in failing hearts, precipitating cardiac muscle stiffness and a consequent decline in contractile efficiency (Boluyt *et al*, 1994).

### **1.7.3. Animal models of hypertension / hypertrophy**

The choice of animal models of hypertension is difficult, primarily because the exact cause of essential hypertension is not known in humans and, secondly, because it is a heterogeneous condition. Hence, no single animal model can exactly mimic the pathophysiology of this condition. Another complicating factor is the fact that cardiovascular diseases like hypertension develop gradually in humans, usually over years, in contrast to the rapid onset in drug or surgically induced animal models of the disease. Nevertheless, over the past 50 years, numerous animal models of hypertension have been developed, principally in the rat, which have substantially increased our understanding of the pathophysiology and treatment of hypertension (Pinto *et al*, 1998).

#### **1.7.3.1. Genetic models of hypertension – the spontaneously hypertensive rat**

The most popular model of hypertension is the spontaneously hypertensive rat (SHR). Okamoto and Aoki developed the SHR by inbreeding wistar rats with the greatest blood pressure (Okamoto & Aoki, 1963). Systolic blood pressure increases at five-six weeks of age and generally reaches a maximum of 200 mmHg by 12-16 weeks of age. Cardiac function is preserved at 12 months of age. However, at 18-24 months of age, most animals develop heart failure, which is accompanied by diminished contractile activity and increased fibrosis (Pinto *et al*, 1998). Although, this model is widely used, the exact mechanisms leading to hypertension remain vague (see chapter five for further details).

#### **1.7.3.2. Dahl salt sensitive rats**

Another genetic model of hypertension is the Dahl salt sensitive rat. In the 1950s Meneely *et al* found that some rats were more sensitive to others in their blood pressure response following salt ingestion. They observed that the salt sensitive Dahl rats developed severe hypertension when given high salt diets, whereas, salt resistant rats did not develop such

severe hypertension following salt ingestion (Meneely & Ball, 1958). In addition to this, authors also found that even when the salt sensitive Dahl rats were placed on a normal salt diet, they still became hypertensive, demonstrating that this is a genetic model of hypertension that is particularly sensitive to salt (Meneely & Ball, 1958). At baseline, Dahl sensitive rats exhibit greater blood pressures. However, when fed a high salt diet (8% NaCl), a huge rise is observed, similar to that seen in SHR. However, in these rats, heart failure begins at a much earlier age typically at four-five months (Pinto *et al*, 1998).

#### 1.7.3.3. *Transgenic (mREN2)27 rats*

The insertion of the murine Ren-2 gene in the rat leads to the generation of the transgenic rat (TGR(mREN2)27) in which severe hypertension occurs, with systolic blood pressure reaching 200 mmHg at eight weeks of age in the heterozygous animal (Langheinrich *et al*, 1996). The homozygous rats develop even higher blood pressures and consequently have a higher mortality rate. The signalling pathways that ultimately lead to hypertension are not known. However, as ACE inhibitors are very effective in lowering blood pressure in these animals, hypertension is most likely to be mediated by AT-II (Langheinrich *et al*, 1996).

#### 1.7.3.4. *Renovascular hypertension*

Goldblatt developed the first animal model of hypertension by clamping the renal arteries in a dog (Goldblatt *et al*, 1934). Subsequently, in 1939, Wilson and Byrom produced a similar model of hypertension in the rat. Chronic hypertension also results when one kidney is clipped and the other is left untouched (two kidney, one clip, 2K1C) in the rat, a species that does not develop efficient collaterals (Pinto *et al*, 1998). Cardiac hypertrophy in the region of 20-50% has been reported, depending on the size of the clip used and the age of the rat. The development of heart failure in this model has not been described. An elevation of

circulating renin and aldosterone are thought to be responsible for initiating hypertension (Pinto *et al*, 1998).

#### 1.7.3.5. *Mineralocorticoid hypertension*

The administration of deoxycorticosterone acetate (DOCA) with a high salt diet for a period of four-six weeks leads to the development of a low renin form of hypertension in rats, with moderate hypertrophy (Doggrell & Brown, 1998). The development of heart failure has not been described in this model, although chronic administration of DOCA and salt is associated with high rates of mortality (see chapter three for further details).

### 1.8. **IPC in the hypertrophied myocardium**

As LVH is a common clinical condition it is important to examine whether IPC can protect the hypertrophied heart. However, eight years have since elapsed following the discovery of IPC in 1986 and the first study investigating IPC in LVH.

Speechly-Dick *et al* were the first to investigate IPC in the hypertrophied myocardium (Speechly-Dick *et al*, 1994). In 1994, these investigators using a DOCA-salt model of hypertrophy reported that IPC reduced infarct size in an *in vivo* model of ischaemia / reperfusion. Control infarct size was reduced from  $67.1 \pm 5.6\%$  to  $19.1 \pm 1.5\%$  following IPC in the hypertrophied myocardium. In the normotensive myocardium, IPC reduced infarct size from  $77.1 \pm 3.8\%$  to  $33.4 \pm 5.5\%$  ( $P < 0.05$ ). Interestingly, the reduction in infarct size following IPC was greater in the hypertensive myocardium compared to the normotensive myocardium (Speechly-Dick *et al*, 1994) with the authors speculating that this enhanced beneficial effect may be due to greater activation of PKC in hypertrophy.

In 1996, Pantos et al also investigated the effects of hypertension on IPC occurrence (Pantos *et al*, 1996). Hypertrophy was induced by suprarenal abdominal aortic constriction for a period of five weeks, following which hearts were Langendorff perfused. These authors reported that in both hypertrophied and normal heart, IPC induced protection using left ventricular developed force as an endpoint. Left ventricular developed pressure (expressed as a % of baseline) was  $39.8 \pm 4.6\%$  in the hypertensive control group and  $70.1 \pm 4.1$  following IPC. Similarly, in the normal myocardium, control recovery was  $49.3 \pm 6.1\%$  and  $76.5 \pm 3.4\%$  after IPC ( $P < 0.01$ ) (Pantos *et al*, 1996). This was the first study to demonstrate the protective effects of IPC in hypertension using an *in vitro* model of ischaemia / reperfusion.

Randall et al examined the effects of IPC in TGR ((mREN-2)27) (four-five month old) hypertensive rat hearts (Randall *et al*, 1997). The authors demonstrated that contractile function following ischaemia was significantly enhanced following IPC in both normotensive and TGR hearts. Interestingly, the authors noted that IPC protected to a greater extent in the hypertensive hearts as opposed to the normotensive hearts (Randall *et al*, 1997), whether this is due to enhanced PKC activation in hypertrophy remains elusive. Similarly, Butler and co-workers reported that IPC improved contractile function following ischaemia in a rat model of hypertension induced by feeding a high salt diet to Dahl salt sensitive rats (Butler *et al*, 1999).

IPC has also been investigated in SHRs. Boutros and Wang demonstrated that IPC, adenosine and bethanechol all induced protection in hearts isolated from SHRs. Hence, these results demonstrate that IPC can not only protect the hypertensive myocardium, but also show that the hypertensive heart can be protected using pharmacological agents (Boutros & Wang, 1995). Secondly, Lu et al also demonstrated that IPC can reduce QT

dispersion and arrhythmia occurrence following ischaemia / reperfusion in 12-14 week old SHR (Lu *et al*, 1999).

In contrast to the studies described above, Moolman *et al* reported that IPC did not protect in a New Zealand model rat of genetic hypertension (Moolman *et al*, 1997). Hearts excised from 12 month old New Zealand genetically hypertensive rats were not protected following IPC, using post ischaemic function in working heart mode and creatine phosphate content as experimental end points (Moolman *et al*, 1997). The primary difference between this study and the ones described above is the duration of hypertension. In the studies that demonstrated a protective effect of IPC, rats were only made hypertensive for a short duration. Protocols used to induce hypertension ranged from two to five weeks in duration. Hence, the effects of long standing hypertension and thus chronic hypertrophy were not addressed. Similarly, in the SHR studies, young adult rats were used which implies they were subjected to hypertension for a short duration as well. Moolman *et al* on the other hand, addressed the effects of chronic hypertension as they used older rats (12 month old) (Moolman *et al*, 1997). Results therefore imply that the duration of hypertension as well as the age of the animal are important factors which may determine the cardioprotective effects of IPC.

### **1.9. Bradykinin in hypertension and hypertrophy**

Madeddu *et al* demonstrated that bradykinin B<sub>2</sub> receptor knock-out mice had higher blood pressures and heart weights than the wild type and heterozygous mice (Madeddu *et al*, 1997). Additionally, these authors showed that chronic blockade of B<sub>2</sub> receptors using Hoe 140, increased blood pressure of wild type mice to levels of B<sub>2</sub> receptor knock-out mice. These data imply that B<sub>2</sub> receptors are essential for the maintenance of normal blood pressure and that defects / blockade of this receptor may lead to hypertension / hypertrophy

(Madeddu *et al*, 1997). These authors also demonstrated that B<sub>2</sub> receptor knock-out mice developed hypertension and hypertrophy associated with chamber dilatation and cardiomyopathy in a subsequent study (Emanuelli *et al*, 1999b). These observations strongly implicate a role for bradykinin in development of hypertension and hypertrophy and subsequent cardiomyopathy. The same group of investigators demonstrated that antisense oligonucleotides targeted to the bradykinin B<sub>1</sub> receptor mRNA decreased blood pressure in the SHR, indicating that this receptor may regulate blood pressure and that its activation could precipitate hypertension in the SHR (Emanuelli *et al*, 1999a). Therefore, results imply that while activation of the bradykinin B<sub>2</sub> receptor exerts blood pressure lowering effects, activation of the bradykinin B<sub>1</sub> receptor leads to an increase in blood pressure.

Studies have demonstrated that levels of bradykinin are attenuated in hypertension (Nakagawa & Nasjletti, 1988; Seino *et al*, 1990). In addition, investigators have also shown that gene delivery of kallikrein lowers blood pressure (Chao *et al*, 1996, 1998; Xiong *et al*, 1995; Yayama *et al*, 1998). In contrast however, Campbell *et al* found increased levels of bradykinin in kidney, lung and heart of young SHRs (Campbell *et al*, 1995b). Reasons for the discordant findings are not known, although, the animal model of hypertension, the age of the animal and techniques used to measure bradykinin may affect the results obtained.

In summary, LVH is associated with high rates of mortality and morbidity. A limited number of studies have demonstrated that IPC occurs in animal models of hypertension and hypertrophy. Bradykinin is thought to be implicated in hypertension and hypertrophy. Circulating levels of bradykinin may be attenuated in hypertension which may in fact contribute to the development of hypertension. In addition, studies have demonstrated that bradykinin B<sub>2</sub> receptor knock-out mice develop hypertension and hypertrophy, implying a

role for this receptor in maintenance of normal vessel tone. It is not known whether bradykinin exerts cardioprotective effects in hypertension and hypertrophy.

## **Chapter One**

### **Part IV: Aims and scope of thesis**

This thesis primarily focuses on IPC and bradykinin induced cardioprotection in the normal and hypertrophied myocardium. Even though IPC has been shown to consistently protect the normal myocardium, does it protect in a model of chronic hypertension? Similarly, bradykinin has been shown to induce cardioprotection in the normal, healthy myocardium. However does it induce cardioprotective effects in the hypertrophied heart? These questions and the following hypotheses were investigated in this thesis.

#### **1.10. Bradykinin induced cardioprotection is impaired in hypertension**

As mentioned previously, bradykinin levels are thought to decline in hypertension, hence it was speculated that bradykinin induced protection may be impaired in this pathology. Bradykinin induced cardioprotection was investigated in a rat model of hypertension. A mild model of hypertension (ie, the DOCA-salt model) was chosen to represent an acute model of hypertrophy associated with short term hypertension. The occurrence of both early and delayed preconditioning (induced using heat shock) were also investigated in the DOCA-salt model of hypertrophy (chapter three).

#### **1.11. Omapatrilat can potentiate a subthreshold preconditioning stimulus via activation of the bradykinin B<sub>2</sub> receptor**

Studies have consistently demonstrated that ACE inhibitors potentiate subthreshold preconditioning by augmenting bradykinin levels. In view of this, it was hypothesised that the dual ACE and NEP inhibitor, omapatrilat would also potentiate subthreshold preconditioning. Hence, in the present study, experiments were designed to determine

whether omapatrilat potentiates IPC and to elucidate whether any protection is mediated by bradykinin B<sub>2</sub> receptor activation (chapter four).

#### **1.12. IPC is diminished in chronic myocardial hypertrophy**

Two studies have reported the protective effects of IPC in the young SHR. Long term myocardial hypertrophy has many detrimental effects on the myocardium. It was therefore hypothesised that IPC may be diminished or even absent in chronic models of hypertrophy. Studies have not examined whether IPC still protects the ageing SHR, which has obviously been exposed to hypertension for a long duration (ie, chronic hypertrophy). Hence, in the present study, IPC was investigated in SHRs at three different stages of development – young, middle aged and ageing (chapter five). Additionally, cardioprotective effects of an ACE inhibitor were investigated in the ageing animals.

#### **1.13. Bradykinin at reperfusion can attenuate infarct size**

Although Massoudy et al demonstrated that bradykinin limited reperfusion injury, using contractile recovery as an experimental end point (Massoudy *et al*, 1994), it is not known whether bradykinin given at reperfusion limits infarct size. In the current study, bradykinin was administered at reperfusion in the isolated heart model using infarct size as an experimental end point. Signalling pathways involved in the protection were also investigated.

Studies have not examined the limitation of reperfusion injury in the hypertrophied heart. In the present study, it was also determined whether bradykinin at reperfusion induced cardioprotection in hearts isolated from ageing SHRs (chapter six).

#### **1.14. Bradykinin elicits delayed preconditioning via generation of nitric oxide**

As described in chapter one (part II), a vast number of studies have demonstrated that bradykinin is able to mimic classical preconditioning. Whether bradykinin induces delayed preconditioning is not known. Bolli's group have provided substantial evidence indicating that early nitric oxide generation is a trigger of delayed ischaemic preconditioning. Thus it was hypothesised that bradykinin induces delayed preconditioning possibly via generation of nitric oxide. This final study examined whether bradykinin can induce a delayed preconditioning like effect and whether any protection is dependent upon nitric oxide synthesis (chapter seven).

**PART TWO**

**EXPERIMENTAL**

## **CHAPTER TWO**

### **General Methods**

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## **2.1. Animals**

Unless stated otherwise, male Sprague-Dawley rats were used in these studies. The rats were obtained from Charles River, Bicester, Oxon and were allowed to acclimatise for a minimum of four-five days prior to use. All rats were caged in groups of four and had free access to fresh water and standard pellet chow (RM1 diet). Animals were subjected to a 12 hour light - dark cycle, and maintained at 19-22°C, and 55±10% humidity. All animals were treated in accordance with the *Guidelines on the Operation of the Animals (Scientific Procedures) Act, 1986*, published by the Stationary Office (London, UK).

## **2.2. Measurement of blood pressure *in vivo***

Rats were anaesthetised via intraperitoneal administration of pentobarbitone sodium (50 mg/kg). A tracheotomy was performed, following which the trachea was intubated with a cannula connected to a rodent ventilator (Harvard apparatus, Edenbridge, UK). Rats were ventilated with room air supplemented with oxygen at 70 to 75 breaths per minute, and a tidal volume of 3-4 ml. The right carotid artery was isolated and cannulated to monitor blood pressure via a lectromed pressure transducer (Lectromed, Letchworth, UK) connected to a pen recorder. Body temperature was maintained between 37.0±1.0 °C with the use of a heating pad. Arterial pH, pCO<sub>2</sub> and pO<sub>2</sub> were monitored using a blood gas system (AVL 995 pH/blood gas analyser, AVL Medical Instruments, Stonebridge, UK). Rats were stabilised for a period of ten minutes and blood pressure recordings were taken at five minute intervals, prior to the excision of the heart for Langendorff perfusion (described below).

## **2.3. The Langendorff perfused heart**

Throughout this research programme, the Langendorff perfused heart technique was used to study responses to ischaemia-reperfusion. Invented by Oscar Langendorff in 1895, the isolated heart perfusion system is one of the most widely used models in cardiovascular

research today (Doring & Dehnert, 1988). This technique involves using the heart of warm-blooded animals (Doring, 1990) (although hearts of cold-blooded animals can also be perfused), which is perfused with either blood or crystalloid buffer through a cannula at constant flow or constant pressure. Perfusion fluid is forced retrogradely into the aorta, closing the aortic valves, directing the perfusion fluid into the coronary arteries, hence perfusing the whole myocardium and draining into the right atrium. Thus, although the entire myocardium is perfused, and the ventricles beat they remain empty throughout the duration of the experiment and hence, do not eject perfusion fluid. An intraventricular balloon can however be inserted into the left ventricle to monitor isovolumic contraction.

Although the rat heart is the commonly selected for Langendorff perfusion, studies involving hearts isolated from rabbit, mouse, guinea pig and hamster have also been reported. Perfusion of hearts obtained from large animals, for instance, pigs, sheep, dogs, monkeys and humans has also been documented (Sutherland & Hearse, 2000). However, such large animals are not regularly used due to obvious reasons of high cost and vast quantities of perfusion fluid that are required. The modification of the Langendorff technique first described by Rigler led to the development of the working heart model (Doring & Dehnert, 1988). In this system, the aorta is cannulated analogous to the Langendorff perfused heart, however, the pulmonary vein or left atrium is also cannulated (Doring & Dehnert, 1988). This allows ventricular filling via the left atrium and ejection of fluid via the aorta. The working heart model is widely used when contractile function is the experimental end point.

The most obvious drawback of the Langendorff technique is the lack of neuronal innervation of the heart and the absence of blood borne mediators. It is therefore devoid of neuronal regulation and systemic circulation and thus cardiac function does not resemble that *in vivo*. However, this prime drawback can be transformed into a possible advantage of the

Langendorff technique. In the absence of other organs, neural reflexes and neurohormonal factors, one can ascertain the direct effects of treatments on the myocardium, without the complication of systemic factors. In addition, the problem associated with the absence of blood-borne mediators can be overcome by perfusing with blood rather than crystalloid buffer solution. Sandhu *et al* compared IPC in blood perfused and buffer perfused isolated rabbit hearts. They reported that IPC was equally protective in both buffer and blood perfused preparations (Sandhu *et al*, 1993). Another possible disadvantage of the technique is the fact that function of the preparation progressively declines. However, it can be maintained for at least three hours (Hearse & Sutherland, 2000; Sutherland & Hearse, 2000). Finally, Paradis and colleagues reported that crystalloid buffer used to perfuse isolated rabbit myocardium is inadequately oxygenated (Paradis *et al*, 1984). However, subsequent experiments by Murashita demonstrated that the isolated rabbit heart perfused with crystalloid buffer is adequately oxygenated (Murashita *et al*, 1991; Opie, 1984)

Advantages of isolated heart preparation include reproducibility of data, and relatively low cost. The Langendorff preparation permits the measurement of a variety of parameters to assess tissue injury including contractile function, biochemical markers (for example, lactate, creatine kinase release) morphological markers (infarct size measurement) and cardiac electrophysiology (for example, arrhythmia detection) (Sutherland & Hearse, 2000)

### **2.3.1. The isolated rat heart perfusion protocol**

Rats were deeply anaesthetised with pentobarbitone sodium (50 mg/kg i.p). Heparin (300 IU) was concurrently administered intraperitoneally. Hearts were excised and placed in ice cold buffer solution to arrest contraction and were rapidly mounted on a Langendorff apparatus. The Langendorff apparatus is represented schematically in figure 2.1.

Hearts were perfused with Krebs's-Henseleit buffer comprised of (in mM); NaCl 118, NaHCO<sub>3</sub> 25, d-glucose 11, KCl 4.7, MgSO<sub>4</sub> 1.22, KH<sub>2</sub>PO<sub>4</sub> 1.21 and CaCl<sub>2</sub> 1.84 (pH 7.3-7.5) when equilibrated with 95% O<sub>2</sub> / 5% CO<sub>2</sub>. Krebs's-Henseleit buffer was filtered using 1 μm filters to remove particulate contamination. Perfusion pressure was maintained at 80 mmHg H<sub>2</sub>O throughout the course of the experiments.

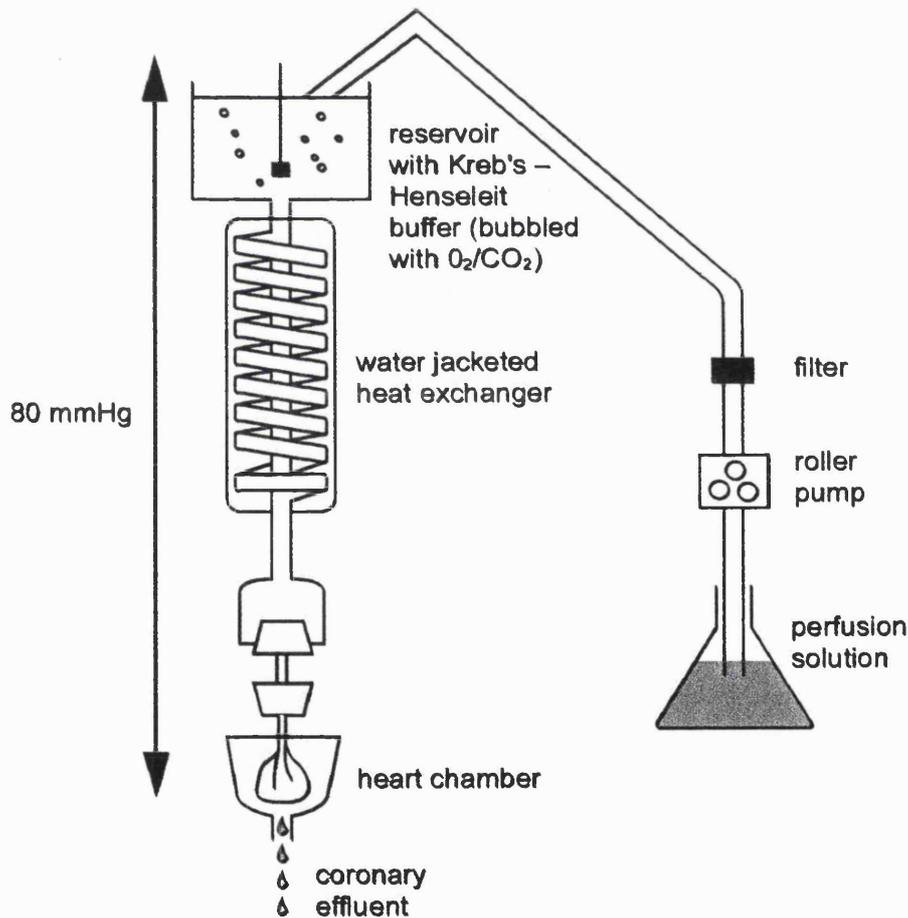


Figure 2.1. Schematic representation of the Langendorff perfusion apparatus

Temperature of the heart was continuously monitored using a thermometer attached to a thermocouple probe that was placed in the pulmonary artery. Temperature was maintained at 37 °C ( $\pm 1$  °C), and was carefully regulated using a thermostatically controlled water jacketed system. In this system, delivery lines of the perfusate, the bubble trap and heart perfusion chamber were surrounded by water flowing at 37-38 °C. In this way, major fluctuations in temperature were avoided, mild increases/decreases were controlled by withdrawing/reapplying the heart perfusion chamber respectively.

A latex isovolumic balloon was inserted into the left ventricle via a small incision in the left atrial appendage and was inflated to give a preload of 5-10 mmHg. The balloon catheter was coupled to a pressure transducer (Lectromed, Letchworth, UK) linked to a pen recorder for measurement of developed pressure and heart rate. Coronary flow rate (CFR) was measured by timed collection of coronary effluent.

### **2.3.2. Regional ischaemia**

All hearts were allowed to stabilise for a minimum period of 15 minutes prior to the induction of infarction. Figure 2.2 demonstrates a Langendorff perfused rat heart during the stabilisation period. A 3/0 silk suture was placed around the left main coronary artery and passed through a plastic tube to form a snare. Coronary occlusion was effected by pulling the ends of the suture taut and clamping the snare onto the epicardial surface, as depicted in figure 2.3. Ischaemia was verified by a 30-50% approximate reduction in CFR and rate pressure product (RPP; heart rate x developed pressure). The artery was occluded for a period of 35 minutes and then reperfused for two hours by loosening the snare. Reperfusion was verified by an immediate increase in CFR following the loosening of the snare.

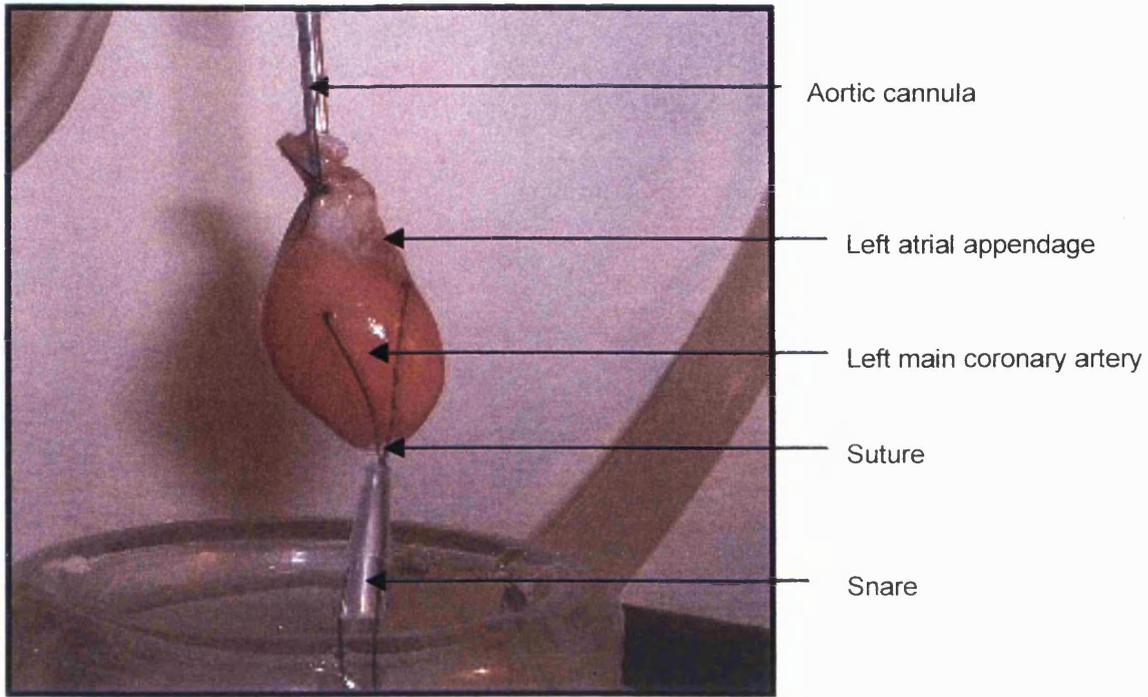


Figure 2.2. A Langendorff perfused rat heart during stabilisation.

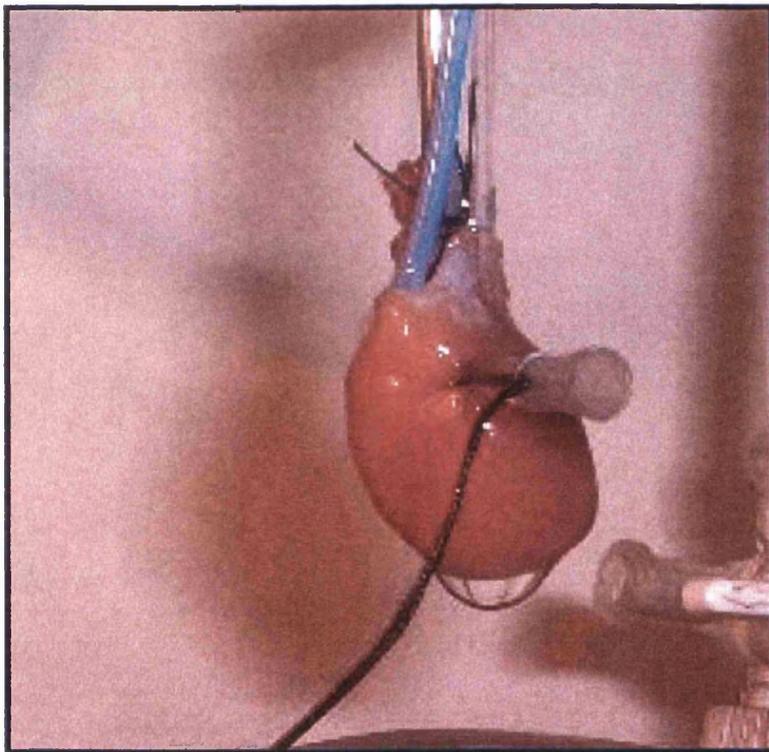


Figure 2.3. A Langendorff perfused rat heart during ischaemia. Ischaemia was induced by pulling the ends of the snare taut and clamping the snare onto the epicardial surface.

### 2.3.3. Infarct size evaluation

Infarct size evaluation was made using the triphenyltetrazolium technique, a method which is widely used to quantify infarct size in experimental models (Schwarz *et al*, 2000; Birnbaum *et al*, 1997a; Ito *et al*, 1997). Triphenyltetrazolium chloride is reduced by NADH in viable tissue, producing a red formazan derivative, which is distinct from the white necrotic tissue (Birnbaum *et al*, 1997b; Ito *et al*, 1997).

On completion of the reperfusion period, the LAD was re-occluded and approximately 1.5 ml of Evans' blue dye was infused via the aorta in order to differentiate the ischaemia zone from the non-ischaemic zone. Figure 2.4 demonstrates the delineation of the risk zone with Evans' blue dye.



Figure 2.4. Delineation of the ischaemic risk zone using Evans' blue dye. Area stained blue corresponds to the non-risk area, red area represents the area at risk.

Following freezing at  $-20^{\circ}\text{C}$  for one-four hours, hearts were sliced into 2 mm transverse sections from apex to base. Slices were then incubated with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) at  $37^{\circ}\text{C}$  for a period of 10-15 minutes. Once fixed in 10% formalin for 24 hours (see figure 2.5), myocardial slices were traced onto transparent sheets in a blinded fashion. This involved giving a colleague in the laboratory heart slices to trace who was not aware of the experimental treatments used. Areas of the infarcted and risk tissue were determined using computer-assisted planimetry (Kurta, Phoenix, AZ). Tissue volumes were then calculated (area x 2 mm thickness) and expressed as the ratio of infarcted-risk tissue (I/R %).

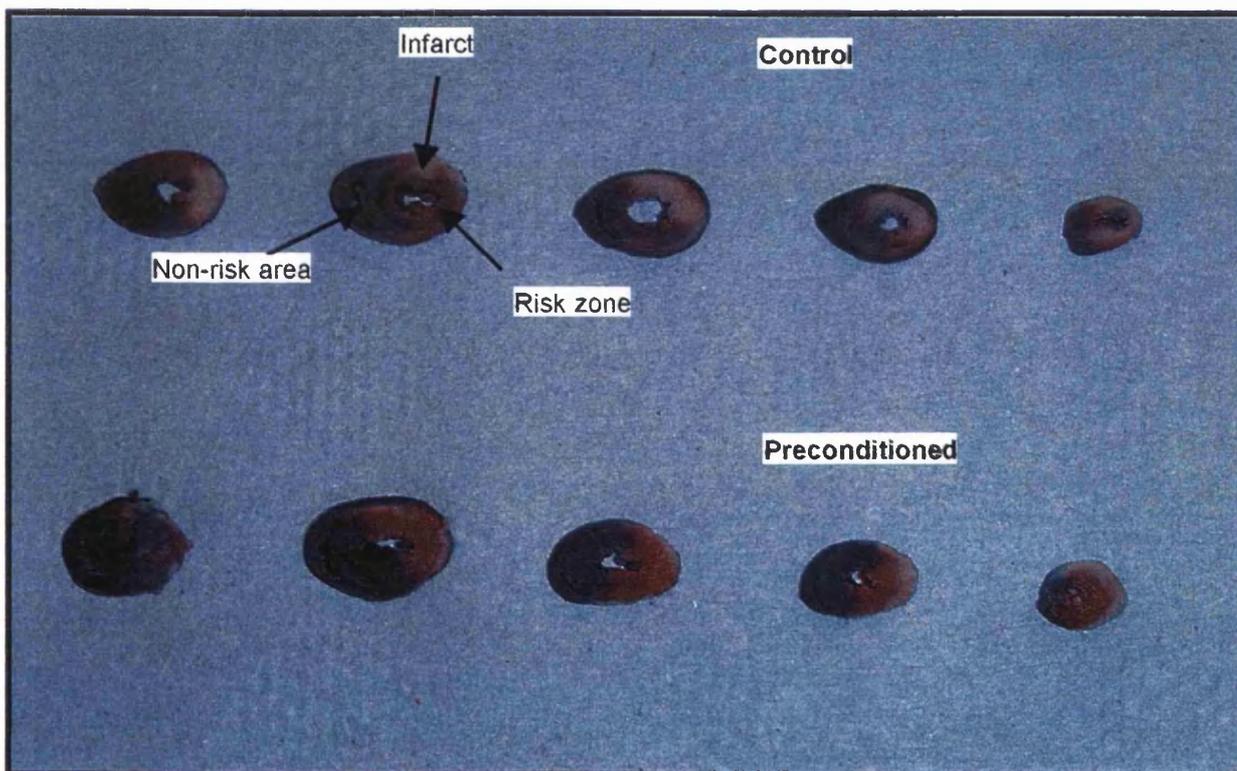


Figure 2.5. Rat heart slices stained with triphenyltetrazolium chloride. These heart slices underwent a 35 minute period of coronary artery occlusion followed by 120 minutes reperfusion. Viable myocardium stains red following triphenyltetrazolium staining, whilst infarcted tissue remains pale. Area stained blue refers to the non risk tissue. The heart on the top panel demonstrates a large infarct. The heart on the bottom panel was preconditioned with two five minute coronary occlusions prior to the 35 minute ischaemia period. Notice the amount of pale infarct tissue is markedly reduced.

#### **2.4. Assessment of left ventricular hypertrophy**

Following Langendorff perfusion, atrial appendages were removed from the hearts and the ascending aorta trimmed. The heart was blotted and weighed prior to freezing. After, the heart was cut from apex to base (ie, just prior to triphenyltetrazolium chloride staining), the right ventricle (RV) was removed and weighed. In this way, the weight of the LV could be calculated by subtracting RV weight from whole heart weight. In the thesis, LVH is expressed as LV/body weight, whilst RVH, is expressed as RV/body weight. Fluctuations in body weight that occur during ageing may make body weight an unreliable reference for normalising LV weight. Thus, some investigators use LV/femur length ratios if weight loss is substantial in hypertensive animals. Heart weight / tibial length ratio has also been used to quantify hypertrophy (Yin *et al*, 1982). However in the present studies as weights of hypertensive and normotensive animals were similar, LV weight was normalised to body weight.

#### **2.5. High performance liquid chromatography**

Plasma noradrenaline levels were determined using high performance liquid chromatography (HPLC), a technique which is commonly used to determine catecholamine levels (Altman *et al*, 1988; Smith *et al*, 1993; Smith & Betteridge, 1984). Dr Chris Smith, department of Medicine, University College London, performed the assay technique. The chromatographic apparatus was obtained from Waters, Division of Millipore Ltd, Middlesex, UK. It consisted of a "Resolve" C<sub>18</sub> reversed-phase column (3.9 mm X 150 mm, particle size 5 µM) and a Waters Model 712 Intelligent Sample processor. Noradrenaline levels were assessed using a Waters Model 460 electrochemical detector with a glassy carbon electrode.

### **2.5.1. Extraction of noradrenaline from plasma and HPLC procedures**

The alumina used for extraction was purchased from Sigma Co (St Louis, MO). The noradrenaline and 3,4-dihydroxybenzylamine (DHBA) standards were obtained from Sigma (DHBA was the internal standard).

Rat blood samples were mixed with ethylene diamine tetraacetic acid (ETDA) (1 mg/ml) and centrifuged at 10,000 rpm for 10 minutes at 4 °C. Supernatant plasma samples were then stored at -80 °C until further use. When samples were analysed, 5 pmol DHBA was added as an internal standard. Plasma volumes in the range of 500-1500 µl were used for noradrenaline extraction. Plasma (500-1500 µl) was added to LP3 tubes containing alumina (10 mg), followed by 400 µl Tris buffer (consisting of 2 mol of Tris base per litre, pH 8.6). Samples were then mixed in a haematological roller for a period of 15 minutes. The alumina was allowed to settle and supernatants were removed. The alumina was washed 3 times with 2 mL water and centrifuged (2000 x g, 2 minutes). Acetic acid solution (150 µl) (consisting of glacial acetic acid [100 µl], 10% sodium disulphite [50 µl] and 5% EDTA [50 µl], made up to 10 ml with water) was used to elute the noradrenaline from the alumina. Extract (100 µl) obtained from this extraction procedure was then injected onto the chromatograph, the flow rate was 1.0 mL/min through the system.

### **2.6. Western blot analysis for HSP 72 and NOS isoforms**

Following relevant treatment protocols (described further in chapters three and seven), hearts were excised and immediately freeze clamped in liquid nitrogen. Approximately 50 mg of frozen myocardial tissue was used for protein extraction. Tissue was homogenised with a homogeniser (IKA Labortechnik T25) in 250 µl suspension buffer containing (in mM): NaCl 100, Tris 10 (pH 7.6), EDTA 1 (pH 8), sodium pyrophosphate 2, sodium fluoride 2, β-glycerophosphate 2; phenyl methyl sulphonyl fluoride (PMSF) 0.1 mg/ml; and 1 µg/ml each

of aprotinin, leupeptin, trypsin inhibitor and protease inhibitor. Thereafter, samples were centrifuged at 11000 rpm for 10 minutes. The pellet was discarded and protein concentrations in the supernatant were determined using bicinchoninic acid (BCA) protein assay reagent (Pierce). Supernatant samples were further diluted in 2x sample buffer (in mM): Tris 100 (pH 6.8), dithiothreitol (DTT) 200; and sodium dodecylsulphate (SDS) 2%, bromophenol blue 0.2% and glycerol 20% and subsequently boiled for a duration of 10 minutes. Sample proteins were loaded and separated on a sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Hybond nitrocellulose membrane (Amersham). Equal protein loading was verified using ponceau red staining (Sigma) of membranes. The membranes were probed with appropriate primary and secondary antibodies. Proteins were detected using enhanced chemiluminescence western blotting detection reagent (Amersham) and bands were visualised using autoradiography.

## **2.7. Statistical analysis**

All data in the thesis are expressed as mean  $\pm$  sem. Differences in mean values of LV and RV weight, rat body weight, infarct volume, risk zone volume, I/R (%), risk volume normalised to LV, expression of various proteins and plasma catecholamine levels, were assessed by one way ANOVA followed by Fisher's protected least significant difference (PLSD).

Cardiodynamic and haemodynamic parameters were examined with repeated measures ANOVA. CFR, RPP and mean arterial blood pressures were all examined using repeated measures ANOVA followed by Bonferroni's test. All the statistical tests were performed using Statview SE + Graphics 1.2 program. The null hypothesis was rejected when  $P < 0.05$ .

## **2.8. Materials**

Triphenyltetrazolium chloride, Evans' blue, bradykinin, L-NAME, captopril and wortmannin were purchased from Sigma (Poole, Dorset, UK). Chemicals required for Krebs'-Henseleit buffer (NaCl, NaHCO<sub>3</sub>, d-glucose, KCl, MgSO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub> and CaCl<sub>2</sub>) were obtained from BDH Laboratory Supplies (Poole, UK). Formaldehyde were also obtained from BDH laboratory Supplies (Poole, UK).

Pentobarbitone Sodium (Sagatal) was obtained from Rhone Merieux (Tallaght, Dublin) and heparin from Leo Laboratories Ltd (Bucks, UK)

## CHAPTER THREE

### Preliminary investigation of cardioprotection in hypertrophied myocardium

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### 3.1. INTRODUCTION

The first study was undertaken to establish a model of mild hypertension induced with DOCA-salt treatment. Classical / delayed preconditioning as well as bradykinin induced cardioprotection were investigated in this model of hypertension.

Kuhlmann et al in 1939 were the first to report that DOCA-salt treatment in dogs induced hypertension (Kuhlmann *et al*, 1939). Subsequently, Grollman et al demonstrated DOCA-salt induced hypertension in rat (Grollman *et al*, 1940). Further investigators showed that DOCA-salt induced hypertension in a variety of animal models, including, chick (Seyle, 1942), guinea pig (Tirtilli & Ruff, 1994), mouse (Honeck *et al*, 2000) and pig (Miller *et al*, 1979). Masson et al, however, showed that the rabbit did not develop hypertension following DOCA-salt treatment. Instead, rabbits developed hypercholesterolaemia (Masson *et al*, 1953). Despite the occurrence of hypertension induced by DOCA-salt in a variety of animal models, the rat is the most widely used species.

DOCA-salt treatment involves the simultaneous administration of DOCA subcutaneously and NaCl (0.9%) / KCl (0.2%) placed in rat drinking water over four weeks. A unilateral nephrectomy is sometimes also performed in conjunction with DOCA-salt treatment to accelerate the development of hypertension. However, reduced renal mass is not essential for hypertension to develop. Rats given DOCA or saline alone do not develop hypertension, only a combination of DOCA and salt produces hypertension (Doggrell & Brown, 1998).

After the SHR, DOCA-salt hypertension is probably the most widely used model to study hypertension and hypertrophy (Pinto *et al*, 1998). Arterial pressure was demonstrated to increase in rat three weeks following the induction of the DOCA-salt treatment (Hebden *et al*, 1990). Systolic blood pressures greater than 165 mmHg have been reported (Baxter &

Yellon, 1992a, 1993). Cardiac hypertrophy (approximately 30% increase in LV mass) has been described in this model (Baxter & Yellon, 1992a, 1993; Besse *et al*, 1994; Pinto *et al*, 1998;). Although animals exhibit LVH, cardiac failure has not been described in DOCA-salt treated animals (Besse *et al*, 1994). Renal changes have been demonstrated following DOCA-salt administration, associated with proteinuria and glomerulosclerosis (Lafferty *et al*, 1991). Analogous to the other models of hypertension, endothelium dependent relaxations are also impaired in the DOCA-salt model of hypertension (Somers *et al*, 2000; Kirchner *et al*, 1993). Myocardial fibrosis due to increased collagen deposition has also been described in DOCA-salt treated rats (Baxter & Yellon, 1992a; Ammarguella *et al*, 2001).

This model has been subject to criticism because mineralocorticoid hyperactivity, especially augmented secretion of deoxycorticosterone, is seldom found in man. However, it must be remembered that no animal model of hypertension fully mimics the human pathology. Apart from developing LVH, the DOCA-salt model, is economical, relatively easy to employ and is associated with low rates of mortality (Doggrell & Brown, 1998). For these reasons, this model was chosen for the preliminary studies.

### **3.1.1. Mechanisms in DOCA-salt hypertension**

This model of hypertension has been extensively characterised, and several mediators of hypertension have been implicated, described below. However, mechanisms leading to hypertension still remain unclear. In the initiation of hypertension, sodium retention may be implicated, whereby increased levels cause volume expansion. However, sodium retention alone is not likely to cause hypertension (Schenk & Mc Neill, 1992). It has been proposed that increased sodium levels alter neurohormonal pressor baroreflexes which then participate in either initiating or maintaining hypertension (Ferrario *et al*, 1987). Some of the

classical, humoral mechanisms involved in the regulation of blood pressure include vasopressin, AT-II, endothelin -1 and catecholamines (Schenk & Mc Neill, 1992).

#### 3.1.1.1. *Vasopressin*

Numerous studies have demonstrated a possible role for vasopressin in the development of DOCA-salt induced hypertension. Crofton et al demonstrated that the urinary excretion of vasopressin in DOCA-salt treated rats was elevated (Crofton *et al*, 1979). Furthermore, they also showed that an i.v. injection of analogues of vasopressin (which abrogate its pressor, but not its antidiuretic activity), lowered blood pressure in DOCA-salt rats. These authors concluded by stating that vasopressin plays both a role in the initiation and maintenance of DOCA-salt hypertension (Crofton *et al*, 1979). Zicha et al demonstrated that the role of vasopressin in DOCA-salt hypertension is also dependent on the age of the animal (Zicha *et al*, 1989). They showed that while vasopressin was not important in regulating blood pressure in young rats with DOCA-salt hypertension, vasopressin contributed to the maintenance of hypertension in the adult rats. The authors presumably did not observe a role for vasopressin in the young rats as they are not capable of synthesising this peptide (Zicha *et al*, 1989). In contrast, Takata et al demonstrated that a vasopressin antagonist caused a hypotensive effect in DOCA-salt rats, which was enhanced with the development of hypertension, at all stages of hypertension, including the prehypertensive stage (Takata *et al*, 1988). Hence, authors implicated a role for vasopressin in the initiation and maintenance of DOCA-salt hypertension. Ouchi et al showed that urinary excretion of vasopressin levels was increased in DOCA-salt rats, they also found that plasma levels of this hormone were elevated in rats subjected to DOCA-salt treatment (Ouchi *et al*, 1987). There was however no difference in the metabolic clearance rate of vasopressin in the DOCA-salt rats compared to the non-treated rats, indicating that

elevated plasma levels of vasopressin in DOCA-salt hypertension are due to the increased release of the hormone, not impaired catabolism (Ouchi *et al*, 1987).

### 3.1.1.2. *Sympathetic nervous system*

The peripheral sympathetic nervous system has been shown to be implicated in the development of DOCA-salt hypertension. Lamprecht *et al* showed that intraventricular injection of 6-hydroxydopamine, normalised blood pressure in DOCA-salt hypertensive rats (Lamprecht *et al*, 1977). The timing of administration of 6-hydroxydopamine was important as, when it was given two weeks following DOCA-salt treatment, it lowered blood pressure. However, when it was given six weeks post DOCA-salt treatment, no reduction in blood pressure was apparent (Lamprecht *et al*, 1977). The results suggested that the sympathetic nervous system is involved in the induction but not in the maintenance of DOCA-salt hypertension. Furthermore, the authors speculated that central sympathetic tracts control the activity of the peripheral sympathetic system and destruction of these tracts would ultimately prevent the participation of the peripheral sympathetic system in the initiation of hypertension. Alterations in plasma catecholamine levels have also been reported in DOCA-salt rats. Bouvier and de Champlain demonstrated that plasma noradrenaline levels were augmented in DOCA-salt treated rats, compared with normotensive control animals (Bouvier & de Champlain, 1986a,1986b). Subsequent studies demonstrated that increased plasma catecholamine levels in DOCA-salt hypertension were not due to defective neuronal reuptake, but rather the consequence of an increase in sympathetic tone in DOCA-salt treated animals (Drolet *et al*, 1989). Furthermore, Sanchez *et al* demonstrated that pharmacological blockade of the  $\alpha$  adrenoceptor prevented hypertension in DOCA-salt rats (Sanchez *et al*, 1989).

#### 3.1.1.3. *Renin - angiotensin system*

The RAS is generally believed not to participate in DOCA-salt hypertension. In fact this model is associated with a depressed plasma renin activity (Gavras *et al*, 1975). Additionally numerous experiments have demonstrated that treatment with ACE inhibitors do not reduce blood pressure in DOCA-salt animals. Karam *et al* demonstrated that enalapril did not lower blood pressure in DOCA-salt hypertensive rats, although it did reduce subendocardial fibrosis (Karam *et al*, 1996). Similarly, Brown *et al* showed that neither captopril or candesartan (AT<sub>1</sub> receptor antagonist) lowered blood pressure in DOCA-salt rats but did attenuate deposition of perivascular and interstitial collagen (Brown *et al*, 1999). More recently, Somers *et al* demonstrated that losartan (AT<sub>1</sub> receptor antagonist) did not reduce blood pressure in DOCA-salt hypertensive rats (Somers *et al*, 2000).

Interestingly, Itaya *et al* reported that intracerebroventricular administration of an ACE inhibitor to DOCA-salt rats, reduced blood pressure (Itaya *et al*, 1986). These results imply a role for brain RAS in the pathogenesis of hypertension in DOCA-salt rats. Furthermore, an increase in renin-like enzyme activity in the brain has been reported during the development of DOCA-salt hypertension (Basso *et al*, 1981).

In summary, experimental evidence indicates that circulating RAS does not participate in the pathogenesis of hypertension in the DOCA-salt model. The precise role of the brain RAS warrants further investigation.

#### 3.1.1.4. *Endothelin -1*

A role for endothelin -1 in DOCA-salt hypertension has been reported. Studies have not only demonstrated overexpression of vascular endothelin -1 (Lariviere *et al*, 1993, 1995; Day *et al*, 1995), but also a reduction in blood pressure following the administration of

bosentan (nonselective endothelin receptor antagonist) in DOCA-salt rats (Li *et al*, 1994).

More recently, Ammarguellat *et al* demonstrated that endothelin A receptor antagonist prevented cardiac fibrosis in DOCA-salt rats (Ammarguellat *et al*, 2001).

#### **3.1.1.5. Alternative mechanisms involved in DOCA-salt hypertension**

Several alternative mechanisms have been implicated in the pathogenesis of DOCA-salt hypertension. Kubo *et al* demonstrated enhanced activity of cholinergic neurones in rostral ventrolateral medulla in DOCA-salt rats. Whether this is involved in the maintenance of hypertension is not fully known (Kubo *et al*, 1998). Basal nitric oxide release may be attenuated in DOCA-salt rats, whether this precipitates hypertension or occurs as a consequence of hypertension is not known (Ayangade-Johnson & Joshua, 2001; Millette *et al*, 2000). A decreased expression of eNOS (the main source of nitric oxide in the myocardium) in the left ventricle of DOCA-salt rats has also been demonstrated (Hara *et al*, 2001).

#### **3.1.2. Aims of present study**

In the present study, cardioprotection was investigated in a model of early LVH, induced by short term treatment with DOCA-salt. Both classical preconditioning and delayed preconditioning were investigated and induced using conventional methods. For classical preconditioning, ischaemia was used as a method of inducing protection, whereas for delayed protection, we used heat shock. Heat shock treatment, whereby basal body temperature is raised to 42 °C for 15 minutes evokes a delayed preconditioning like effect (Marber *et al*, 1993; Currie *et al*, 1993). IPC (Randall *et al*, 1997; Butler *et al*, 1999; Boutros & Wang, 1995; Speechly-Dick *et al*, 1994; Pantos *et al*, 1996) and heat shock (Cornelussen *et al*, 1994,1997; Joyeux M *et al*, 1998b) have previously shown to protect the hypertrophied heart. Hence, the present study verified the occurrence of IPC and heat shock in the

DOCA-salt model of hypertrophy. Bradykinin has been widely demonstrated to induce cardioprotection in many animal models of ischaemia-reperfusion (Bugge & Ytrehus, 1996; Goto *et al*, 1995; Leesar *et al*, 1999), however, whether bradykinin can induce protection in the hypertrophied heart is not known. Accordingly, the present study investigated whether bradykinin induced protection is preserved in hypertension. Indeed, bradykinin has been implicated in the pathogenesis of hypertension. Studies have proposed that activity of the kallikrein-kinin system is attenuated in hypertension (Favaro *et al*, 1975; Ader *et al*, 1987; Sharma *et al*, 1996). Furthermore, studies have also demonstrated that bradykinin levels are decreased in hypertension which may in fact contribute to the induction of hypertension (Nakagawa & Nasjletti, 1988; Sharma *et al*, 1996). Thus, bradykinin induced cardioprotection may well be impaired in hypertension.

## **3.2. METHODS AND MATERIALS**

### **3.2.1. DOCA -salt regimen**

A rapid DOCA-salt regimen was used to induce hypertrophy in rats. This method has previously shown to induce hypertrophy four weeks following DOCA-salt administration (Baxter & Yellon, 1992a, 1993). DOCA was obtained from Sigma (Poole, Dorset). When preparing the DOCA suspension, the vegetable gum, tragacanth was used, which is known to provide adequate dispersion of DOCA following its administration. The following formula was used to prepare the DOCA suspension for s.c. injection:

DOCA	100 mg
Tragacanth	6 mg
Ethanol	several drops
Saline (0.9%) to	1 ml

Using a pestle and mortar, the DOCA and tragacanth were triturated, with, a few drops of ethanol added to moisten the mixture. Appropriate volumes of saline (0.9%) were then

added to the mixture. This suspension was stored at less than 0 °C and made on a weekly basis.

Adult rats (weight approximately 250-300 g) were injected with the DOCA mixture (10 mg, s.c.) two or three times a week and 0.9% NaCl / 0.2% KCl solution was substituted for normal tap water for a period of four weeks. Following four weeks treatment, normal tap water was administered to the animals. Rats were not used for approximately seven-ten days following the termination of DOCA-salt treatment. Normotensive control animals received no treatment.

### **3.2.2. Experimental protocols**

In the first study, hearts (obtained from both normotensive and DOCA-salt hypertensive rats) were randomly assigned to one of the following treatment protocols, following an initial stabilisation period (figure 3.1).

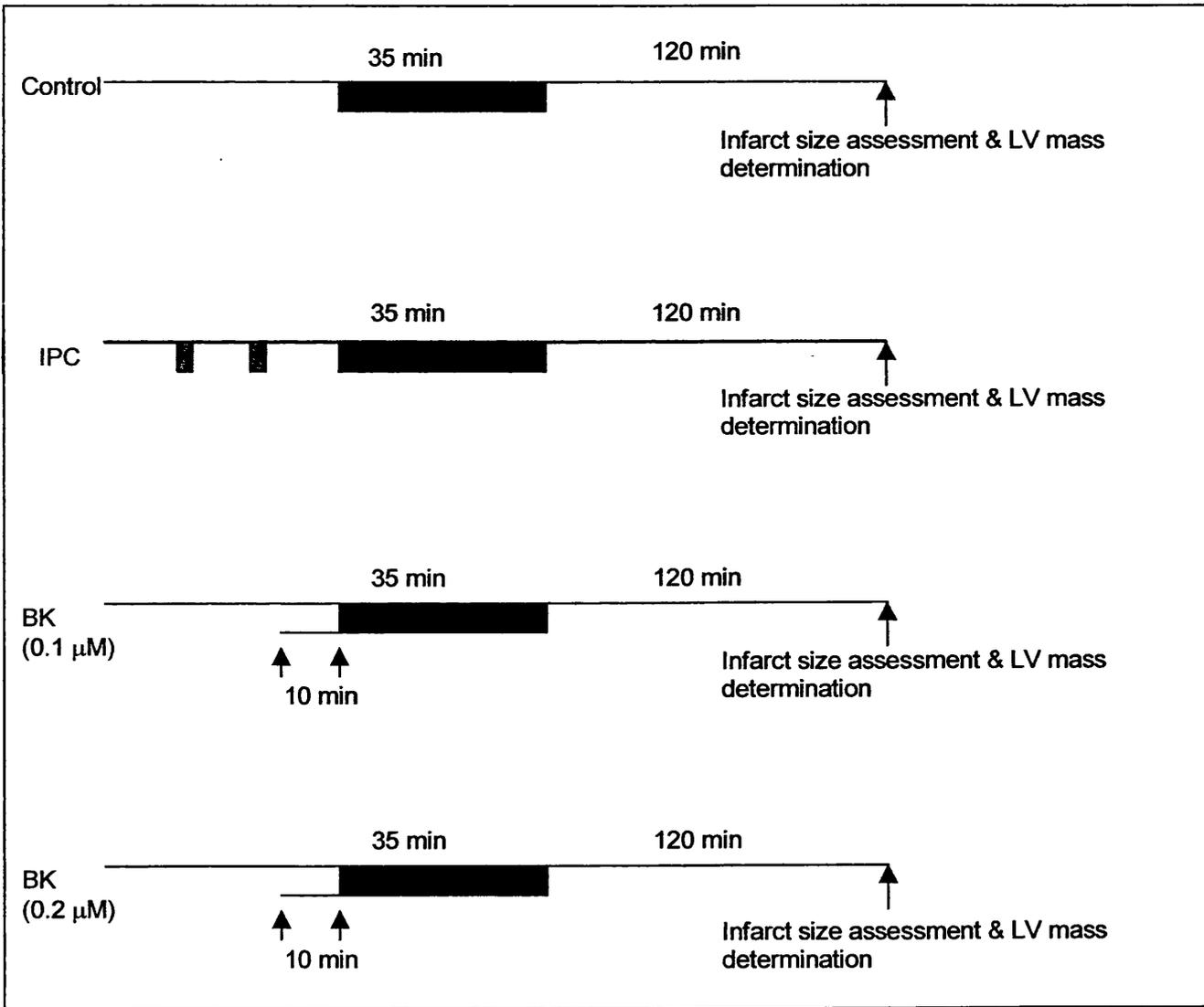
Group 1; Control. Hearts underwent 35 minutes regional ischaemia and 120 minutes reperfusion.

Group 2; IPC. Hearts were subjected to 2 cycles of 5 minutes global ischaemia interspersed by 10 minutes reperfusion.

Group 3; Bradykinin (0.1 µM) treatment. Hearts were subjected to bradykinin (0.1 µM) for 10 minutes prior to infarct induction.

Group 4; Bradykinin (0.2 µM) treatment. Hearts were subjected to bradykinin (0.2 µM) for 10 minutes prior to infarct induction.

All of the above experiments were performed using hearts obtained from both normotensive and hypertensive animals, therefore, there was total of eight experimental groups. The concentration of bradykinin was determined from previous reports which demonstrated that bradykinin at a concentration of 0.1  $\mu\text{M}$  was cardioprotective (Zhu *et al*, 1995; Jin & Chen,1998).



■ = global ischaemia      ■ = regional ischaemia

Figure 3.1. Experimental treatment protocols. Hearts were subjected to either IPC or bradykinin prior to infarct induction (study one).

In the second study, animals were subjected to heat shock treatment 24 hours prior to infarct induction. Rats (both DOCA-salt hypertensive and normotensive controls) were assigned to one of the following protocols (ie, there was a total of four experimental groups).

Group 1; Sham treatment. Rats were given intraperitoneal sodium pentobarbitone (50 mg/kg), but were not subjected to heating.

Group 2; Heat shock treatment. Rats were anaesthetised (50 mg/kg) and placed in a heating blanket to raise core body temperature. Temperature was monitored using a digital rectal thermometer and was maintained at 41.5 °C to 42 °C for 15 minutes, following which animals were allowed to recover at room temperature. During this early recovery period, rats were encouraged to drink water to compensate for the fluid loss during the heat shock procedure. After animals had fully recovered from heat stress, they were returned to their cages for 24 hours prior to the infarct protocol.

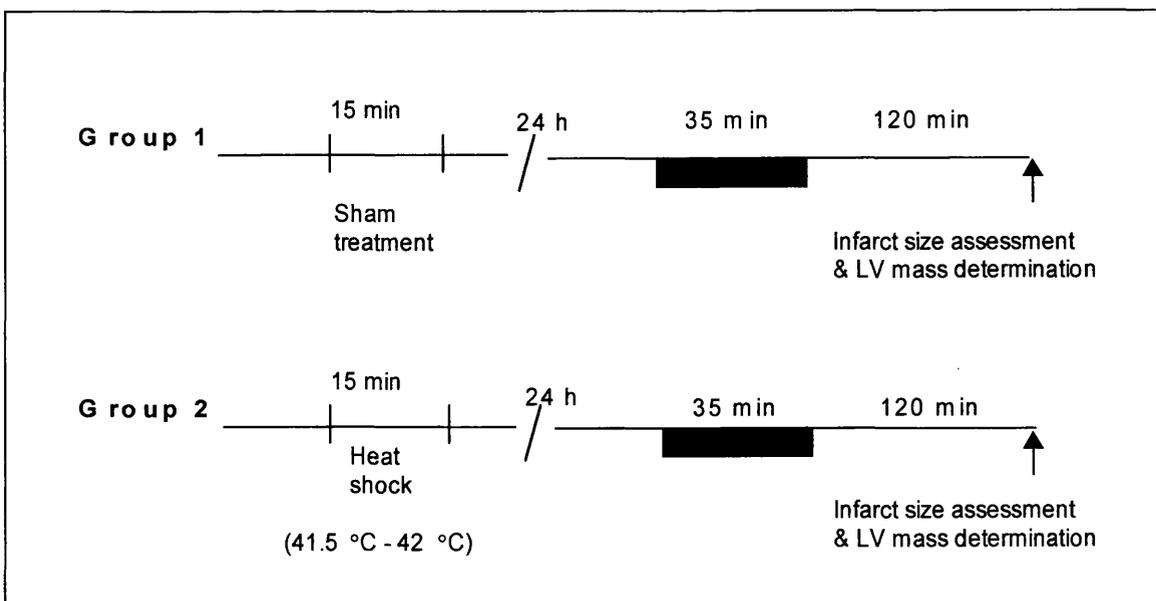


Figure 3.2. Experimental treatment protocols (study two). Rats were subjected to either sham or heat shock treatment 24 hours prior to infarct induction.

### **3.2.3. Blood pressure measurement**

The effects of DOCA-salt treatment on blood pressure were assessed in a separate cohort of anaesthetised animals. The methods for determination of blood pressure are described in section 2.2.

### **3.2.4. Detection of HSP 72 following heat shock**

Separate groups of animals were prepared for biochemical analysis of HSP 72. Twenty-four hours following heat shock or sham treatment, hearts were excised and immediately freeze clamped in liquid nitrogen and stored at -80 °C. A Western blot was performed according to the technique described in section 2.7. A 12.5% gel was prepared and the membrane was probed with primary mouse HSP 72 monoclonal antibody at 1:1000 dilution followed by anti mouse horseradish peroxidase-linked antibody (1: 2500 dilution).

## **3.3. RESULTS**

### **3.3.1. Exclusions**

A total of 129 animals were used in these studies. 106 rats were used for infarct studies. A total of four hearts were excluded; two due to inadequate delineation of the ischaemic risk zone; one due to no reduction in CFR following coronary artery ligation; one due to bradyarrhythmia during stabilisation and rhythm disturbances that persisted throughout the course of the experiment. Three DOCA-salt animals died following heat shock. Therefore, data for 99 successfully conducted infarct studies are reported (57 for the IPC / bradykinin study and 42 for the heat shock study). Eleven rats were used for assessment of blood pressure; 12 animals were sacrificed for Western blot analysis of HSP 72.

### **3.3.2. Characterisation of DOCA-salt rats**

#### **3.3.2.1. Blood pressure data**

Systolic and diastolic blood pressures are reported in table 3.1. The rats subjected to DOCA-salt treatment (ie, hypertensive hearts) displayed significantly higher systolic and diastolic blood pressure compared with the normotensive rats (ie, rats not subjected to DOCA-salt treatment).

Table 3.1. Summary of blood pressure

Group	n	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)
DOCA-salt hypertensive	6	172±13 *	131±11 *
Normotensive	5	116±5	93±5

\* = P <0.05 versus normotensive rats (one way ANOVA)

### 3.3.2.2. *Body weight, risk zone volume and hypertrophy index*

Rat body weights were generally greater in the normotensive control rats compared with DOCA-salt rats, and reached statistical difference among some groups as shown in table 3.3. Previous studies have also demonstrated that DOCA-salt rats do not gain as much weight as the normotensive animals (Karam *et al*, 1996; Tomanek & Barlow, 1990).

Myocardial risk volume was significantly greater in all hypertrophied groups (ie, DOCA-salt treated rats) compared to normotensive groups (table 3.2 / 3.3). When risk zone was normalised to the volume of the left ventricle, there were no significant differences among any of the groups.

LV body weight ratios are depicted in table 3.2 and 3.3. DOCA-salt treated rats exhibited greater LV body weight ratios when compared to the non-treated rats. The hypertrophy index was 26%, consistent with previous reports (Baxter & Yellon, 1992a, 1993; Besse *et al*, 1994). RVH did not occur in the DOCA-salt treated rats.

Table 3.2. Rat body weight, risk zone volume, risk zone volume normalised to LV volume, LV/body weight ratios, and RV/body weight ratios

Group	n	rat body weight (g)	risk zone volume (cm <sup>3</sup> )	risk zone / LV volume (%)	LV/ body weight (mg/g)	RV/body weight (mg/g)
Hypertensive control	7	457±24	0.91±0.08 *	53.6±3.2	3.6±0.13 *	0.34±0.04
Normotensive control	9	530±26	0.65±0.06	50.0±2.4	2.7±0.12	0.33±0.03
Hypertensive IPC	7	486±19	0.85±0.07 *	50.0±1.2	3.5±0.10 *	0.36±0.02
Normotensive IPC	8	507±15	0.60±0.05	49.0±3.7	2.6±0.30	0.37±0.03
Hypertensive bradykinin (0.1 µM)	6	461±9	0.83±0.06 *	52.7±3.9	3.6±0.16 *	0.32±0.02
Normotensive bradykinin (0.1 µM)	7	496±12	0.63±0.08	46.8±2.7	2.6±0.06	0.32±0.02
Hypertensive bradykinin (0.2 µM)	7	430±15	0.75±0.10 *	46.3±4.1	3.9±0.20 *	0.40±0.03
Normotensive bradykinin (0.2 µM)	6	450±10	0.54±0.03	46.6±3.3	2.9±0.12	0.34±0.02

\* = P < 0.05 versus corresponding normotensive group (one way ANOVA)

Table 3.3. Rat body weight, risk zone volume, risk zone volume normalised to LV volume, LV/body weight ratios, and RV/body weight ratios

Group	n	rat body weight (g)	risk zone volume (cm <sup>3</sup> )	risk zone / LV volume (%)	LV/ body weight (mg/g)	RV/body weight (mg/g)
Hypertensive sham	10	421±12	0.71±0.04 *	50.1±1.4	4.0±0.14 *	0.38±0.03
Normotensive sham	12	457±14	0.53±0.04	47.9±1.3	2.8±0.09	0.35±0.02
Hypertensive heat shock	11	421±13	0.67±0.05 *	47.7±2.1	4.1±0.13 *	0.38±0.02
Normotensive heat shock	9	481±16 †	0.51±0.02	48.6±1.4	2.9±0.08	0.33±0.01

\* = P < 0.05 versus corresponding normotensive group (one way ANOVA)

† = P < 0.05 versus hypertensive sham and hypertensive heat shock groups (one way ANOVA)

3.3.2.3. *Plasma noradrenaline concentration*

Consistent with previous reports (Bouvier & de Champlain, 1986a, 1986b), DOCA-salt treated rats had a higher plasma concentration of noradrenaline compared to the normotensive animals, this difference however did not reach statistical significance ( $P = 0.3$ ).

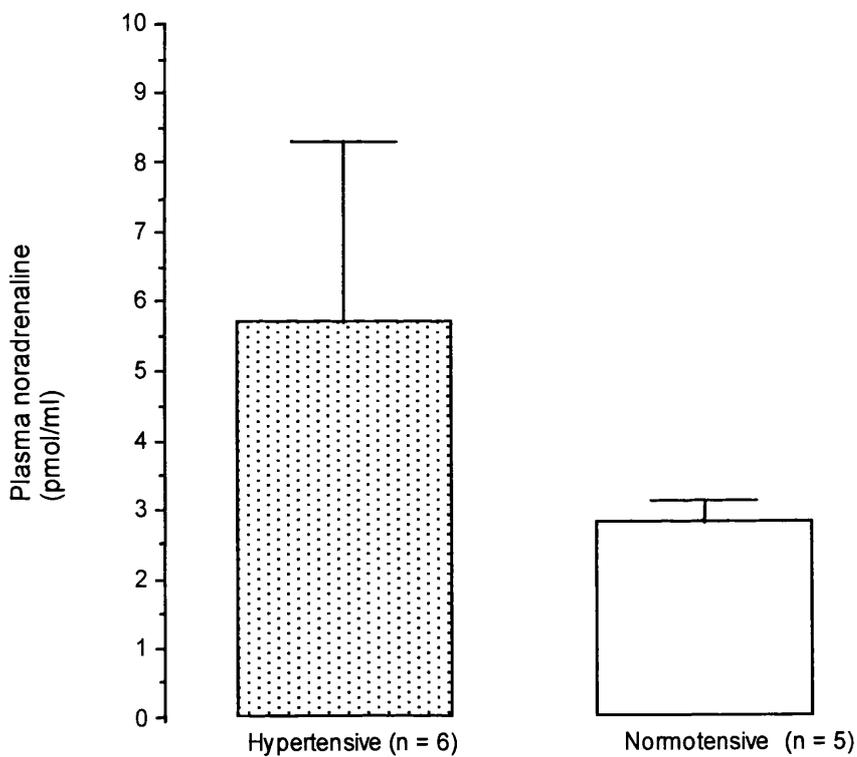


Figure 3.3. Plasma noradrenaline concentration in the hypertensive and normotensive animals

### 3.3.3. Infarct limiting effects of IPC

Figure 3.4. summarises the infarct size data, expressed as a percentage of the risk zone following IPC. Normotensive control infarct size was  $51.2 \pm 3.0\%$  and  $18.9 \pm 3.4\%$  ( $P < 0.01$ ) following IPC. Similarly, hypertensive control infarct size was  $47.1 \pm 3.2\%$  and  $18.5 \pm 2.6\%$  following IPC ( $P < 0.01$ ). Thus, IPC produced a similar cardioprotective effect in both normotensive and hypertensive hearts.

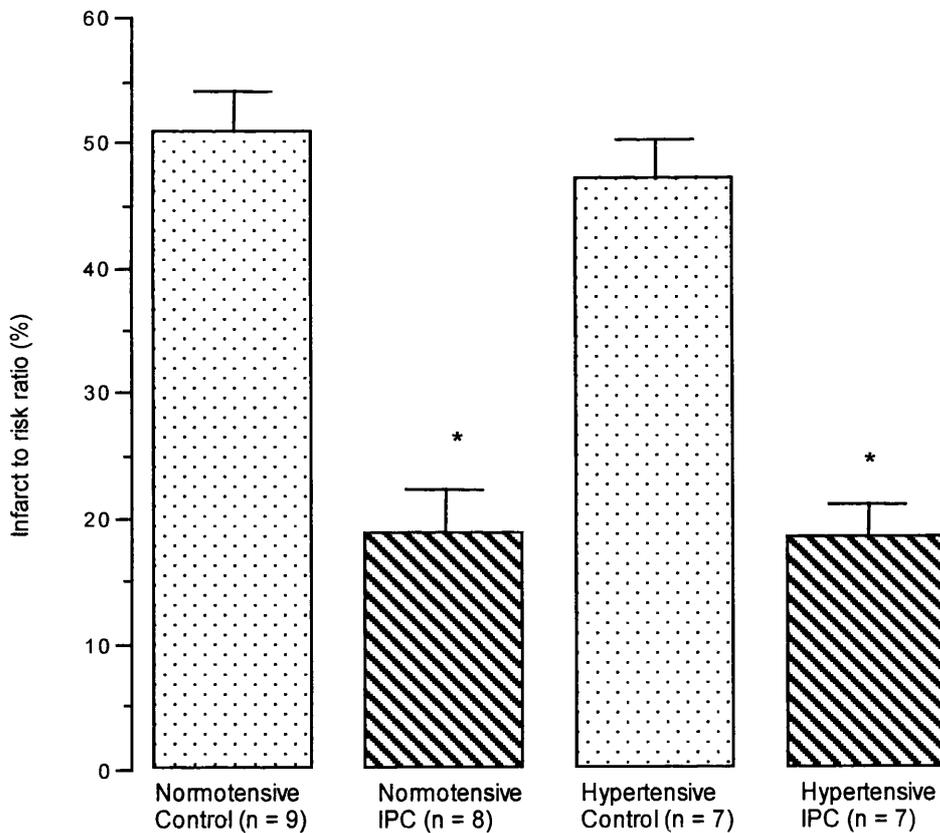


Figure 3.4. Infarct to risk zone ratio (%). IPC limited infarct size in both normotensive and hypertensive hearts. \* =  $P < 0.01$  versus controls groups (one way ANOVA)

### 3.3.4. Infarct limiting effects of bradykinin

Pre-treatment with bradykinin (0.1  $\mu\text{M}$ ) caused a reduction in infarct size in the normotensive animals from  $51.2 \pm 3.0\%$  to  $21.0 \pm 2.7\%$  ( $P < 0.01$ ). In the hypertensive hearts, bradykinin (0.1  $\mu\text{M}$ ) caused a reduction in infarct size from  $47.1 \pm 3.2\%$  to  $33.7 \pm 4.2\%$  ( $P < 0.05$ ). Thus, even though bradykinin induced cardioprotection in both normotensive and hypertensive hearts, the protection was clearly attenuated in the hypertensive heart compared with the protection observed in the normotensive heart. When a higher concentration of bradykinin (0.2  $\mu\text{M}$ ) was used, no further cardioprotection was observed in the hypertensive hearts. Bradykinin (0.2  $\mu\text{M}$ ) reduced infarct size from  $51.2 \pm 3.0\%$  to  $18.2 \pm 4.9\%$  ( $P < 0.01$ ) in normotensive rat hearts. Bradykinin (0.2  $\mu\text{M}$ ) reduced infarct size from  $47.1 \pm 3.2\%$  to  $29.5 \pm 5.3\%$  ( $P < 0.01$ ) in hypertensive hearts. Therefore, even at a higher concentration, bradykinin induced protection was significantly diminished in DOCA-salt rat hearts compared with normotensive hearts (figure 3.5).

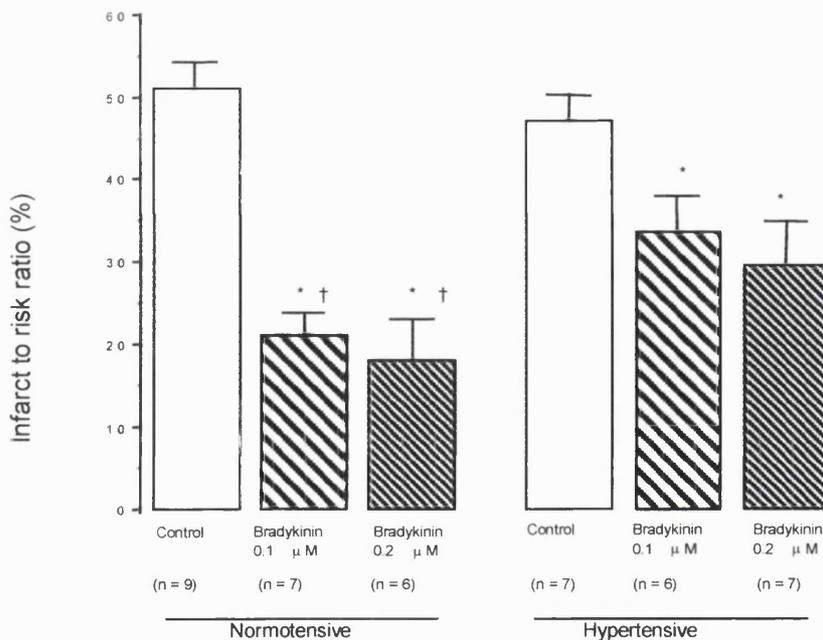


Figure 3.5. Infarct to risk ratio (%). Bradykinin limited infarct size in both normotensive and hypertensive hearts. However, greater protection was observed in the normotensive hearts. \* =  $P < 0.05$  versus control group. † =  $P < 0.05$  versus corresponding hypertensive groups (one way ANOVA).

### 3.3.6. Infarct limiting effects of heat shock

Figure 3.6. highlights the infarct size data, expressed as a percentage of the risk zone following heat shock treatment. Normotensive animals subjected to sham treatment exhibited an infarct size of  $43.1 \pm 3.6\%$ . Heat shock treatment, reduced infarct size to  $24.3 \pm 4.0\%$  ( $P < 0.01$ ) in the normotensive animals. Similarly, in the hypertensive hearts, infarct size following sham treatment was  $39.3 \pm 3.9\%$ . Heat shock treatment reduced infarct size to  $23.7 \pm 3.5\%$  ( $P < 0.01$ ) in these animals.

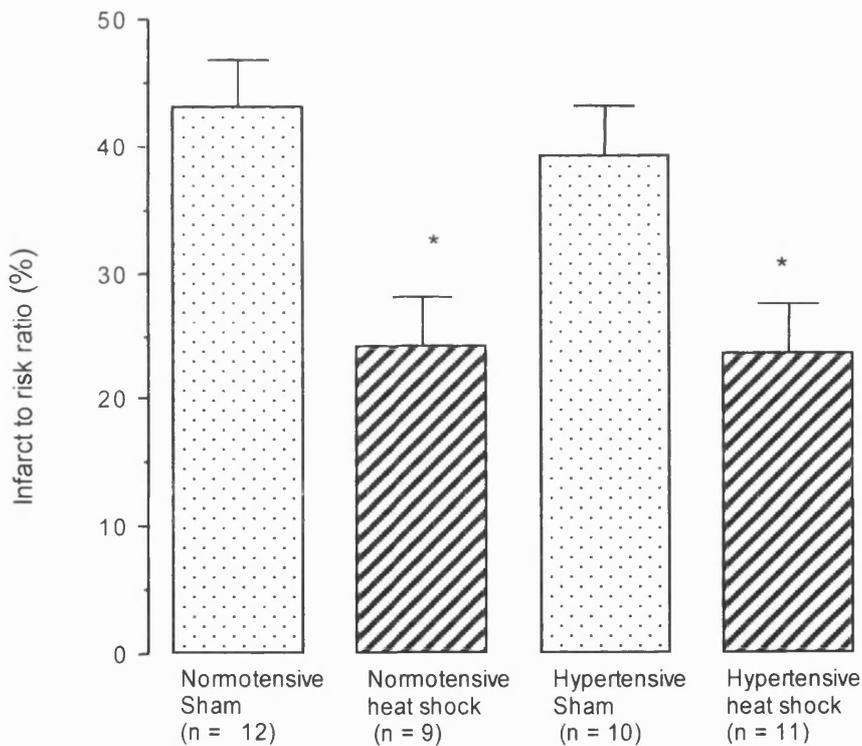


Figure 3.6. Infarct size expressed as a percentage of the risk zone (%). The cardioprotective effects of heat shock 24 hours prior to infarct induction was present both in the normotensive control and hypertensive hearts. \* =  $P < 0.01$  versus sham groups (one way ANOVA)

3.3.6.1. Expression of HSP 72 following heat shock

Expression of HSP 72 was assessed 24 hours following heat shock treatment. Figure 3.7. demonstrates that heat shock caused a significant induction of HSP 72 protein levels in both normal and hypertrophied myocardium. Interestingly, upregulation of HSP 72 following heat shock was greater in normotensive animals compared with the hypertensive rat hearts.

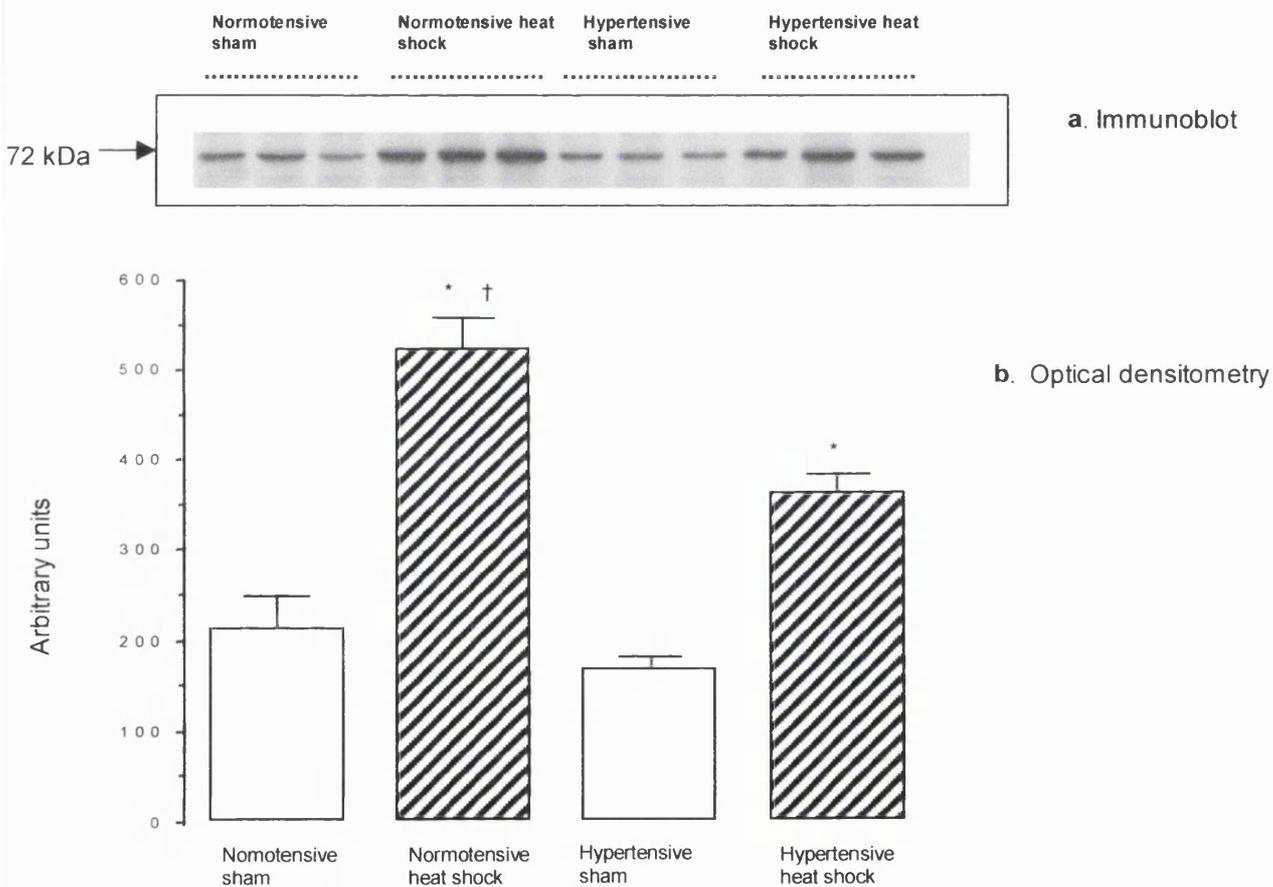


Figure 3.7.

**Panel a-** Western blot demonstrating expression of HSP 72 following heat shock. Lanes 1-3 represent samples subjected to sham treatment (normotensive). Lanes 4-6 represent samples subjected to heat shock treatment (normotensive). Lanes 7-9 represent samples subjected to sham treatment (hypertensive). Lanes 10-12 correspond to samples subjected to heat shock treatment (hypertensive).

**Panel b-** Expression of HSP 72 in arbitrary units. Heat shock caused a significant induction of HSP 72 in both normotensive and hypertensive hearts.

\* =  $P < 0.05$  versus sham group. † =  $P < 0.05$  versus hypertensive heat shock (One way ANOVA)

### **3.3.7. Coronary flow and contractility data**

CFR and RPP are presented in tables 3.4 - 3.7. No differences in CFR were observed among the groups at baseline. Following administration of bradykinin (both 0.1  $\mu\text{M}$  and 0.2  $\mu\text{M}$ ), a significant increase in CFR was observed in the normotensive animals. A vasodilatory effect was however, not observed in the hypertrophied rat hearts. CFR decreased significantly following coronary occlusion in all groups and increased following reperfusion. There were no significant differences among groups during ischaemia and reperfusion. The hypertrophied hearts generally displayed higher RPP at stabilisation and during the experimental procedure compared with normotensive animals. This is presumably due to the increase in mass of the LV which leads to an enhancement of contractility. RPP declined considerably following coronary artery occlusion in all groups, and gradually declined during reperfusion.

Table 3.4. Summary of CFR (ml/min/g)

Group	Basal	Pre-Ischaemia (+ bradykinin)	Ischaemia		reperfusion		
			5 min	30 min	5 min	60 min	120 min
Hypertensive control	10.2±0.6	10.4±0.6	5.2±0.5	5.4±0.4	7.6±0.4	5.8±0.5	5.0±0.5
Normotensive control	10.4±0.6	10.0±0.9	5.6±0.9	5.6±1.0	7.1±1.2	6.5±1.3	4.8±1.2
Hypertensive IPC	10.5±0.6	9.3±0.5	5.4±0.3	5.6±0.4	7.7±0.9	6.3±0.6	5.2±0.8
Normotensive IPC	9.3±0.6	9.0±0.3	5.4±0.4	5.3±0.6	8.1±0.7	6.3±0.8	4.7±0.6
Hypertensive bradykinin (0.1 µM)	10.5±0.6	10.5±0.9	5.4±0.5	5.1±0.5	7.3±1.4	5.3±0.7	4.4±0.7
Normotensive bradykinin (0.1 µM)	9.4±0.8	12.5±1.3 *	4.9±0.8	5.0±1.0	7.3±1.2	5.1±1.2	4.2±1.0
Hypertensive bradykinin (0.2 µM)	10.0±0.6	8.1±0.9	4.7±0.5	4.8±0.4	7.4±0.6	5.0±0.4	4.0±0.3
Normotensive bradykinin (0.2 µM)	11.4±0.6	14.0±0.5 *	6.1±0.5	5.7±0.7	8.9±0.7	6.9±0.9	5.6±1.0

\* = P < 0.05 versus normotensive control group. (Repeated measures ANOVA)

Table 3.5. Summary of RPP (mmHg/min X 10<sup>3</sup>)

Group	Basal	Pre- Ischaemia (+ bradykinin)	-----Ischaemia-----		-----reperfusion-----		
			5 min	30 min	5 min	60 min	120 min
Hypertensive control	41.1±2.7	38.1±2.5	10.6±2.6	23.2±2.8	34.2±5.4	25.3±0.73	21.9±1.9
Normotensive control	31.6±4.4	30.3±3.3	11.5±2.6	18.7±2.6	20.5±2.5	18.8±2.3	15.9±2.1
Hypertensive IPC	43.6±4.9	33.3±4.4	16.6±3.3	24.5±4.0	35.4±0.5	26.6±3.8	24.5±4.6
Normotensive IPC	31.6±4.4	23.2±3.4	12.5±2.1	13.1±2.9	22.9±2.9	18.8±3.0	16.6±2.5
Hypertensive bradykinin (0.1 µM)	41.0±0.9	32.3±1.6	13.6±1.7	21.3±1.8	23.5±0.9	25.1±2.4	20.1±1.8
Normotensive bradykinin (0.1 µM)	35.4±3.3	24.0±1.9	14.0±2.0	20.0±1.3	20.0±2.5	15.0±1.9	12.6±1.7
Hypertensive bradykinin (0.2 µM)	47.2±5.6	33.8±5.5	14.7±2.1	22.2±2.9	27.0±3.0	24.3±3.3	22.7±3.6
Normotensive bradykinin (0.2 µM)	45.8±6.0	38.7±5.3	15.2±4.3	25.3±5.4	21.5±5.0	19.2±5.3	12.5±4.8

Table 3.6. Summary of CFR (ml/min/g)

Group	Basal	Pre-Ischaemia	-----Ischaemia-----		-----reperfusion-----		
			5 min	30 min	5 min	60 min	120 min
Hypertensive sham	11.4±0.6	11.3±0.8	6.0±0.5	6.2±0.6	8.8±1.2	7.3±1.3	6.0±1.1
Normotensive sham	11.9±0.8	11.5±0.8	7.1±0.7	6.9±0.8	10.0±0.8	8.4±0.8	7.1±0.7
Hypertensive heat shock	11.4±0.5	10.9±0.5	6.2±0.4	6.1±0.4	9.3±0.5	7.6±0.5	6.5±0.5
Normotensive heat shock	11.6±0.6	11.3±0.8	6.7±0.4	6.9±0.8	9.2±0.8	8.1±0.5	7.4±0.7

Table 3.7. Summary of RPP (mmHg/min X 10<sup>3</sup>)

Group	Basal	Pre-Ischaemia	-----Ischaemia-----		-----reperfusion-----		
			5 min	30 min	5 min	60 min	120 min
Hypertensive sham	46.5±3.0	42.0±2.7	17.7±2.8	26.7±3.2	29.7±3.5	25.7±2.1	21.4±2.1
Normotensive sham	41.2±3.3	39.3±3.0	16.3±2.4	24.0±2.7	25.9±3.2	23.0±2.3	22.1±3.1
Hypertensive heat shock	50.3±2.6	46.9±3.2	20.0±2.8	31.2±2.7	32.4±4.7	33.9±2.9	28.7±2.9
Normotensive heat shock	41.0±3.8	36.9±3.4	13.9±1.5	22.9±1.7	25.9±3.3	25.6±1.6	23.5±2.8

\* = P < 0.05 versus normotensive control group. (Repeated measures ANOVA)

### **3.4. DISCUSSION**

The main findings of the present study are; (1) IPC induced using two x five minute cycles of preconditioning ischaemia evoked cardioprotection in both normotensive and DOCA-salt hypertensive rat hearts. (2) A novel finding of the present study was the observation that although bradykinin induced protection in the hypertrophied hearts, the protection was significantly less than that observed in normal rat hearts. Previous studies have not examined the cardioprotective effects of bradykinin in hypertrophy. (3) Heat shock 24 hours prior to infarct induction significantly attenuated infarct size in both DOCA-salt hypertensive and normotensive animals.

The DOCA-salt model of hypertrophy used in these studies produced a stable, consistent model of hypertension associated with mild LVH. No major differences in LVH were observed among any of the DOCA-salt treated animals, a mean hypertrophy index of 26% was observed. The DOCA-salt regimen was not only easy to implement, but was also associated with very low rates of mortality. In fact, all animals survived the DOCA-salt treatment.

The occurrence of IPC in the hypertrophied myocardium has already been discussed in section 1.8.

#### **3.4.1. Bradykinin and protection of the hypertrophied myocardium**

Despite an extensive literature investigating the protective effects of bradykinin in the normal, healthy myocardium, to our knowledge, bradykinin induced cardioprotection has not been investigated in the hypertrophied myocardium. In the present study although bradykinin evoked a limitation of infarct size in the hypertensive heart, it was significantly less than that observed in the normotensive heart. Reasons for this are not known,

however, many investigators have reported alterations in the kallikrein-kinin system in hypertension (Favaro *et al*, 1975; Ader *et al*, 1987; Nakagawa & Nasjletti, 1988; Sharma *et al*, 1996). While the majority of studies report that bradykinin levels are attenuated in hypertension (Nakagawa & Nasjletti, 1988; Seino *et al*, 1990; Sharma *et al*, 1996), a limited number of studies argue that plasma kinins are increased in hypertension (Campbell *et al*, 1995a; Campbell *et al*, 1995b). Nakagawa and Nasjletti reported that arterial plasma bradykinin was lower in DOCA-salt hypertensive rats, compared with untreated normal rats (Nakagawa & Nasjletti, 1988). Similarly, Seino *et al* also demonstrated that bradykinin is depressed in the established phase of hypertension in DOCA-salt hypertensive rats (Seino *et al*, 1990). Furthermore, studies have demonstrated that adenoviral gene delivery of the kallikrein gene in hypertension attenuates hypertension and hypertrophy (Jin *et al*, 1997; Wang *et al*, 1995; Chao *et al*, 1996; Xiong *et al*, 1995; Yayama *et al*, 1998). Whether decreased basal levels of bradykinin are somehow linked with the attenuated cardioprotection observed in this study is not known. In contrast, Campbell *et al* have demonstrated that increased levels of bradykinin are present in tissues of young SHR (Campbell *et al*, 1995a) and in brown adipose tissue of TGR(mRen-2)<sup>2</sup> rat (Campbell *et al*, 1995b). It is not known whether increased basal levels of bradykinin are linked with reduced bradykinin induced cardioprotection, although bradykinin B<sub>2</sub> receptor desensitisation has been shown following repeated stimulation (Luckhoff *et al*, 1988). Circulating bradykinin levels were not measured here, but the majority of studies favour the notion that bradykinin levels are reduced in hypertension. Therefore, increased bradykinin levels leading to bradykinin B<sub>2</sub> receptor desensitisation are not likely to account for the reduced bradykinin induced cardioprotection observed in this study.

G-protein abnormalities have also been reported to occur in hypertension (Johnson & Friedman, 1993). It is not known whether, G-protein receptor downregulation or uncoupling is responsible for the reduced protective actions of bradykinin observed in this study.

Another interesting observation of this study was the lack of coronary vasodilator effect of bradykinin in the hypertrophied hearts. While bradykinin caused a significant increase in CFR when administered to normotensive hearts, it did not induce vasodilatation in hypertensive hearts. These results are consistent with the finding that endothelium dependent relaxation is perturbed in hypertension (Drexler & Homig, 1999). Studies have shown that endothelium dependent relaxations are impaired in DOCA-salt hypertension (Kirchner *et al*, 1993; Anderson *et al*, 1988). Further, Millette *et al* demonstrated that bradykinin induced vasodilatation was diminished in isolated DOCA-salt rat hearts (Millette *et al*, 2000). Perfusion with superoxide dismutase restored bradykinin induced vasodilation, suggesting that increased production of free radicals in hypertension may scavenge nitric oxide, explaining why there are diminished basal levels of nitric oxide in hypertension. Additionally, Anderson *et al* demonstrated that DOCA-salt rats had a reduced CFR following adenosine administration compared with normotensive animals (Anderson *et al*, 1988). Bradykinin has been shown to augment CFR via the release of both nitric oxide and PGI<sub>2</sub> (Hatta *et al*, 1997). Basal nitric oxide levels are thought to be decreased in DOCA-salt rats and additionally, reduced activity of eNOS has been demonstrated in the myocardium of DOCA-salt rats (Hara *et al*, 2001). Bradykinin induced PGI<sub>2</sub> production is not affected in DOCA-salt hypertensive rats (Millette *et al*, 2000), implying that the absence of the vasodilatory effect of bradykinin may be a consequence of diminished nitric oxide production.

### **3.4.2. Heat shock and cardioprotection**

Several studies have demonstrated that heat shock 24 hours prior to ischaemia-reperfusion induces a cardioprotective effect. Currie et al were the first to demonstrate the protective effects of heat stress (Currie *et al*, 1998). They showed that 24 hours following heat shock, contractile function was enhanced and creatine kinase release was decreased following ischaemia-reperfusion in the isolated rat heart (Currie *et al*, 1988). Currie et al subsequently demonstrated that heat shock attenuated infarct size in rabbit heart (Currie *et al*, 1993). Similarly, Walker et al also demonstrated that heat shock was able to protect the rabbit heart (Walker *et al*, 1993). Protective effects of heat shock have been shown in rat heart as well. Cornelussen et al demonstrated that post ischaemic functional recovery was improved following heat shock in rat (Cornelussen *et al*, 1998). Steare and Yellon showed that heat stress protected against reperfusion arrhythmias in rat heart (Steare & Yellon, 1993).

A limited number of studies have examined heat shock induced protection in the hypertrophied myocardium. Cornelussen et al reported that heat shock led to an improvement of functional recovery following ischaemia in a rat model of hypertrophy induced by aortic banding (Cornelussen *et al*, 1994). Subsequently, it was reported that heat shock protected the aged hypertrophied rat heart (15 month old) (Cornelussen *et al*, 1997). Finally, Joyeux et al demonstrated that heat shock protected in the transgenic [(mREN-2)27] hypertensive rats (Joyeux *et al*, 1998b). These authors found that heat shock attenuated infarct size in hypertrophied animals from  $48.0 \pm 3.8\%$  to  $20.0 \pm 1.0\%$ . A similar reduction in infarct size was observed in the normotensive rats. Similarly, in the current study, it was found that heat shock reduced infarct size in DOCA-salt hypertrophied rat hearts.

The effects of heat shock in alternative disease states has not been examined extensively. In streptozotocin-induced diabetic rat hearts, heat shock prior to infarction failed to evoke cardioprotection (Joyeux *et al*, 1999). Reasons for the lack of protective effect of heat shock in the streptozotocin model of diabetes remain elusive.

#### 3.4.2.1. *Molecular mechanisms of heat shock induced cardioprotection*

The exact mechanism by which heat shock induces protection against ischaemia-reperfusion 24 hours later are not fully understood. Numerous investigators have demonstrated an upregulation of HSP 72 following heat shock induced protection and delayed preconditioning induced with ischaemia (Joyeux *et al*, 1998b; Currie *et al*, 1993; Steare & Yellon, 1993; Morris *et al*, 1996; Yellon & Marber, 1994; Marber *et al*, 1993). Indeed, in the present study, we found that HSP 72 was upregulated significantly following heat shock in both normal and hypertrophied hearts. Exactly how the upregulation of HSP 72 leads to cardioprotection is not known. However, HSP 72 is a molecular chaperone that aids the folding, assembly and disassembly of proteins (Benjamin & McMillan, 1998; Morris *et al*, 1996). Even though studies have implied a role HSP 72 in the protective effects of heat shock, some studies do not support this idea. While some studies in transgenic mice overexpressing HSP 72 have demonstrated that this protein induced protection against ischaemia-reperfusion (Marber *et al*, 1995; Radford *et al*, 1996), studies involving delayed preconditioning induced pharmacologically with CCPA (Baxter & Yellon, 1997b) or MLA (Yoshida *et al*, 1996) did not observe induction of HSP 72 synthesis following drug treatments. The role of HSP 72 in mediating delayed preconditioning clearly requires further investigation. In the present study it was observed that HSP 72 was induced following heat shock. However, this does not necessarily mean that this protein was involved in mediating the protection induced by heat shock. Indeed studies have reported that, despite upregulation of HSP 72 following various experimental strategies, no protection

against ischaemia-reperfusion was observed (Qian *et al*, 1999; Sagnek *et al*, 1997; Tanaka *et al*, 1994a). HSP 72 may merely be upregulated due to a stress effect of heat shock.

Interestingly, in the present study we observed that induction of HSP 72 was greater in normotensive rats, compared to hypertensive rat hearts following heat shock, although, a similar reduction in infarct size was seen in both normal and hypertrophied hearts. Consistent with these findings, Tajima *et al* demonstrated that HSP 72 expression induced by coronary artery occlusion is attenuated in hypertrophied hearts, compared with normal hearts (Tajima *et al*, 1997). Reasons for this are unknown, although, HSP 72 has been implicated in hypertension. Hypertension can be regarded as a stressful stimulus. Hence variations in basal expression of HSP 72 may occur in this pathology, indeed studies have demonstrated that basal levels of HSP 72 are upregulated in hypertrophy (Xu *et al*, 1995; Gaia *et al*, 1995; Delcayre *et al*, 1988; Hamet *et al*, 1990). This however, was not the case in this study where basal expression of HSP 72 was similar in both hypertrophied and normotensive hearts.

Alternative mechanisms involved in heat shock induced protection have been demonstrated. Yamashita *et al* demonstrated that activation of Mn-SOD is critical in heat shock protection in rat (Yamashita *et al*, 1998a). Joyeux and co-workers showed that the infarct limiting effect of heat shock was abrogated by SB 203580, implying a role for p38 MAPK pathway (Joyeux *et al*, 2000). Additionally, although a role for tyrosine kinase was not found in heat shock induced protection, PKC activation was found to be essential (Joyeux *et al*, 1997). More recently, NOS has also been implicated in heat shock induced delayed protection (Arnaud *et al*, 2001).

### **3.5. CONCLUSION**

In preliminary studies, the DOCA-salt model of hypertension was used to induce cardiac hypertrophy in rat hearts. Consistent with previous reports, DOCA-salt treatment produced a stable form of mild LVH. Both IPC and heat shock induced protection in the hypertrophied heart. However, bradykinin induced limitation of infarct size was attenuated in the hypertensive heart compared to the normotensive heart, reasons for which remain elusive. It is important to emphasise this finding - even in a mild model of hypertrophy, bradykinin induced cardioprotection and vasodilatation are absent. Clearly, bradykinin induced cardioprotection warrants further investigation in a model of chronic hypertrophy. Therefore, in subsequent studies, IPC and bradykinin induced protection were examined in the chronically hypertensive myocardium.

## CHAPTER FOUR

### Effects of inhibiting bradykinin breakdown in the ischaemic myocardium

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#### 4.1. INTRODUCTION

In the previous chapter, we demonstrated that bradykinin induced protection was attenuated in the hypertrophied myocardium. In subsequent studies described in this and the following chapter, bradykinin induced cardioprotection was further investigated in the normal and hypertrophied myocardium using a pharmacological approach whereby enzymes that degrade bradykinin are inhibited to augment bradykinin levels. In the present study, bradykinin degradation was inhibited using a dual ACE and NEP inhibitor in the normal myocardium.

Despite the vast number of therapeutic modalities available for the treatment of hypertension, it still remains a major public health problem (Weber, 1999). If untreated or treated inadequately, there is the risk of serious cardiovascular events, for example congestive heart failure (Weber, 1999; Himmelmann, 1999; Cleland, 1999). One class of drugs that have proved to be beneficial in the treatment of hypertension and heart failure are the ACE inhibitors. ACE inhibitors have been shown to lower morbidity and mortality post myocardial infarction and in patients with heart failure of any aetiology in several major trials (Yusuf & Lonn, 2000). Most recently, the ACE inhibitor ramipril has exhibited beneficial effects in patients with coronary artery disease as seen in the HOPE study (The HOPE investigators, 2000).

As mentioned in chapter one, omapatrilat (BMS 186716) is a member of a novel class of therapeutic agents termed "vasopeptidase inhibitors" developed for the treatment of hypertension and heart failure (Weber, 1999; Trippodo *et al*, 1999; Burnett, 1999; Intengan & Schiffrin, 2000; Thomas *et al*, 1998; Duncan *et al*, 1999). The inhibition of either ACE or NEP prevents the enzymatic breakdown of bradykinin. Omapatrilat, through combined

inhibition of both ACE and NEP, would be expected to augment tissue concentrations and the physiological actions of both bradykinin and natriuretic peptides.

In addition to its potent coronary vasodilator properties, bradykinin exerts cardioprotective actions in the ischaemic myocardium (Wall *et al*, 1994; Starkopf *et al*, 1997; Goto *et al*, 1995; Leesar *et al*, 1999; Zhu *et al*, 1995), as reviewed in chapter one. Bradykinin is a key trigger of IPC. Pharmacological antagonism of the B<sub>2</sub> receptor abrogated the protection afforded by preconditioning in rabbit (Goto *et al*, 1995). Conversely, administration of exogenous bradykinin mimicked the effects of IPC and resulted in profound limitation of infarct size following coronary artery occlusion (Goto *et al*, 1995). In addition, IPC was not demonstrable in B<sub>2</sub> receptor knock out mice (Yang *et al*, 1997c).

In the present study, the "threshold hypothesis" of IPC advanced by Downey and co-workers was adapted (Goto *et al*, 1995). A brief period of ischaemia (two minutes) causes the generation of several mediators of IPC, including bradykinin, but the tissue concentrations achieved during subthreshold IPC are insufficient to trigger the full protective response. It was hypothesised that under these subthreshold conditions, omapatrilat would maintain bradykinin levels (by preventing its degradation) sufficiently to elicit the full IPC response. Accordingly, the primary aim of this study was to determine if omapatrilat lowers the threshold for the induction of IPC. Secondly, we evaluated if any beneficial effect observed was mediated by activation of the bradykinin B<sub>2</sub> receptor by using Hoe 140, a selective B<sub>2</sub> receptor antagonist. Comparative studies were also performed with the conventional ACE inhibitor, captopril.

## **4.2. METHODS**

Hearts were excised and Langendorff perfused. Following stabilisation, hearts were assigned to one of the following 11 treatment groups illustrated in figure 4.1. Experiments were performed sequentially with randomisation throughout groups one and two. Concentrations of omapatrilat, captopril and Hoe 140 were determined from previous reports (Dogan *et al*, 1998b; Bugge & Ytrehus, 1996; Starkopf *et al*, 1997).

### **4.2.1. Treatment protocols**

Group 1; Control. Hearts underwent 35 minutes regional ischaemia and 120 minutes reperfusion.

Group 2; IPC. Hearts were subjected to 5 minutes global ischaemia followed by 10 minutes reperfusion prior to regional ischaemia induction.

Group 3; Subthreshold IPC (Sub-IPC). Hearts were subjected to 2 minutes global ischaemia followed by 10 minutes reperfusion prior to regional ischaemia induction.

Group 4; Subthreshold IPC + omapatrilat. Hearts were subjected to 2 minutes global ischaemia, preceded by and followed by 5 minute perfusions with omapatrilat 10  $\mu$ M. Following perfusion with standard Kreb's-Henseleit buffer for 5 minutes regional ischaemia was induced.

Group 5; Subthreshold IPC + captopril. Hearts were subjected to 2 minutes global ischaemia, preceded by and followed by 5 minute perfusions with captopril 200  $\mu$ M. Following perfusion with standard Kreb's-Henseleit buffer for 5 minutes, regional ischaemia was induced.

Group 6; Omapatrilat alone. Omapatrilat 10  $\mu\text{M}$  was perfused for 12 minutes, followed by 5 minutes perfusion period with standard Krebs's-Henseleit buffer prior to regional ischaemia.

Group 7; Captopril alone. Captopril 200  $\mu\text{M}$  was perfused for 12 minutes, followed by 5 minutes perfusion with standard Krebs's-Henseleit solution prior to regional ischaemia.

Group 8; Subthreshold IPC + Omapatrilat + Hoe 140. Hearts were treated with 2 minutes global ischaemia as described for group 4. Hoe 140 1  $\mu\text{M}$  was co-perfused with omapatrilat 10  $\mu\text{M}$  for 5 minutes prior to global ischaemia and 5 minutes following global ischaemia, followed by perfusion with Hoe 140 alone, immediately prior to regional ischaemia.

Group 9; Subthreshold IPC + captopril + Hoe 140. Hearts were treated with 2 minutes global ischaemia as described for group 4. Hoe 140 1  $\mu\text{M}$  was co-perfused with captopril 200  $\mu\text{M}$  for 5 minutes prior to global ischaemia and 5 minutes following global ischaemia, followed by 5 minutes perfusion with Hoe 140 alone, immediately prior to regional ischaemia.

Group 10; Omapatrilat + Hoe 140. Omapatrilat 10  $\mu\text{M}$  and Hoe 140 1  $\mu\text{M}$  were co-perfused for 12 minutes followed by 5 minutes of Hoe 140 alone prior to infarct induction.

Group 11; Hoe 140 alone. Hoe 140 1  $\mu\text{M}$  was perfused for 17 minutes prior to regional ischaemia.

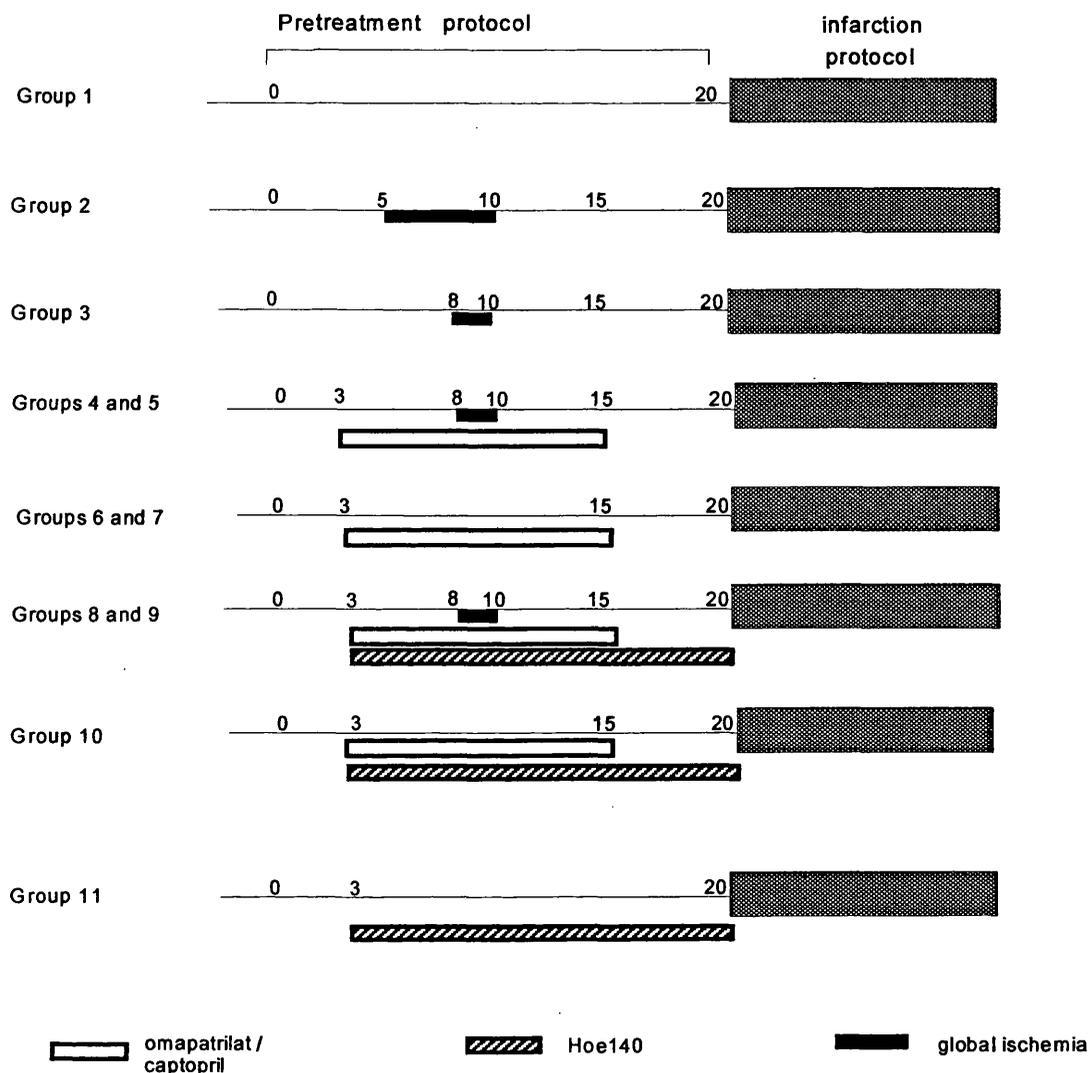


Figure 4.1. Experimental treatment protocols. In the 20 minute period preceding the infarction protocol, hearts were subjected to the pre-treatment protocols illustrated: control hearts (group 1) received no treatment; standard preconditioning (group 2); subthreshold preconditioning (group 3); subthreshold preconditioning with perfusion of omapatrilat (group 4); subthreshold preconditioning with perfusion of captopril (group 5); omapatrilat perfused alone (group 6); captopril perfused alone (group 7); subthreshold preconditioning with co-perfused omapatrilat and Hoe 140 (group 8); subthreshold preconditioning with co-perfused captopril and Hoe140 (group 9); co-perfused omapatrilat and Hoe 140 (group 10); Hoe 140 alone (group 11). Preconditioning and subthreshold preconditioning were induced with global (no flow, normothermic) ischaemia for 5 minutes and 2 minutes respectively.

#### **4.2.2. Determination of bradykinin concentration in coronary effluent**

Hearts were excised and Langendorff perfused. Following stabilisation for 10 minutes, hearts were assigned to one of the following three treatment groups.

Group 1; Control. Hearts underwent 2 minutes of global ischaemia and 5 minutes reperfusion.

Group 2; Omapatrilat. Hearts underwent 2 minutes of global ischaemia and 5 minutes reperfusion. Omapatrilat (10  $\mu\text{M}$ ) was perfused throughout the experimental procedure.

Group 3; Captopril. Hearts underwent 2 minutes of global ischaemia and 5 minutes reperfusion. Captopril (200  $\mu\text{M}$ ) was perfused throughout the experimental procedure.

Coronary effluent was collected at various time points and immediately frozen in liquid nitrogen. Bradykinin levels in the coronary effluent were determined using radio-immunoassay. Professor Gerd Heusch's department, in Germany performed the assay technique. Bradykinin was measured with a commercially available kit (Peninsula Laboratories Inc). This kit is designed to measure bradykinin levels specifically by a competitive radio-immunoassay. The anti-serum used in this assay was raised against synthetic bradykinin.

Briefly, all bradykinin assay samples were reconstituted with radio-immunoassay buffer. A standard curve was constructed and samples were incubated overnight at 4 °C. Rehydrated  $^{125}\text{I}$ -bradykinin (100  $\mu\text{l}$ ) was added to each sample tube and again incubated overnight at 4 °C. Rehydrated goat anti-rabbit IgG serum (100  $\mu\text{l}$ ) and normal rabbit serum were added to the tubes. Samples were vortexed and centrifuged at 3000 rpm for 20

minutes. Supernatant was removed and discarded and cpm in the pellet were measured from the standard curve.

#### **4.2.3. Materials**

Omapatrilat was a gift from Dr J Powell, Bristol Myers Squibb (Princeton, NJ, USA). Hoe 140 was a gift from Aventis Pharma (Frankfurt, Germany).

### **4.3. RESULTS**

#### **4.3.1. Exclusions**

A total of 111 animals were used in this study. Sixteen animals were used for radio-immunoassay measurement of bradykinin in the coronary effluent. 95 animals were used for the infarct studies. Six hearts were excluded; Three due to low heart rates during the stabilisation period, two due to persistent ventricular fibrillation during ischaemia and reperfusion and one due to physical trauma to the coronary artery during the induction of regional ischaemia. Therefore, the data for 89 successfully completed infarct experiments are reported. Final numbers are shown in table 4.1.

#### **4.3.2. Body weight and risk zone volume**

Rat body weights and wet hearts weights were comparable among groups (table 4.1). Myocardial ischaemic risk zone volume, which is a major determinant of infarct size, was similar in all the experimental groups at approximately 0.5 cm<sup>3</sup>, with no statistically significant differences.

Table 4.1. Body weight, wet heart weight and risk zone volume.

Group	n	Body weight (g)	Wet heart weight (g)	Risk zone volume (cm <sup>3</sup> )
Group 1	17	425±9	1.45±0.03	0.501±0.021
Group 2	11	407±8	1.41±0.063	0.490±0.035
Group 3	9	429±13	1.51±0.04	0.500±0.028
Group 4	7	421±8	1.43±0.082	0.508±0.073
Group 5	6	398±5	1.46±0.044	0.480±0.040
Group 6	6	416±16	1.43±0.08	0.500±0.049
Group 7	6	398±7	1.50±0.05	0.472±0.020
Group 8	7	427±16	1.47±0.07	0.590±0.052
Group 9	6	403±10	1.44±0.02	0.530±0.046
Group 10	6	400±3	1.43±0.05	0.540±0.027
Group 11	8	421±12	1.50±0.042	0.510±0.029

#### **4.3.3. Infarct-limiting effects of preconditioning, omapatrilat and captopril**

Figures 4.2 - 4.4 summarise infarct size data, normalised as a percentage of the ischaemic risk zone. Control (group 1) infarct size was  $53.4 \pm 2.0\%$ . A standard IPC protocol (5 minutes global ischaemia and 10 minutes reperfusion, group 2) caused a prominent reduction in infarct size to  $21.5 \pm 3.5\%$ , ( $P < 0.01$  versus control). This protective effect of IPC is consistent with previously published reports in this model of ischaemia-reperfusion. Subthreshold IPC (2 minutes global ischaemia and 10 minutes reperfusion, group 3) caused no protective effect ( $48.4 \pm 3.8\%$ ,  $P$  not significant versus control). However, when omapatrilat  $10 \mu\text{M}$  was perfused during the subthreshold IPC stimulus (group 4), a reduction in infarct size was observed, comparable to that seen with the full IPC stimulus ( $19 \pm 2.5\%$ ,  $P < 0.01$  versus control and subthreshold IPC group). In the absence of a subthreshold IPC stimulus, omapatrilat alone exhibited some infarct limiting potential (group 6). Infarct size was  $34.6 \pm 1.5\%$  ( $P < 0.01$  versus control).

Captopril exhibited a similar protective effect when administered during subthreshold IPC (group 5), with infarct size reduced to  $20.3 \pm 4.9\%$  ( $P < 0.01$  versus control and subthreshold IPC). However, in contrast to the finding with omapatrilat, captopril administered alone (group 7) did not evoke any infarct-sparing effect ( $48.5 \pm 3.1\%$ ,  $P$  not significant versus control).

#### **4.3.4. Effects of bradykinin B<sub>2</sub> receptor blockade**

To assess the contribution of bradykinin in the cardioprotection observed, the specific and selective bradykinin B<sub>2</sub> receptor antagonist, Hoe 140 was co-administered during the preconditioning and drug pre-treatments. Hoe 140 administered alone (group 11) did not influence infarct size in non-preconditioned hearts (infarct to risk ratio  $51.5 \pm 4.2\%$ ). However, the limitation of infarct size observed with omapatrilat plus subthreshold IPC, and

omapatrilat alone, was abrogated in the presence of Hoe 140 (groups 8,9,10), suggesting that bradykinin B<sub>2</sub> receptor activation is an obligatory mechanism for the protective effect of omapatrilat alone or in combination with subthreshold IPC. The protective effect of captopril in combination with subthreshold IPC was also abolished in the presence of Hoe 140, pointing to bradykinin B<sub>2</sub> receptor activation.

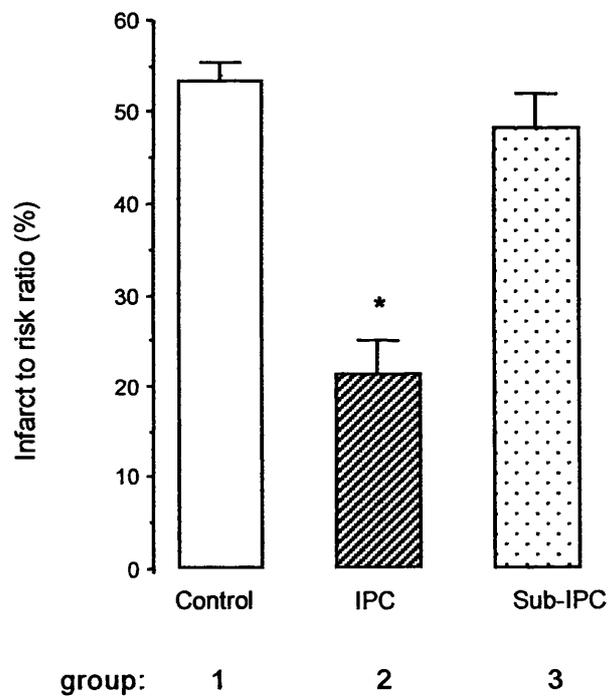


Figure 4.2. Infarct to risk ratio (%). Mean  $\pm$  s.e.m. \* P < 0.01 versus control (group 1) (one way ANOVA). IPC with one cycle of 5 minutes ischaemia/10 minutes reperfusion significantly attenuated infarct size. Subthreshold IPC with 2 minutes of ischaemia/10 minutes reperfusion had no significant protective effect.

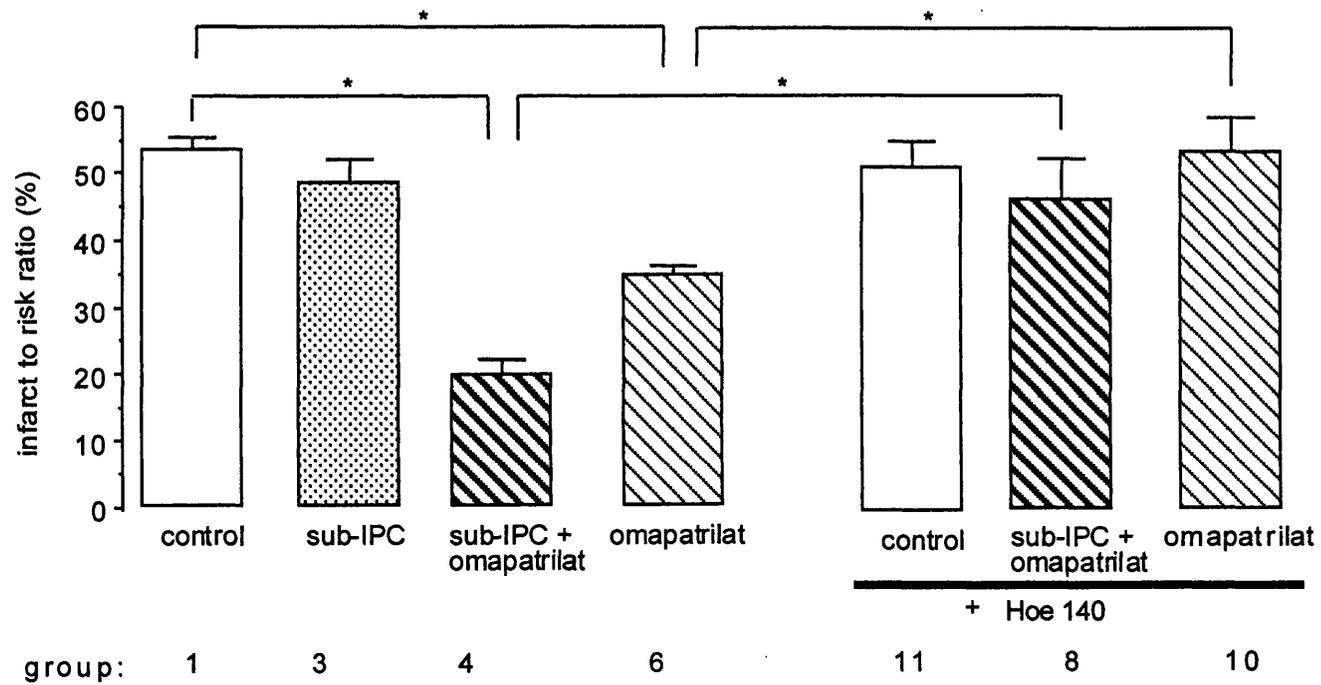


Figure 4.3. Infarct to risk ratio (%). Mean  $\pm$  s.e.m. \*  $P < 0.01$  (one way ANOVA). The combination of omapatrilat and subthreshold IPC produced a significant reduction in infarct size. Omapatrilat given alone, also produced a cardioprotective effect. Hoe 140 abolished both of these protective effects.

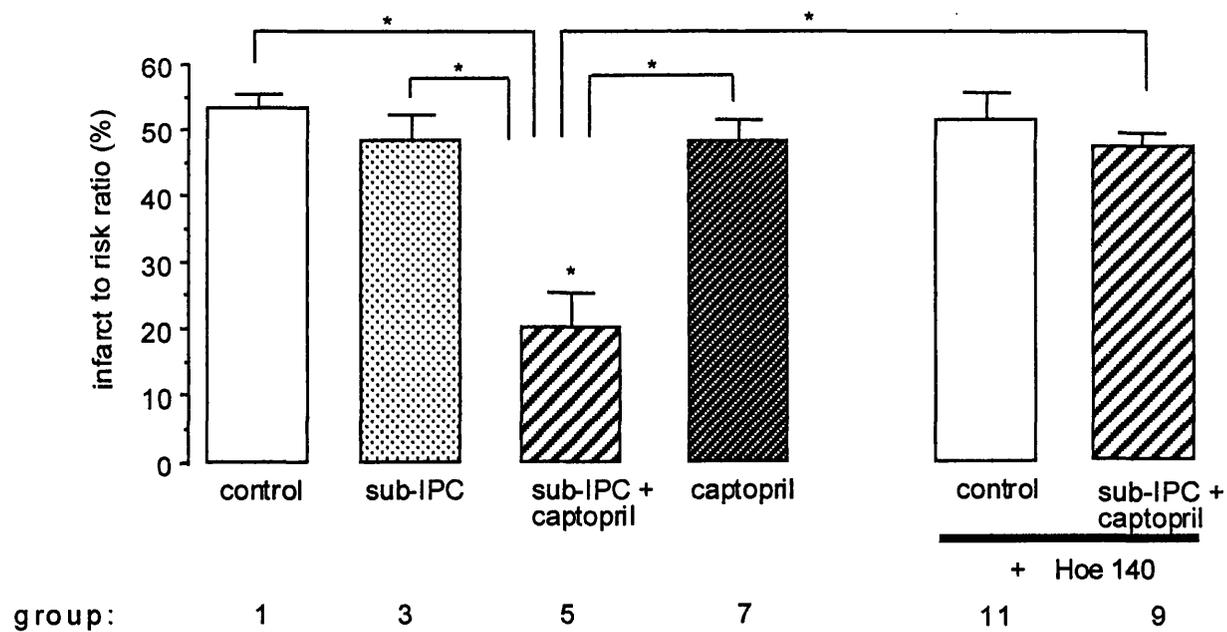


Figure 4.4. Infarct to risk ratio (%). Mean  $\pm$  s.e.m. \*  $P < 0.01$  (one way ANOVA). The combination of captopril and subthreshold IPC produced a significant reduction in infarct size, an effect abrogated in the presence of Hoe 140.

#### **4.3.5. Bradykinin concentration in coronary effluent**

There were no significant differences in bradykinin concentration in the coronary effluent at baseline among the groups. At reperfusion, there was a significant release of bradykinin. When captopril was present in the perfusion medium, a further increase in bradykinin concentration was observed. However, when omapatrilat was present in the perfusion medium, an increase in bradykinin concentration was not observed. In fact, omapatrilat did not influence bradykinin concentration during the entire experimental protocol (figure 4.5).

#### **4.3.6. Coronary flow and contractility data**

CFR and RPP are presented in tables 4.2 and 4.3. Immediately after coronary artery occlusion, flow rate decreased by around 40-50% of the pre-ischaemic value. Upon reperfusion, flow rate increased immediately and then gradually declined throughout the remaining reperfusion period, reflecting 'run down' in the preparation. None of the experimental treatments influenced CFR measurements. RPP declined markedly in all groups following coronary artery occlusion. There was a tendency towards recovery of RPP during early reperfusion but gradual decline in contractile function, which was similar in all groups during the reperfusion period. There were no statistically significant differences among the groups throughout the experimental procedure. The long reperfusion protocol required for infarct determination entails considerable functional 'run-down' which precludes interpretation of the contractile data beyond early reperfusion.

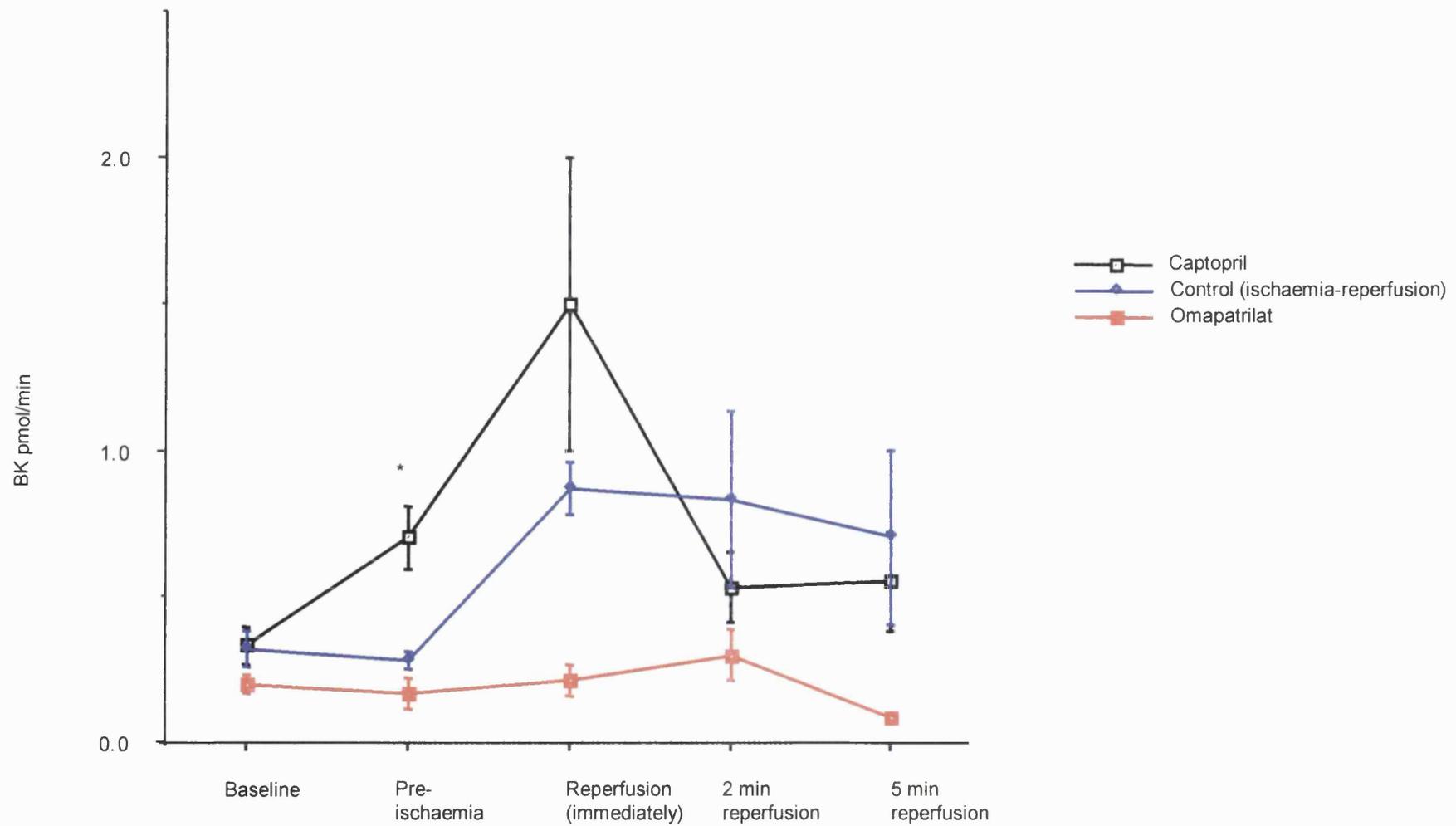


Figure 4.5. Bradykinin concentration in coronary effluent. Bradykinin release was augmented following ischaemia. Captopril further enhanced bradykinin release following ischaemia. However, surprisingly, omapatrilat had no effects upon the concentration of bradykinin in the coronary effluent throughout the experimental procedure. \* =  $P < 0.05$  versus control group (one way ANOVA).  $n = 5/6$  /group (BK - bradykinin).

Table 4.2. Coronary Flow Rate (ml/min)

Group	basal	Pre- ischaemia	ischaemia		reperfusion		
			5 min	30 min	5 min	60 min	120 min
Group 1	16.5±0.4	16.4±0.4	9.6±0.4	9.6±0.4	14.3±0.4	11.1±0.4	9.2±0.5
Group 2	17.0±0.7	16.0±0.6	8.7±0.5	9.7±0.9	14.8±0.7	12.3±0.7	10.5±0.8
Group 3	16.4±0.8	16.9±0.7	9.7±0.6	10.2±0.5	15.8±0.5	12.7±0.8	10.9±1.1
Group 4	15.0±0.7	14.5±1.0	8.0±0.6	8.7±0.7	13.6±1.0	11.2±0.9	9.3±0.4
Group 5	16.8±0.7	15.7±0.5	8.3±0.6	9.2±0.6	15.0±0.9	11.8±1.1	9.3±1.1
Group 6	17.2±0.5	16.5±0.7	9.1±0.8	9.3±1.1	14.7±1.2	12.3±1.2	11.0±1.3
Group 7	17.0±1.3	14.8±1.1	9.0±0.8	9.3±1.1	13.8±1.0	10.5±0.7	8.8±2.0
Group 8	16.9±1.2	16.8±1.6	8.4±1.0	9.6±0.9	14.1±1.0	11.6±1.0	9.3±0.7
Group 9	16.0±0.8	15.0±1.2	7.7±0.3	9.7±1.5	13.2±1.4	11.0±1.6	8.3±0.7
Group 10	16.5±1.1	15.0±1.1	10.0±1.4	9.5±1.6	14.7±1.6	11.0±1.1	8.7±0.9
Group 11	16.5±1.0	15.3±0.9	9.8±1.0	10.7±1.1	15.0±1.0	11.1±0.6	9.1±0.6

Table 4.3. Rate Pressure Product (mmHg/min x 10<sup>3</sup>)

Group	basal	Pre- ischemia	ischaemia		reperfusion		
			5 min	30 min	5 min	60 min	120 min
Group 1	41.5±2.2	39.7±2.1	17.5±1.8	23.2±1.6	26.5±1.3	21.3±1.2	17.6±1.6
Group 2	41.1±4.3	38.6±3.0	14.5±1.8	21.1±2.0	25.1±3.0	22.0±2.5	19.6±2.3
Group 3	40.3±3.8	38.6±4.1	16.0±1.8	22.1±1.2	29.0±1.4	21.3±2.0	20.1±3.1
Group 4	40.0±4.3	42.6±8.7	13.0±1.1	18.0±0.9	21.8±3.0	18.4±2.4	17.1±2.7
Group 5	46.9±4.6	36.6±4.2	18.7±2.5	21.1±4.0	27.0±3.3	24.5±1.9	20.7±2.5
Group 6	38.3±7.1	36.0±6.0	14.3±1.9	17.0±1.6	25.0±5.0	17.6±2.8	17.0±3.6
Group 7	44.3±4.0	34.1±4.5	19.6±3.4	25.7±2.3	23.2±3.1	22.2±3.1	16.0±2.6
Group 8	38.6±4.6	43.1±3.0	14.4±2.5	21.0±4.2	27.6±4.3	19.4±3.7	15.5±3.6
Group 9	45.2±5.5	40.4±3.2	19.0±3.9	22.5±7.0	23.8±4.5	21.3±2.8	19.6±3.8
Group 10	42.0±7.2	30.0±3.5	18.2±1.6	23.1±3.4	20.3±4.6	17.4±3.2	15.6±1.8
Group 11	41.2±3.8	33.1±3.4	18.8±3.8	23.0±7.0	23.8±4.5	21.3±2.8	19.6±3.8

## **4.4 DISCUSSION**

The present study has demonstrated that omapatrilat reduced the threshold for IPC of the myocardium. A subthreshold preconditioning stimulus that was not sufficient to elicit a cardioprotective response was used. However, in conjunction with omapatrilat significant protection against infarction was observed, similar to that observed with a full preconditioning stimulus. This cardioprotective effect of the combination of omapatrilat and subthreshold preconditioning was abrogated with the specific bradykinin B<sub>2</sub> receptor antagonist, Hoe 140, showing that the protection was mediated by bradykinin. This effect of omapatrilat was comparable to that observed with the ACE inhibitor, captopril. However, in contrast to captopril, omapatrilat administered prior to coronary artery occlusion without an IPC stimulus was found to be protective *per se* and resulted in modest infarct size limitation. The direct action of the agent was also abolished by Hoe 140.

### **4.4.1. ACE inhibitors and subthreshold preconditioning**

The ability of omapatrilat to reduce myocardial infarct size when combined with subthreshold preconditioning ischaemia is consistent with previous studies using ACE inhibitors. Miura's group demonstrated that captopril potentiated IPC through a bradykinin dependent mechanism in rabbit heart (Miki *et al*, 1996). Similarly, in our laboratory, Morris and Yellon showed that both lisinopril and captopril augmented a subthreshold preconditioning stimulus in isolated human atrial trabeculae muscle (Morris & Yellon, 1997). The cardioprotective effects of an ACE inhibitor and subthreshold preconditioning were abolished by Hoe 140. Nozawa *et al* demonstrated that chronic treatment with the ACE inhibitor, temocapril potentiated preconditioning (Nozawa *et al*, 1999). The investigators administered temocapril or placebo to rabbits orally for two weeks, following which hearts were subjected to ischaemia-reperfusion. Temocapril in conjunction with a subthreshold preconditioning stimulus (2 minutes global ischaemia / 5 minutes of reperfusion) attenuated

infarct size, whereas subthreshold preconditioning alone had no effect (Nozawa *et al*, 1999). Even though the authors did not provide direct evidence for the involvement of the bradykinin B<sub>2</sub> receptor in the protection, a role for this receptor in mediating protection is most likely.

In the present study it was found that both captopril and omapatrilat were able to potentiate IPC, an effect abrogated with Hoe 140, implying that bradykinin B<sub>2</sub> receptor is implicated in the protection. Bradykinin levels were determined using radio-immunoassay. Even though captopril enhanced bradykinin levels at reperfusion, the addition of omapatrilat to the perfusion fluid had no effects on bradykinin concentration. These results are somewhat puzzling; similar results to that observed with captopril should theoretically have also been obtained with omapatrilat. It is not known why omapatrilat did not augment bradykinin levels at reperfusion, however, the following can be suggested; the omapatrilat itself may have somehow interfered with the radio-immunoassay technique used to quantify bradykinin levels. There is a strong possibility that this may be the case, as the increase in bradykinin concentration seen even at reperfusion in the control hearts was not observed in the omapatrilat treated hearts. Alternatively, omapatrilat may inhibit only tissue ACE as opposed to plasma ACE, implying that bradykinin concentration should have been determined in whole heart samples as opposed to the coronary effluent. However, plasma is absent in the isolated Langendorff perfused heart, again ruling out this possibility. Additionally, Dumoulin *et al* demonstrated that omapatrilat augmented exogenous bradykinin levels in rat coronary effluent samples (Dumoulin *et al*, 2001). Unfortunately, due to lack of time we could not determine reasons as to why omapatrilat did not augment bradykinin levels. Clearly, future experiments are required to investigate this issue in greater depth.

#### **4.4.2. Direct cardioprotective effects of omapatrilat**

In addition to its ability to potentiate a preconditioning stimulus, omapatrilat was found to directly limit infarct size (ie, in the absence of preconditioning ischaemia). This effect was dependent on B<sub>2</sub> receptor activation since it was abolished by Hoe 140. To reiterate, even though omapatrilat was washed out prior to the ischaemic insult, it exerted a modest limitation of infarct size. A washout period was used to ensure that omapatrilat (and therefore, bradykinin) was not present in myocardium during the ischaemic insult. As triggers of IPC have to be washed out prior to the ischaemic event, omapatrilat which theoretically augments bradykinin levels was washed out prior to infarction. Indeed, bradykinin can, not only trigger IPC but can additionally exert direct cardioprotective effects, implying that even if omapatrilat was not washed out prior to infarction, it should in theory have limited infarct size as well. This is the first demonstration of omapatrilat's ability to limit infarct size although previous investigations have found that combined ACE/NEP inhibition to be cardioprotective using other end points. For example, Rastegar et al have recently documented the direct cardioprotective properties of the combined ACE/NEP inhibitor, Z13752A. In a canine model of coronary artery occlusion, they showed that pre-ischaemic treatment with Z13752A reduced the occurrence of ventricular arrhythmias in ischaemia and reperfusion. This beneficial effect was also abrogated with Hoe 140, implying a role for B<sub>2</sub> receptor activation (Rastegar *et al*, 2000a). Schriefer et al reported that direct administration of a NEP inhibitor just prior to reperfusion led to a prominent reduction in infarct size in rabbit heart. They also observed this using an ACE inhibitor alone and a combination of an ACE inhibitor and NEP inhibitor. Again, the protection was abolished with Hoe 140, indicating the involvement of B<sub>2</sub> receptor activation (Schriefer *et al*, 1996).

In contrast to protection observed with omapatrilat, when the conventional ACE inhibitor captopril was administered alone, no limitation of infarct size was seen. In other words, when captopril was administered alone (ie, in the absence of preconditioning ischaemia), and washed out prior to infarction, it did not reduce infarct size. It is not known whether protection would have been observed if captopril was not washed out prior to infarction. If captopril was not washed out, bradykinin would presumably accumulate in the myocardium during ischaemia, which may well exert beneficial effects in reperfusion. The lack of protection observed with captopril is consistent with previous reports. For example, Miki et al showed that captopril alone did not limit infarct size in rabbit heart (Miki et al, 1996). Similarly, Nozawa et al demonstrated that temocapril alone did not reduce infarct size in rabbit heart (Nozawa et al, 1999). In contrast, Ertl et al were the first to report that captopril could limit infarct size in the canine heart (Ertl et al, 1982). Weidenbach et al have demonstrated recently that ramiprilat limited infarct size in the porcine heart (Weidenbach et al, 2000). Similarly, Shimada and Avkiran have demonstrated that ramiprilat administered prior to ischaemia reduced the incidence of sustained ventricular fibrillation in isolated rat heart (Shimada & Avkiran, 1996). These investigators also showed that Hoe 140 abolished these effects, implying a role for the B<sub>2</sub> receptor in the observed cardioprotection. Matoba et al reported that the ACE inhibitor, cilazaprilat protected directly against hypoxia / reoxygenation injury in cultured rat myocytes (Matoba et al, 1999). They also demonstrated that cilazaprilat enhanced bradykinin levels in the culture media of the myocytes. The reasons for such discordancies in the experimental literature relating to ACE inhibitors and infarction are not clearly defined. It has been proposed that ACE inhibitors that possess a SH moiety, such as captopril, act as scavengers of ROS and this might be the basis of any cardioprotective action (Birincioglu et al, 1997; Theres et al, 2000). However, this seems to be an unsatisfactory explanation. Birincioglu et al reported both lisinopril and captopril limited the occurrence of arrhythmias in an *in vivo* rat model of coronary artery occlusion but

lisinopril is devoid of a SH group (Birincioglu *et al*, 1997). The discrepancies in the experimental literature with conventional ACE inhibitors are unresolved (Przyklenk & Kloner, 1993).

#### **4.4.3. Role of natriuretic peptides**

As omapatrilat inhibits both ACE and NEP, it may lead to the accumulation of higher levels of bradykinin, compared with captopril due to inhibition of both ACE and NEP. Indeed, very recently, Dumoulin *et al* demonstrated that short term and long term omapatrilat treatment inhibited bradykinin degradation to a greater extent than ACE or NEP inhibitors alone in the rat coronary bed (Dumoulin *et al*, 2001). They showed that bradykinin degradation rate (at a coronary perfusion of 1 ml/min) was  $4.56 \pm 0.39$  1/min/g without inhibitors during a single passage through the coronary bed. This was significantly reduced to  $2.57 \pm 0.19$  1/min/g with enalaprilat, to  $2.97 \pm 0.38$  1/min/g with a NEP inhibitor (retrothiorphan), to  $1.82 \pm 0.17$  1/min/g with both enalaprilat and retrothiorphan, and to  $1.14 \pm 0.35$  1/min/g with omapatrilat. Additionally, they demonstrated that 14 day treatment of rats with either ACE inhibitors (enalapril, quinapril and ramipril), a NEP inhibitor (candoxatril) or omapatrilat had a similar effect on bradykinin degradation. In the untreated rats, bradykinin degradation (at a coronary perfusion of 1 ml/min) was  $4.35 \pm 0.41$  1/min/g. This value was reduced by 30% for the NEP inhibitor, by 50% for the ACE inhibitors and by 75% for omapatrilat. These data clearly demonstrate that omapatrilat is able to inhibit bradykinin degradation to a greater degree than either ACE or NEP inhibitors alone or in combination (Dumoulin *et al*, 2001).

The release of ANP and BNP has also been shown to occur during ischaemia (Uusimaa *et al*, 1992; Arad *et al*, 1994; Lochner *et al*, 1992; Talwar *et al*, 2000). Arad *et al* demonstrated elevated levels of ANP in coronary effluent following global ischaemia in isolated rat heart (Arad *et al*, 1994). Indeed, the ability of ANP to elicit myocardial protection has been

documented. For example, Takata et al demonstrated that the administration of ANP was cardioprotective in dog myocardium, preventing reperfusion arrhythmias and preserving high energy phosphates in the ischaemic myocardium (Takata *et al*, 1996). More recently, Rastegar et al also reported the cardioprotective properties of ANP using arrhythmia prevalence as an experimental end point in dog (Rastegar *et al*, 2000b). Furthermore, Takagi et al, demonstrated that carperitide (a recombinant form of  $\alpha$ -hANP) attenuated infarct size in dog myocardium (Takagi *et al*, 2000). The ability of BNP and CNP to induce cardioprotection must not be excluded, these natriuretic peptides also being substrates for NEP. However, whether they induce cardioprotection has not been investigated. Since natriuretic peptides are primary substrates for NEP, it is possible that these peptides may co-operate with bradykinin in eliciting the cardioprotective actions of omapatrilat. Yang et al reported that the NEP inhibitor, CGS24592 directly protected against ischaemia-reperfusion injury in an *in vivo* rat model, an effect also reversed with Hoe 140 (Yang *et al*, 1997a). However, cardioprotection was not abolished using a natriuretic peptide receptor antagonist (HS-142-1), suggesting that natriuretic peptides are unlikely to account for the cardioprotection induced by NEP inhibitors. The issue of natriuretic peptide contribution in attenuating ischaemia-reperfusion injury remains to be more fully investigated.

Combined inhibition of circulating proteases, termed "vasopeptidases" which modify the activity of vasoactive peptides, is a novel concept aimed at the treatment of cardiovascular anomalies like hypertension and chronic heart failure. However, the present study suggests that this pharmacological approach may have cardioprotective benefits that go beyond any cardiac and systemic haemodynamic actions. Inhibiting ACE and NEP not only leads to a reduction in the degradation of bradykinin, but also augments circulating and tissue levels of natriuretic peptides. Thus, there are theoretical reasons for believing that combined inhibition of ACE and NEP may be particularly beneficial in clinical settings where

concurrent or intermittent myocardial ischaemia is a feature. Agents such as omapatrilat might therefore offer particular advantage to patients with coronary artery disease who are at risk of experiencing myocardial infarction.

#### **4.5. CONCLUSION**

In summary, this study has demonstrated that omapatrilat potentiates IPC through a kinin dependent mechanism. Additionally, omapatrilat, unlike captopril was found to be directly protective against ischaemia-reperfusion injury and conferred some direct protection against myocardial infarction which was also bradykinin-mediated. Further work is required to establish the benefit of omapatrilat in myocardial ischaemia in experimental models *in vivo* and in clinical investigations.

## CHAPTER FIVE

### Ischaemic Preconditioning and ACE inhibition in the chronically hypertensive myocardium

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## **5.1. INTRODUCTION**

### **5.1.1. The Spontaneously hypertensive rat**

Okamoto and Aoki in 1963 developed a strain of genetically hypertensive rats by inbreeding Wistar rats with the greatest blood pressure (Okamoto & Aoki, 1963). These investigators selected male Wistar rats with a systolic blood pressure in the region of 150 to 175 mmHg and female Wistar rats that were slightly hypertensive (ie, systolic blood pressure in the region of 130 to 140 mmHg) which were mated to produce F<sub>1</sub> rats. From the F<sub>1</sub> rats, males and females with hypertension (blood pressure > 150 mmHg) persisting for more than one month were mated to obtain F<sub>2</sub> rats. This procedure was then repeated to obtain F<sub>3</sub>, F<sub>4</sub>, F<sub>5</sub>, and F<sub>6</sub> rats. The incidence of severe hypertension increased with each generation. In the F<sub>3</sub> to F<sub>6</sub> rats, 100% of the animals developed spontaneous hypertension. The authors named these rats "spontaneously hypertensive rats" (Okamoto & Aoki, 1963).

In the SHR, systolic blood pressure increases at around five-six weeks of age and reaches a maximum of around 200-220 mmHg at 12-16 weeks of age. The SHR is the most popular model used to study hypertension today (Pinto *et al*, 1998). The high blood pressures in the SHR are associated with significant cardiac hypertrophy. Hence, the SHR provides a suitable model for the study of myocardial hypertrophy linked with chronic hypertension. The SHR provides a stable, consistent model of hypertension, and permits the measurement of various cardiac, biochemical and haemodynamic parameters (Doggrell & Brown, 1998). Indeed, the SHR is believed to resemble human essential hypertension more closely than any other animal model of hypertension. The reported life span of the SHR is thought to be in the range of 10-21 months, with 60% developing heart failure at around 18 months of age (Pinto *et al*, 1998). Generally, a 30% increase in cardiac mass is observed. Pfeffer *et al* demonstrated that cardiac output and stroke volume was only markedly reduced in the SHR at 90 weeks of age (not at 13, 15 and 52 weeks) (Pfeffer *et al*, 1979).

Augmented apoptosis has also been demonstrated in the myocardium of SHR. Liu *et al* found a significant increase in apoptosis in SHR at four weeks of age, which plateaued at 16 weeks, but remained high up to 64 weeks of age (Liu *et al*, 2000). Ratios of Bcl-2/Bax were reduced and fibroblasts were also significantly increased in the heart of SHR. Treatment with ramipril reduced apoptosis and fibroblasts and increased the ratio of Bcl-2/Bax.

Endothelial dependent relaxations are impaired in the SHR (at approximately 13-15 weeks of age) (Arribas *et al*, 1997; Pinto *et al*, 1998). Renal damage (proteinuria and reduced creatinine clearance) has been observed in older SHRs, although studies have not described frank renal failure (Feld *et al*, 1977; Komatsu *et al*, 1995). Excess deposition of fibrillar collagen within the cardiac interstitium has been reported in the SHR (Conrad *et al*, 1995; Brilla *et al*, 1996). Structural remodelling of the myocardial collagen matrix is thought to be responsible for abnormalities in myocardial stiffness.

Two further developed sub-strains of SHRs exist; The SHR stroke prone (SHRSP), which develops even higher blood pressures and usually dies from stroke and the SHR which develops heart failure (SHHF) before 18 months of age (Hasenfuss, 1998; Pinto *et al*, 1998).

### **5.1.2. Mechanisms of hypertension in the SHR**

Although the SHR model of hypertension is extensively used, mechanisms leading to hypertension remain elusive. Evidence implicates a role for the RAS, (Ferrone & Antonaccio, 1979; Harrap, 1992; Zelezna *et al*, 1999; Chen *et al*, 1998; Hefti *et al*, 1986; Harrap *et al*, 1990) nitric oxide / free radicals (Grunfield *et al*, 1995; Chou *et al*, 1998; Shirasaki *et al*, 1986; Suzuki *et al*, 1998; Ito *et al*, 1995; Dominiczak & Bohr, 1995), PGI<sub>2</sub> (Numaguchi *et al*, 1999) and the sympathetic nervous system (Pacak *et al*, 1993). A role for

the Y chromosome has also been proposed (Ely *et al*, 1997, 2000; Hilbert *et al*, 1991; Jacob *et al*, 1991). Interestingly, Bradykinin may also be implicated in the development of hypertension in the SHR. Favaro *et al* reported that kallikrein content in kidneys of SHRs was significantly lower than the normotensive WKY rats (Favaro *et al*, 1975). Additionally, adenoviral kallikrein gene delivery has demonstrated to exert blood pressure lowering effects in the SHR (Jin *et al*, 1997; Wang *et al*, 1995). These observations suggest that bradykinin levels may be attenuated in the SHR, which could contribute to the development of hypertension.

### **5.1.3. Part A IPC in the chronically hypertensive myocardium**

Despite extensive research in the field of IPC, most studies have focused on the "healthy", non-diseased myocardium. A very limited number of studies have examined IPC in the diseased myocardium, which would ultimately provide greatest clinical benefit. In particular, few studies have investigated the effects long standing hypertensive cardiac hypertrophy has on IPC occurrence. As cardiac hypertrophy is associated with a vast array of disturbances (eg, impaired endothelium dependent relaxations, interstitial and perivascular fibrosis of intramyocardial coronary arteries, increased cardiomyocyte apoptosis, increased susceptibility to various end points of ischaemia), it is important to examine whether IPC still occurs in this pathology. In addition to this, even though it was observed that IPC was protective in a mild degree of hypertrophy (chapter three), bradykinin induced cardioprotection was attenuated. Bradykinin is an important trigger of IPC and if bradykinin induced protection is affected even in mild hypertrophy, IPC may well be affected in a model of chronic hypertrophy. Furthermore, studies have implied that bradykinin levels maybe attenuated in hypertension (Favaro *et al*, 1975; Ader *et al*, 1987; Nakagawa & Nasjletti, 1988; Sharma *et al*, 1996; Seino *et al*, 1990). Accordingly, the primary aim of this study was to determine if the IPC response is still preserved in the SHR at various stages of

development. Young SHR (ySHR; 3-4 months old), middle aged SHR (mSHR; 7-8 months old) and ageing SHR (aSHR; 11-13 months) were investigated. Comparative studies were also performed with age matched normotensive wistar kyoto rats (WKY).

## 5.2. METHODS

### 5.2.1. Treatment protocols

Prior to the excision of hearts for Langendorff perfusion, some animals were randomised for blood pressure measurement (see section 2.2). Following stabilisation, hearts were assigned to one of the following groups, illustrated in figure 5.1.

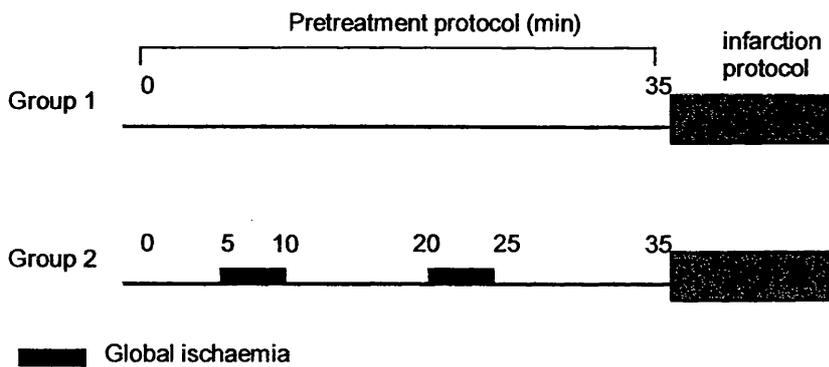


Figure 5.1. Experimental treatment protocols. Hearts were randomised to either group 1 (control) or group 2 (IPC)

Group 1; Control. Hearts underwent 35 minutes regional ischaemia and 120 minutes reperfusion.

Group 2; IPC. Hearts were subjected to 2 cycles of 5 minutes global ischaemia interspersed by 10 minutes reperfusion.

## **5.3. RESULTS**

### **5.3.1. Exclusions**

A total of 94 rats were used in this study. Three hearts were excluded; two due to inadequate delineation of the risk zone; one due to severe bradycardia throughout the course of the experiment. Five SHRs died spontaneously - four at approximately ten months of age and one at five months of age. Therefore, data for 86 successfully completed infarct experiments are reported. Final numbers in each group are shown in table 5.2.

### **5.3.2. Characterisation of SHRs**

#### **5.3.2.1. *Blood pressure data***

Systolic and diastolic blood pressures are depicted in table 5.1. At every age, SHRs displayed significantly higher systolic / diastolic blood pressures compared to WKY rats. There was however, no significant increase of systolic / diastolic blood pressure across the age range studies in either SHR or WKY rats.

Table 5.1. Summary of systolic and diastolic blood pressure

Group	n	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)
<b>Young 3-4 months</b>			
y SHR Cont	6	211±20 *	168±9 *
y SHR IPC	5	198±14 *	154±8 *
y WKY Cont	6	112±13	96±10
y WKY IPC	6	119±9	96±12
<b>Middle aged 7-8 months</b>			
m SHR Cont	4	209±10 *	183±7 *
m SHR IPC	5	193±8 *	176±9 *
m WKY Cont	5	120±6	105±3
m WKY IPC	4	113±6	103±6
<b>Ageing 11-13 months</b>			
a SHR Cont	5	224±22 *	193±19 *
a SHR IPC	3	190±18 *	170±5 *
a WKY Cont	3	120±11	100±16
a WKY IPC	5	136±8	115±5

Cont; control IPC; ischaemic preconditioning Cap; captopril

\* = P<0.05 versus corresponding age matched WKY group (one way ANOVA)

### 5.3.2.2 *Body weight, risk zone volume and hypertrophy index*

Rat body weights showed a progressive increase with age in both SHRs and WKY rats (table 5.2). There were no significant differences in body weight between SHR and WKY groups at any given age.

Myocardial risk zone volume was significantly greater in SHR compared to WKY at all three ages, with a progressive increase with age (ie, as the size of the myocardium increased, so

did risk zone volume). However, when risk zone was normalised to the volume of the left ventricle, there were no significant differences between the animals at any given age (table 5.2.)

LV body weight ratios are depicted in table 5.2. At all ages, SHR exhibited significantly greater LV body weight ratios when compared to WKY rats. The hypertrophy index generally increased with age in the SHR. In the young SHR, the mean hypertrophy index was 17%. In the middle aged SHR, the mean hypertrophy index was 24% and finally, in the ageing (11-13 months old) SHRs, it was 29%. This value is consistent with previous reports, indicating that cardiac hypertrophy in the region of 30% is observed in the SHR (Conrad *et al*, 1991).

RVH increased with age in the SHR. In the young SHR, the RV was not significantly larger than in the age matched WKY rats. However, in the middle aged and ageing animals, the RV was significantly larger compared to the corresponding age matched WKY groups.

Table 5.2. Rat body weight, risk zone volume, risk zone volume normalised to LV volume, LV/body weight ratios, and RV/body weight ratios

	Group	n	rat body weight (g)	risk zone volume (cm <sup>3</sup> )	risk zone / LV volume(%)	LV/ body weight (mg/g)	RV/body weight (mg/g)
Young	y SHR Cont	7	290±5.3	0.52±0.03 *	52.8±2.0	4.4±0.94 *	0.41±0.20
	y SHR IPC	6	288±9.8	0.53±0.03 *	53.9±1.8	4.6±0.14 *	0.43±0.30
	y WKY Cont	6	256±8.2	0.36±0.02	53.1±2.5	3.9±0.14	0.40±0.20
	y WKY IPC	6	265±5.7	0.35±0.03	53.5±3.1	3.6±0.07	0.39±0.31
Middle Aged	m SHR Cont	8	379±9.7	0.78±0.04 *	54.8±1.2	5.1±0.12 *	0.43±0.23 *
	m SHR IPC	8	378±9.3	0.74±0.04 *	54.2±2.8	4.7±0.14 *	0.41±0.24 *
	m WKY Cont	8	388±1.0	0.51±0.04	54.1±1.6	3.7±0.09	0.34±0.22
	m WKY IPC	8	371±6.1	0.45±0.03	52.9±1.8	3.7±0.11	0.33±0.13
Ageing	a SHR Cont	8	434±16.7	1.01±0.05 *	55.0±1.7	5.0±0.14 *	0.50±0.60 *
	a SHR IPC	5	452±20.1	1.09±0.08 *	54.5±1.0	5.2±0.40 *	0.51±0.80 *
	a WKY Cont	7	434±18.2	0.67±0.06	53.5±2.0	3.5±0.08	0.32±0.02
	a WKY IPC	9	411±6.4	0.70±0.04	55.4±2.2	3.8±0.07	0.34±0.12

Cont; control IPC; Ischaemic preconditioning Cap; captopril . \* = P <0.05 versus corresponding aged matched WKY group (One way ANOVA)

### 5.3.2.3. Plasma noradrenaline concentration

At all three ages, plasma noradrenaline concentration was increased in SHRs. In the young SHR plasma noradrenaline concentration was somewhat higher compared to the young WKY rat. However, the difference was not statistically significant. Similarly, in the middle aged animals, although the SHR had a higher plasma noradrenaline concentration, this was not statistically significant. However, in the ageing SHR, plasma noradrenaline concentration was significantly greater compared to the ageing WKY rat. In the WKY rat, there was no significant difference in plasma noradrenaline concentration with age. In the SHR, although there was a decline in levels from young to middle aged animals, there was a steep increase in the ageing SHRs. As previously mentioned, sympathetic nervous system activity is increased during hypertension, both clinical and experimental studies report higher levels of catecholamines in hypertension (Akers *et al*, 2000; de Champlain *et al*, 1976).

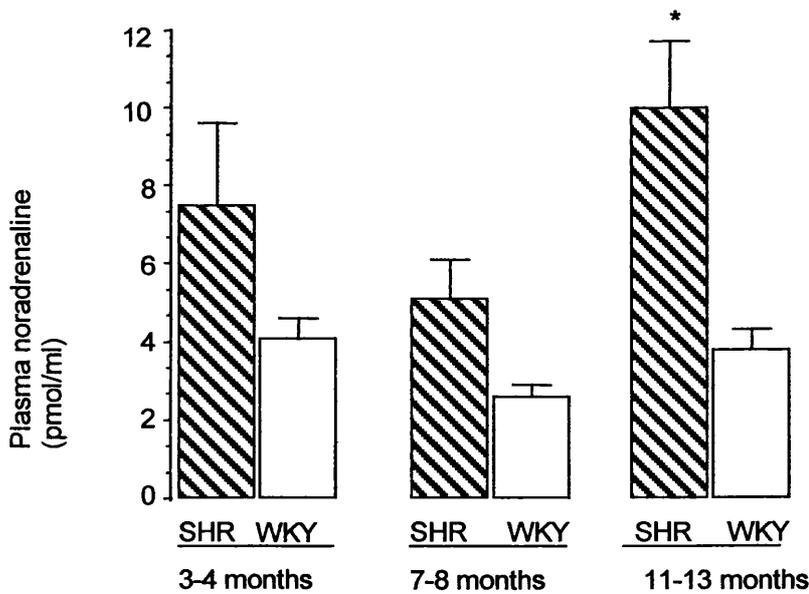


Figure 5.2. Plasma noradrenaline levels. \* =  $P < 0.05$  versus ageing WKY group. (One way ANOVA,  $n = 4-8$ /group).

### 5.3.3. Infarct limiting effects of IPC

Figure 5.3. summarises infarct size data, expressed as a percentage of the risk zone. In the young SHR, control infarct size was  $56.0 \pm 5.5\%$ . IPC caused a significant reduction in infarct size to  $23.6 \pm 4.3\%$  ( $P < 0.01$ ). Similarly, in the young WKY rats, control infarct size of  $56.3 \pm 5.3\%$  was reduced to  $23.0 \pm 3.3\%$  following IPC ( $P < 0.01$ ). In the middle aged rats, a similar trend was observed; control infarct size in SHR was  $54.5 \pm 3.3\%$ , IPC reduced this to  $26.6 \pm 3.7\%$  ( $P < 0.01$ ). WKY control infarct size was  $55.0 \pm 4.9\%$  and  $20.9 \pm 4.4\%$  following IPC ( $P < 0.01$ ). In the ageing rats, however, a different pattern of results was obtained; in the SHR, control infarct size was  $58.7 \pm 4.6\%$  and IPC did not produce any cardioprotective effect ( $54.7 \pm 5.1\%$ ,  $P > 0.05$ ). Interestingly, the ageing WKY rats displayed similar results; control infarct size was  $52.0 \pm 3.1\%$ , following IPC, infarct size was  $43.4 \pm 5.7\%$ ,  $P > 0.05$ . Thus, although IPC afforded protection in young and middle aged SHR / WKY rats, no protection was observed in either ageing SHR or WKY rats.

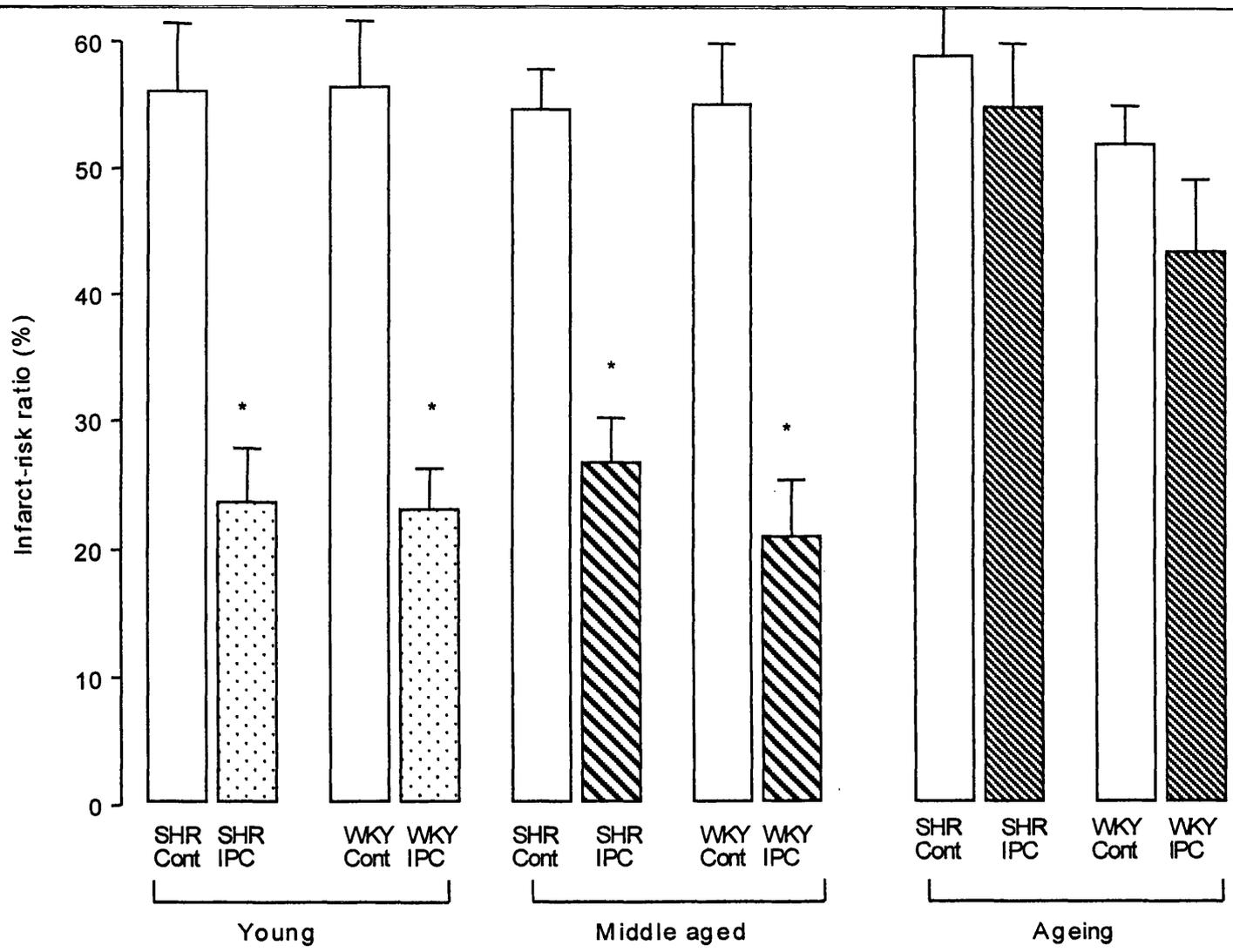


Figure 5.3. Infarct size data represented as infarct to risk ratio. IPC caused a significant reduction of infarct size in young and middle aged SHR/WKY rat hearts. IPC did not however cause a significant limitation of infarct size in either the ageing SHR or WKY rat hearts.  
 \* = P < 0.01 versus corresponding control groups. (One way ANOVA) (Cont - control; IPC - ischaemic preconditioning)

#### **5.3.4. Coronary flow and contractility data**

CFR and RPP are presented in table 5.3 and 5.4. Differences in CFR were observed between the various ages at baseline and throughout the experimental protocols. Both young SHR / WKY rats exhibited higher basal CFR when compared to middle aged and ageing animals. In other words there was a progressive age related decline in CFR. Nevertheless, there was a similar significant reduction in flow rate upon coronary artery occlusion and recovery immediately following reperfusion. However, younger hearts exhibited a better recovery of flow upon reperfusion than older animals. Additionally, variations in RPP were found at baseline and throughout the experiments. Generally, SHR showed greater RPP compared to WKY rats at baseline. RPP appeared to be greater in the middle aged SHRs. RPP also declined with age in the WKY rat. Nevertheless, RPP declined considerably following coronary artery occlusion in all groups, and exhibited the gradual "run down" pattern usually observed during reperfusion.

Table 5.3. Coronary Flow Rate (ml/min/g)

Group	Basal	Pre-Ischaemia	ischaemia		reperfusion			
			5 min	30 min	5 min	60 min	120 min	
Young	y SHR Cont	12.5±0.7 *	10.5±0.8 *	5.7±0.8 *	5.4±0.8 *	9.4±0.8 *	6.8±0.8 *	5.2±0.6
	y SHR IPC	11.1±0.5	8.8±0.8	4.7±0.7	4.3±0.6	8.0±0.8	5.8±0.6	4.2±4.5
	y WKY Cont	11.5±1.3	10.3±1.2	5.6±0.7	5.4±0.7	9.9±0.8 *	6.8±0.6	5.3±0.7
	y WKY IPC	12.8±0.8 *	10.4±0.8	5.6±0.6	5.6±0.6	9.8±0.7*	6.9±0.6	5.3±0.5
Middle Aged	m SHR Cont	9.70±0.6	8.5±0.6	4.4±0.3	4.3±0.4	7.2±0.5	5.3±0.5	4.2±0.5
	m SHR IPC	9.4±0.4	8.4±0.4	4.0±0.4	4.1±0.4	7.1±0.4	5.3±0.4	4.2±0.3
	m WKY Cont	9.50±0.3	8.9±0.3	5.2±0.3	5.5±0.2	9.0±0.5	6.6±0.4	5.3±0.3
	m WKY IPC	9.4±0.3	9.0±0.4	4.8±0.3	4.9±0.4	8.0±0.8	6.3±0.5	5.2±0.4
Ageing	a SHR Cont	9.1±0.2	8.1±0.3	3.9±0.2	3.7±0.4	6.3±0.3	4.5±0.3	4.0±0.3
	a SHR IPC	9.6±0.7	8.0±1.1	4.4±0.8	4.2±0.8	6.8±0.9	5.0±0.7	4.2±0.7
	a WKY Cont	9.5±0.9	8.6±1.0	5.3±0.7	4.8±0.6	7.9±0.8	6.7±0.8	5.3±0.9
	a WKY IPC	9.7±0.4	8.6±0.7	4.8±0.7	4.4±0.7	7.8±0.8	5.7±0.7	4.3±0.7

Cont - control; IPC - Ischaemic preconditioning; Cap – captopril

\* = P <0.05

Table 5.4. Rate Pressure Product (mmHg/min X 10<sup>3</sup>)

Group	Basal	Pre-Ischaemia	ischaemia		reperfusion			
			5 min	30 min	5 min	60 min	120 min	
Young	y SHR Cont	46.7±3.8 *	37.5±4.2	15.6±2.0	20.3±2.8	23.1±2.1	20.1±2.3	17.3±2.3
	y SHR IPC	49.7±3.1	35.9±3.6	16.8±1.5	18.3±1.3	20.0±2.2	18.3±1.4	15.0±1.6
	y WKY Cont	44.7±4.6 *	32.7±2.8	14.4±2.3	16.3±2.1	17.0±2.3	15.0±1.8	12.0±1.3
	y WKY IPC	46.8±5.2	34.8±3.7	17.0±2.3	20.0±1.9	22.2±4.2	20.0±2.2	16.0±3.1
Middle Aged	m SHR Cont	51.2±4.3 *	46.1±3.5 *	21.6±1.2	25.6±1.8	27.3±2.0	21.9±2.4	18.1±2.2
	m SHR IPC	50.8±2.3	40.4±4.4 *	18.5±2.3	20.0±2.5	27.0±3.8	22.4±3.2	17.5±3.7
	m WKY Cont	38.3±2.7	33.1±1.7	18.0±1.6	19.7±1.9	23.0±2.3	20.3±1.4	17.4±1.3
	m WKY IPC	35.5±1.1	32.3±2.7	19.0±2.0	20.0±1.9	24.4±2.5 *	20.7±1.6	14.5±1.5
Ageing	a SHR Cont	33.2±3.6	33.6±4.6	17.3±3.0	22.1±2.8	20.1±4.0	15.4±3.0	11.0±1.5
	a SHR IPC	44.3±6.0	29.0±3.4	16.9±2.4	18.0±0.8	20.0±2.3	16.0±1.1	14.2±2.3
	a WKY Cont	30.7±6.0	25.0±5.0	15.4±2.4	17.2±3.8	17.2±3.1	19.2±3.0	11.3±1.4
	a WKY IPC	30.8±3.9	26.7±3.1	11.3±1.3	15.3±2.3	14.7±2.1	14.8±1.8	12.0±1.8

Cont - control; IPC - Ischaemic preconditioning; Cap - captopril

\* = P < 0.05

#### **5.4. Part B                      ACE inhibition in the chronically hypertensive myocardium**

Reasons for the lack of protective effect of IPC in both the ageing SHR and WKY are not known. However, bradykinin levels are thought to be diminished in hypertension (Nakagawa & Nasjletti, 1988; Seino *et al*, 1990), thus, it was investigated if an ACE inhibitor in combination with the IPC protocol is able to augment bradykinin levels sufficiently and restore the preconditioning response. Previous studies have demonstrated that ACE inhibitors potentiate IPC by increasing bradykinin levels. Similarly, in chapter four, it was reported that captopril potentiated subthreshold preconditioning via activation of the bradykinin B<sub>2</sub> receptor in normal hearts. Accordingly, in the second part of this study, captopril was used in conjunction with the standard IPC protocol to examine if bradykinin levels can be augmented sufficiently to elicit the preconditioning response in the ageing animals. Indeed, it was found in an earlier study that bradykinin induced cardioprotection was diminished in a mild model of hypertrophy (chapter three). Thus it remains to be investigated if elevating endogenous bradykinin levels with an ACE inhibitor is able to restore the preconditioning response in the ageing SHR/WKY rat hearts. This approach to harnessing bradykinin in hypertension might have obvious therapeutic relevance.

## 5.5. Treatment protocols

Hearts were subjected to captopril treatment with or without the IPC protocol, shown in figure 5.4. Control experiments were randomised in this part of the study as well.

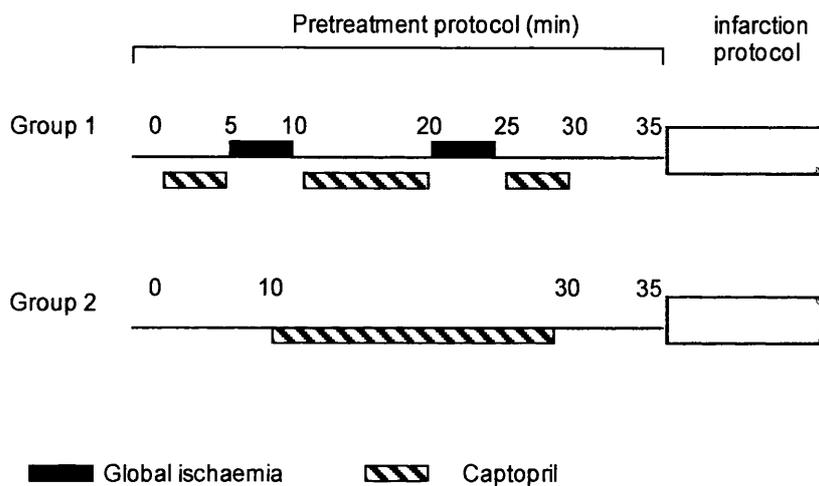


Figure 5.4. Experimental treatment protocols. 35 minutes prior to the infarct protocol, hearts were subjected to either captopril and IPC (group 1) or captopril alone (group 2). The control protocol was the same as that used in part A of the study, see figure 5.1.

Group 1: IPC & Captopril (IPC & Cap). Captopril (200  $\mu$ M) was perfused for 5 minutes before and for 10 minutes after the first preconditioning cycle and for 5 minutes after the second preconditioning cycle.

Group 2: Captopril (Cap). Captopril (200  $\mu$ M) was perfused alone for 20 minutes prior to the induction of ischaemia.

## 5.6. RESULTS

An additional 23 animals (SHR and WKY) were used for this part of the study. Results of the control experiments are the same as that presented in part A of the study.

### 5.6.1. Blood pressure data

Systolic and diastolic blood pressures are depicted in table 5.5. The SHRs exhibited greater systolic / diastolic blood pressures compared to WKY rats.

Table 5.5. Summary of systolic and diastolic blood pressure

Group	n	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)
a SHR Cont	5	224±22 *	193±19 *
a SHR IPC + Cap	6	210±8 *	170±14 *
a SHR Cap	4	209±13 *	173±22 *
a WKY Cont	3	120±11	100±16
a WKY IPC + Cap	4	120±10	99±5
a WKY Cap	5	127±11	109±10

Cont; control IPC; ischaemic preconditioning Cap; captopril. \* = P<0.05 versus corresponding age matched WKY group (one way ANOVA)

### 5.6.2. Body weight, risk zone volume and hypertrophy index

No significant differences in rat body weight were observed. Myocardial risk zone volume was significantly greater in the SHR compared to WKY hearts. When risk zone was normalised to the volume of the left ventricle, there were no significant differences between the animals (table 5.6).

LVH and RVH patterns similar to that reported in part A of the study were seen (table 5.6).

Table 5.6. Rat body weight, risk zone volume, risk zone volume normalised to LV volume, LV/body weight ratios, and RV/body weight ratios

Group	n	rat body weight (g)	risk zone volume (cm <sup>3</sup> )	risk zone / LV volume(%)	LV/ body weight (mg/g)	RV/body weight (mg/g)
a SHR Cont	8	434±16.7	1.01±0.05 *	55.0±1.7	5.0±0.14 *	0.50±0.60 *
a SHR IPC + Cap	6	448±20.1	1.20±0.12 *	59.2±3.0	5.1±0.30 *	0.51±0.60 *
a SHR Cap	5	417±17.3	1.20±0.06 *	57.5±1.6	5.4±0.18 *	0.53±0.50 *
a WKY Cont	7	434±18.2	0.67±0.06	53.5±2.0	3.5±0.08	0.32±0.02
a WKY IPC + Cap	6	451±16.0	0.70±0.08	53.0±3.7	3.8±0.14	0.36±0.30
a WKY Cap	6	419±6.7	0.73±0.03	57.0±2.2	3.7±0.09	0.36±0.01

Cont - control; IPC - Ischaemic preconditioning; Cap - captopril . \* = P <0.05 versus corresponding age matched WKY group (One way ANOVA)

### 5.6.3. Effects of captopril on infarct size

The combination of captopril and IPC in the ageing SHR did not produce a cardioprotective effect ( $59.4 \pm 4.2\%$ ,  $P$  not significant versus control). However in the ageing WKY group, infarct size was significantly reduced to  $36.4 \pm 4.8\%$  following captopril and IPC treatment ( $P < 0.05$ ). The administration of captopril alone did not influence infarct size in either SHR or WKY respectively ( $55.6 \pm 4.5\%$  /  $54.5 \pm 1.3\%$ ,  $P$  not significant versus control) (figure 5.5).

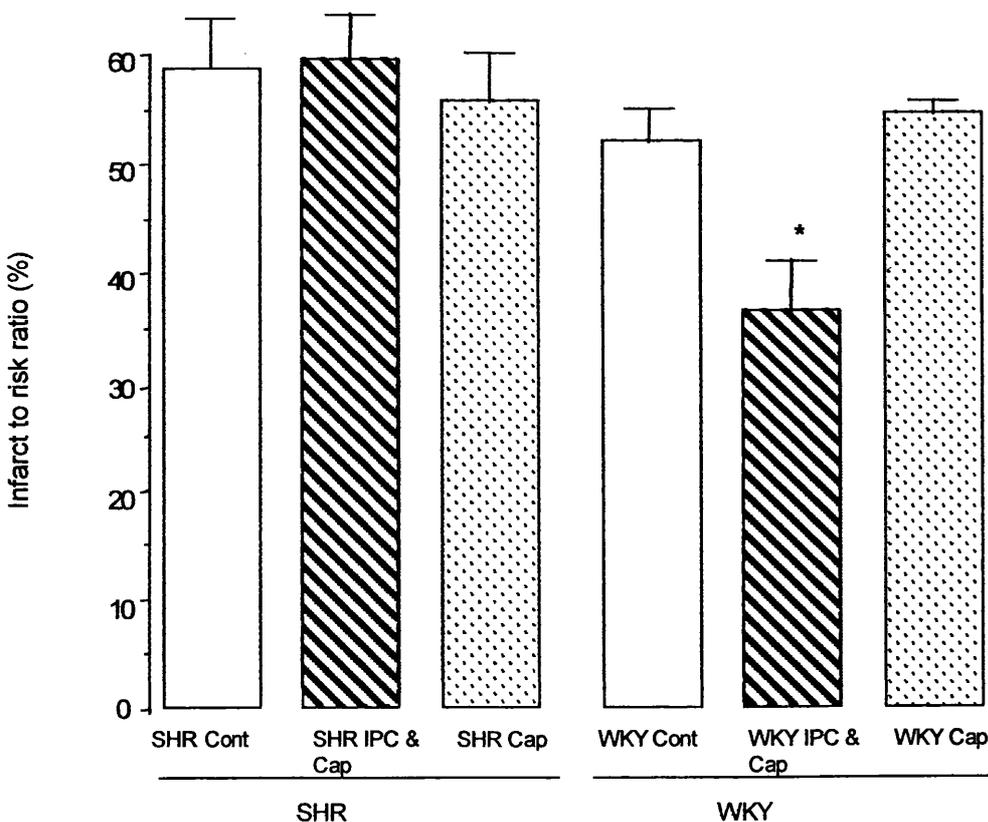


Figure 5.5. Infarct size data represented as infarct to risk ratio. Captopril in conjunction with the IPC protocol did not attenuate infarct size in the ageing SHR. A modest reduction was however observed in the ageing WKY rats. \* =  $P < 0.05$  versus WKY control (One way ANOVA). Cont - control; Cap = captopril

#### **5.6.4. Coronary flow and contractility data**

CFR and RPP data are summarised in table 5.7 and 5.8 respectively. Captopril treatment did not influence CFR or RPP. A reduction in CFR was observed following coronary artery ligation which recovered during initial reperfusion, but gradually declined in the later stages of reperfusion.

Table 5.7. Coronary Flow Rate (ml/min/g)

Group	Basal	Pre-Ischaemia	-----ischaemia-----		-----reperfusion-----		
			5 min	30 min	5 min	60 min	120 min
a SHR Cont	9.1±0.2	8.1±0.3	3.9±0.2	3.7±0.4	6.3±0.3	4.5±0.3	4.0±0.3
a SHR IPC + Cap	9.4±0.8	8.0±0.6	3.3±0.3	3.5±0.2	6.0±0.3	4.7±0.3	3.5±0.5
a SHR Cap	8.5±0.6	7.4±0.4	4.0±0.6	4.0±0.4	6.0±0.7	4.5±0.2	3.5±0.3
a WKY Cont	9.5±0.9	8.6±1.0	5.3±0.7	4.8±0.6	7.9±0.8	6.7±0.8	5.3±0.9
a WKY IPC + Cap	9.3±0.5	8.0±0.7	3.8±0.5	4.2±0.5	6.6±0.8	5.2±0.9	4.5±0.8
a WKY Cap	9.4±0.4	8.7±0.8	5.0±0.4	5.0±0.6	8.0±0.8	5.8±0.6	4.7±1.0

Table 5.8. Rate Pressure Product (mmHg/min X 10<sup>3</sup>)

Group	Basal	Pre-Ischaemia	-----ischaemia-----		-----reperfusion-----		
			5 min	30 min	5 min	60 min	120 min
a SHR Cont	33.2±3.6	33.6±4.6	17.3±3.0	22.1±2.8	20.1±4.0	15.4±3.0	11.0±1.5
a SHR IPC + Cap	44.6±6.8	34.0±6.0	15.3±2.7	17.8±2.0	21.5±2.4	21.1±5.0	15.6±4.2
a SHR Cap	33.9±5.7	30.2±4.2	14.3±3.6	18.0±4.8	20.5±3.8	14.8±3.7	15.2±1.4
a WKY Cont	30.7±6.0	25.0±5.0	15.4±2.4	17.2±3.8	17.2±3.1	19.2±3.0	11.3±1.4
a WKY IPC + Cap	36.0±3.2	33.0±3.3	13.4±3.8	12.8±3.5	15.6±3.8	14.1±3.7	11.3±2.4
a WKY Cap	37.8±3.2	28.0±3.3	13.0±3.8	13.0±3.5	20.6±0.2	12.7±1.5	9.9±1.1

Cont - control; IPC - Ischaemic preconditioning; Cap - captopril

## **5.7. DISCUSSION**

The data presented here demonstrated that IPC caused a significant limitation of infarct size both in the young and middle aged SHR and normotensive WKY rat hearts. IPC did not however, induce protection in ageing SHR myocardium. Interestingly, it was also found that IPC did not reduce infarct size significantly in ageing normotensive WKY rat hearts. This implies that events occurring between age 7/8 months to 11/13 months in the SHR / WKY rats interfere with the mechanisms of IPC. Hence, during the 4-6 months of additional ageing with or without hypertension, animals lose the capacity to respond to IPC. Captopril in conjunction with the IPC protocol failed to restore the preconditioning response in the ageing SHR, however, produced a modest limitation of infarct size in the ageing normotensive WKY rat.

In this work, for simplicity, 11-13 month old animals are referred to as "ageing", and 7-8 month old animals as "middle aged" but, it is important to point out that the lifespan of the SHR / WKY rat is approximately 10-21 months (Pinto *et al*, 1998). Therefore, it may be more accurate to refer to the middle aged animals as "mature adult" and ageing animals as "middle aged". The life span of a rat varies from one colony to another, however the average life span of SHRs is thought to be shorter than that of "normal" rats. From the age of 8 months, SHRs appeared poorly groomed and less active than younger counterparts. In fact, five SHRs died spontaneously before 12 months of age. The normotensive WKY rats appeared less active and poorly groomed at approximately 10 months of age, however, no animals died spontaneously.

### **5.7.1. Tolerance to ischaemia-reperfusion in hypertrophy**

Studies have demonstrated that hypertrophied hearts are more susceptible to ischaemia-reperfusion injury compared to normal hearts. Anderson *et al* demonstrated that

hypertrophied rat hearts developed ischaemic contracture sooner than normal hearts following global ischaemia (Anderson *et al*, 1987). Ventricular fibrillation upon reperfusion in DOCA-salt hypertrophied hearts was more severe than in normal hearts following ten minutes of coronary artery occlusion (Baxter & Yellon, 1992b). Similarly, the SHR was more susceptible to damage evoked by 45 minutes of global ischaemia compared with age matched WKY rats (Snoeckx *et al*, 1993). Additionally, Koyanagi *et al* reported that dogs with LVH exhibited a larger infarct size compared with normotensive controls (Koyanagi *et al*, 1982). In contrast, in these experiments, no increase in control infarct size was observed in the SHR at any age compared to the WKY rat. Similarly, Speechly-Dick *et al* did not observe an increase in control infarct size in the DOCA-salt hypertrophied hearts compared with normotensive controls (Speechly-Dick *et al*, 1994). The reasons for the discordant findings are not known, although, models of hypertrophy, experimental end points, methods of ischaemia induction (ie, regional/global) and duration of ischaemia all vary, which may help to explain divergent results. In these studies, perfusion pressure (80 mmHg) was kept constant throughout the course of the experiments (discussed below). It is not known whether blood pressure (as opposed to myocardial hypertrophy) influences infarct size. As there was no significant difference in infarct size between SHR and WKY rat hearts (at any given age), it is not likely that blood pressure influenced infarct size. However, it must be remembered that blood pressure was measured *in vivo* and infarction was induced in the *in vitro* setting in which perfusion pressure was kept constant.

### **5.7.2. Ageing and ischaemia-reperfusion**

Similarly, in these experiments, control infarct sizes were not larger in older animals (ie, in either SHR or WKY). In contrast, Azhar *et al*, reported that the aged mouse heart displayed larger infarct sizes compared to younger counterparts (Azhar *et al*, 1999). Several investigators agree with this observation (Tani *et al*, 1997; Mariani *et al*, 2000), although,

once again, the reasons for divergent results are not known. However, compatible with these data, Schulman et al, did not find an increase control infarct size in aged animals (Schulman *et al*, 2001).

CFR was reduced in ageing animals compared to younger counterparts at baseline and during reperfusion in both SHR and WKY rats. CFR declined with age presumably because of greater muscle mass in proportion to coronary vessels leading to a reduction in coronary perfusion in hypertrophy and ageing. Therefore, the middle aged and ageing hearts may have been underperfused relative to the young hearts. Perfusion pressure (80 mmHg) in these studies was kept constant throughout the experiments and was the same for all the animals. If a higher perfusion pressure was used in the middle aged and ageing animals, differences in CFR may have been corrected. Even though middle aged and ageing animals exhibited lower flow rates, the hearts did not appear to be ischaemically underperfused (ie, they were functioning well, albeit less than the younger animals and temperature was stable). I opted to use the same coronary perfusion pressure in all the animals due to various reasons. Firstly, it is thought that coronary flow rate may be unphysiologically high in the isolated perfused heart due to coronary vasodilatation and due to the decreased viscosity of Krebs-Henseleit buffer compared with blood. Secondly, due to practical limitations of the Langendorff apparatus used, it was not possible to increase the perfusion pressure by large degrees. It was therefore decided that the same coronary perfusion pressure would be used for all the animals. Blood pressure was not significantly higher in ageing versus young WKY rats, (although there was a trend towards higher blood pressures in the ageing animals). It is therefore likely that the middle aged and ageing normotensive animals were adequately perfused at 80 mmHg perfusion pressure.

In agreement with these findings, Snoeckx et al reported that the recovery of CFR and contractility following ischaemia was depressed in aged SHR and WKY rats, compared to adult SHRWKY rats (Snoeckx *et al*, 1993). The authors postulated that this may be due to depressed perfusion of the subendocardial layers during reperfusion. This underperfusion is likely to be caused by an increase in coronary resistance in the inner layers of the LV wall. In both hypertension and ageing, function of the endothelium is impaired, whereby coronary vasodilator reserve is reduced, which could also explain lower coronary flows in aged SHR and WKY rats.

Mariani et al demonstrated that recovery of contractile function was attenuated following hypoxia in aged human myocardium, compared to the recovery in younger myocardium (Mariani *et al*, 2000). Similarly, Misare et al reported that postischaemic systolic functional recovery was worse in the aged sheep compared with younger counterparts (Miasre *et al*, 1992). Hence, numerous studies have reported reduced functional capacity of the ageing myocardium. Similarly, in this study, we found that the contractility was reduced at baseline and throughout the experimental protocol in the ageing WKY rat compared to the young WKY animals. Whether the RPP is reduced in ageing animals due to a reduction in relative flow per gram is not known. As previously mentioned, blood pressure did not increase with age in the WKY animals, implying that the middle aged and ageing WKY rat hearts were not likely to be underperfused relative to the young hearts at a perfusion pressure of 80 mmHg. However, in SHR, generally, the contractile function was equally preserved in the ageing myocardium compared with the young, presumably due to LVH which is able to maintain cardiac output (compensated hypertrophy).

The occurrence of IPC in myocardial hypertrophy has already been discussed in section 1.8.

### 5.7.3. IPC and ageing

This study has demonstrated that hearts isolated from ageing SHR<sub>s</sub> could not be preconditioned. At approximately 18 months of age, SHR<sub>s</sub> have been reported to develop heart failure (Conrad *et al*, 1991). It is not known whether the ageing SHR<sub>s</sub> in this study had heart failure. There was evidence of lung target organ injury, as areas of haemorrhage and bullae which reflect high blood pressure in the pulmonary circulation were observed in the ageing SHR<sub>s</sub>. However, there was no evidence of ascites to suggest that the animals were suffering from right-sided heart failure. In addition, animals did not appear cyanosed, and even appeared pink when anaesthetised. Further, hearts isolated from ageing SHR<sub>s</sub> functioned well when Langendorff perfused, albeit, not as well as their younger counterparts. These data imply that the ageing SHR<sub>s</sub> used in this study may not have been suffering from heart failure. However, RVH had occurred in these animals and plasma noradrenaline concentration was elevated, both of which are indicative of heart failure. Additionally, as mentioned previously, animals were poorly groomed and five animals (SHR) died spontaneously before 12 months of age and generally appeared less active than younger counterparts. Similarly, ageing WKY rats also appeared poorly groomed, with a coarse yellowish coat as opposed to a soft white coat. Another interesting finding of this study was that hearts isolated from age matched WKY rats were also unresponsive to IPC. Hence, one cannot predict whether the long-standing hypertensive cardiac hypertrophy or ageing or even a combination of the two factors interferes with the molecular mechanisms of IPC. Indeed several studies have demonstrated that IPC does not elicit cardioprotection in aged animals. Abete *et al* demonstrated that IPC did not prevent postischaemic dysfunction in senescent wistar rats (Abete *et al*, 1996). However, exogenous noradrenaline was able to mimic IPC in the senescent rats. These investigators found that even though IPC caused the release of noradrenaline in adult heart and IPC was abolished with  $\alpha$  adrenergic blockade, IPC did not augment noradrenaline levels in the senescent heart.

Thus, they speculated that IPC may be absent in the elderly animals due to a reduction of noradrenaline release. Further, in a subsequent study this group demonstrated exercise training restored the preconditioning response in the elderly animals by increasing noradrenaline release following the preconditioning ischaemia (Abete *et al*, 2000).

Fenton *et al* recently documented that IPC was absent in 22 month old Fischer 344 rats using both infarct size and contractile function as experimental end points (Fenton *et al*, 2000). Although these animals are obviously somewhat older than the ones used in this study, it has also been reported that IPC failed to evoke cardioprotection in 12 month old Fischer 344 rats (Tani *et al*, 2001). More recently, Schulman *et al* demonstrated that 12 month old Sprague Dawley rat hearts were not responsive to IPC with one x five minute cycle of ischaemia-reperfusion (Schulman *et al*, 2001). In contrast to these studies, Burns *et al* reported that the preconditioning response was preserved in senescent sheep myocardium (aged 5.7 to 8.0 years) (Burns *et al*, 1996). These investigators demonstrated that IPC reduced infarct size to a similar extent in both the adult and ageing sheep heart (by 54% in adult sheep and by 47% in senescent sheep respectively). The reason for the discordant finding is not clear. However, it is relevant to point out that the "senescent" Dorset or Suffolk sheep used were only of 5.7 to 8 years of age and the lifespan of these sheep is up to 20 years. Thus whether the animals used in these studies were sufficiently aged is debatable. The second window of preconditioning has not been extensively examined in ageing animals. However, Gray and co-workers recently reported that heat stress did not protect aged (16 month old) Sprague Dawley rat hearts (Gray *et al*, 2000).

#### **5.7.4. IPC in other disease states**

The investigation of IPC in alternative cardiac disease states, namely, heart failure, diabetes mellitus and hypercholesterolaemia also remain largely under-investigated.

Miki et al investigated the effects of postinfarct remodelling on IPC. Myocardial infarction was induced in rabbits by ligation of the coronary artery for two weeks. Although IPC reduced infarct size in the sham operated rabbits, it had no effect in the remodelled hearts. Diazoxide, however, reduced infarct size in the remodelled myocardium implying that the lack of IPC in the remodelled myocardium is due to defects in the signalling cascade upstream of the mitochondrial  $K_{ATP}$  channel (Miki *et al*, 2000).

Dekker et al showed that in papillary muscles isolated from failing hearts, IPC increased  $[Ca^{2+}]_i$  rise, contracture and electrical uncoupling during sustained ischaemia. Hence, IPC has adverse effects in failing papillary muscles (Dekker *et al*, 1998). Whether this is relevant to the ageing SHR used in this study is not known, although the SHRs probably did not have heart failure but were heading towards the decompensated stage of hypertrophy.

Kersten et al demonstrated that IPC did not reduce infarct size in diabetic dogs (Kersten *et al*, 2000). In contrast, other investigators have demonstrated that IPC still protects the diabetic myocardium (Ravingerova *et al*, 2000; Tatsumi *et al*, 1998; Moon *et al*, 1999; Bouchard & Lamontagne, 1998).

Szilvassy et al showed that pacing-induced preconditioning was abolished in atherosclerotic rabbits (Szilvassy *et al*, 1995). Interestingly, when serum cholesterol levels dropped (with no change in atherosclerotic lesions), the IPC response was reinstated. These findings indicate that atherosclerosis *per se* without hypercholesterolaemia does not influence IPC. The observation that IPC is abrogated by hypercholesterolaemia but not atherosclerosis also appears to be consistent with the finding that patients with coronary artery stenosis demonstrate the preconditioning response (Kloner & Yellon, 1994).

More studies are clearly required to fully investigate the preconditioning response in various cardiac diseases. For a summary of IPC in the diseased myocardium, see review by Ferdinandy *et al*, 1998.

#### **5.7.5. Signalling mechanisms in preconditioning**

Reasons for the lack of protective effect of IPC can be postulated. Hypertension and ageing induce a host of disturbances which may interfere with IPC. Endothelial dysfunction has been shown to occur in both cases. Particular emphasis has been laid on the diminished availability of vasodilators, for example, nitric oxide, PGI<sub>2</sub>, endothelium-derived hyperpolarising factor (EDHF) and bradykinin and an increase in vasoconstrictors for example, thromboxane A<sub>2</sub>, endothelin -1 and oxygen free radicals (Drexler & Hornig, 1999; Mombouli & Vanhoutte, 1999) (Nakagawa & Nasjletti, 1988; Seino *et al*, 1990). Previous studies have shown endothelium-dependent vasodilation to acetylcholine is blunted in ageing and hypertension in both humans and animals (Drexler & Hornig, 1999; Mombouli & Vanhoutte, 1999). An important trigger of IPC, bradykinin, is primarily synthesised by endothelial cells. Hence, endothelial dysfunction may hinder the liberation of bradykinin explaining why IPC is blunted in hypertension / ageing. Indeed, alterations in the kallikrein-kinin system have been reported in hypertension (Favaro *et al*, 1975; Ader *et al*, 1987; Nakagawa & Nasjletti, 1988; Sharma *et al*, 1996). Studies have demonstrated that the production of bradykinin is attenuated during hypertension (Nakagawa & Nasjletti, 1988; Seino *et al*, 1990). Additionally, studies have also demonstrated that adenoviral gene delivery of the kallikrein gene in hypertension attenuates hypertension and hypertrophy (Wang *et al*, 1995; Chao *et al*, 1998; Xiong *et al*, 1995; Yayama *et al*, 1998). These results imply that bradykinin production may be attenuated during hypertension and that restoration of bradykinin levels ameliorate hypertension. In addition, bradykinin associated abnormalities have been reported to occur in ageing. Bradykinin induced vasodilation is

converted to a vasoconstrictor response in vessels of aged animals (Mantelli *et al*, 1995). Further, bradykinin induced vasodilatation is impaired in the basilar arteries of old animals (Arribas *et al*, 1997).

Abnormalities in G-proteins, have been reported in hypertension and ageing (Johnson & Friedman, 1993). Adequate functioning of G-proteins is crucial as triggers of IPC initiate the protective signalling cascade by activating G-protein linked receptors (Nakano *et al*, 2000b). Disturbances in PKC signalling have also been implicated in hypertension and ageing. Investigators have demonstrated that the translocation of PKC  $\epsilon$  and PKC  $\alpha$  is impaired in ageing following ischaemia / reperfusion (Korzick *et al*, 2000); both isoforms have been implicated in IPC (Nakano *et al*, 2000b; Ping *et al*, 2001). Recently, Tani *et al* showed in the senescent rat heart, IPC did not translocate PKC  $\alpha/\delta$  whereas both isoforms were translocated in young heart following IPC (Tani *et al*, 2001). PKC  $\epsilon$  has additionally been implicated in hypertrophy, where enhanced activity was linked with myocyte hypertrophy (Sil *et al*, 1998). Finally, alterations in mitochondrial respiration have been demonstrated to occur, during senescence. Fannin *et al* demonstrated an age related decline in cytochrome oxidase activity in interfibrillar mitochondria (Fannin *et al*, 1999). Alterations in mitochondrial energy production have also been found to occur in hypertension (Seccia *et al*, 1999). A vast number of studies favour a role for the mitochondrial  $K_{ATP}$  channel in IPC (Nakano *et al*, 2000b), however, alterations in mitochondria may lead to a dysfunctional / modified  $K_{ATP}$  channel. Therefore, all pathways that have been implicated in IPC, including triggers, mediator (PKC) and the possible end effector site (mitochondria) are affected during the processes of hypertension and ageing, which may help to explain why IPC is lacking in these conditions. Indeed, Schulman *et al* demonstrated that CCPA, dioctanoyl-sn-glycerol or diazoxide all failed to induce cardioprotection in isolated aged (18 month old) rat hearts, whilst these agents clearly protected young hearts (Schulman *et al*, 2001). Results imply

that adenosine receptors (ie, G-protein linked receptors), PKC and the mitochondrial  $K_{ATP}$  channel are affected during the process of ageing, hence, no cardioprotection was observed. Exactly, how they are affected remains elusive.

Therefore, molecular mechanisms implicated in IPC also appear to be involved in hypertrophy / ageing, hence mechanisms may be downregulated in long standing cardiac hypertrophy, so that IPC cannot initiate further activation of the pathways that are already activated in this pathology. Furthermore, levels triggers of IPC, namely adenosine (Dobson *et al*, 1990) and opioids (Boluyt *et al*, 1993) have been shown to increase in the senescent myocardium, again implying that high levels of these substances may lead to receptor downregulation.

#### **5.7.6. ACE inhibitors and cardioprotection**

When captopril was combined with the IPC protocol, in the ageing animals, no limitation of infarct size was observed in the SHR. In contrast, a modest but significant protective effect was seen in hearts isolated from age matched normotensive animals.

The reasons for the lack of protective effect of captopril in ageing SHR are unknown, although one can speculate that as both ageing and hypertension induce endothelial dysfunction, there is a reduced production of bradykinin. As already mentioned, studies have demonstrated that bradykinin levels are attenuated in hypertension. In ageing alone the degree of endothelial dysfunction may not be as severe such that captopril can augment bradykinin levels to elicit cardioprotection. However, in hypertension, one can speculate that endothelial dysfunction may be so severe that captopril is unable to enhance bradykinin levels sufficiently. Nevertheless, bradykinin levels were not measured in this study, hence, it is not known whether this is actually the case. Interestingly, Mantelli *et al* demonstrated

that while bradykinin caused vasoconstriction as opposed to vasodilatation in old SHR/WKY vessels, this vasoconstriction was more pronounced in the old SHRs (Mantelli *et al*, 1995). This is just a possibility, many other factors may be involved. Interestingly, as described in chapter three, the kallikrein-kinin system has been implicated in hypertension. Much controversy exists regarding the precise activity of the kallikrein-kinin system in hypertension. While most studies report activity of kallikrein-kinin system is diminished in hypertension (Favaro *et al*, 1975; Ader *et al*, 1987; Nakagawa & Nasjletti, 1988; Sharma *et al*, 1996), some studies report that bradykinin levels are augmented in this pathology. For example, Campbell *et al* found increased levels of bradykinin in SHR myocardium (Campbell *et al*, 1995a). This implies that the bradykinin B<sub>2</sub> receptor may be desensitised in hypertension due to the presence of increased bradykinin levels. Indeed, Luckhoff *et al* demonstrated that B<sub>2</sub> receptor desensitisation occurs following repeated stimulation (Luckhoff *et al*, 1988). Hence, raised levels of bradykinin in the presence of captopril may not be able to induce cardioprotection due to bradykinin B<sub>2</sub> receptor desensitisation. Defects in other parts of the preconditioning mechanism for example, intracellular signalling and or the distal end effector site (discussed in section 5.7.5) in chronic hypertrophy may also interfere with ACE inhibitor induced protection.

#### **5.7.7. Limitations of study**

A major limitation of this study is the fact that bradykinin levels were not measured. Hence, we cannot propose for certain that basal bradykinin levels are attenuated in the SHR and that captopril does not augment bradykinin levels to the same extent as it does in the normotensive animals. Additionally, captopril was administered acutely. If on the other hand, captopril was administered chronically, presumably, hypertension and LVH would be reduced and endothelial function restored. Future studies must examine whether long-term treatment with captopril restores the preconditioning response in these SHRs. Captopril at

a concentration of 200  $\mu\text{M}$  was used. Although this dose successfully augmented the subthreshold preconditioning response in young animals, this concentration may not have been sufficient in the elderly animals. However, the activity of ACE is not altered in the SHR, hence captopril 200  $\mu\text{M}$  should be sufficient to inhibit ACE activity in hypertrophy (Dendorfer *et al*, 2001).

## **5.8. CONCLUSION**

In summary, since the initial discovery of IPC some 15 years ago, a very limited number of studies have examined the effects of this phenomenon in diseased myocardium. It was demonstrated in this study that IPC does not protect the ageing SHR or normotensive WKY rat hearts even though protection was clearly apparent in young and middle aged rat hearts. The reasons for this are not clear and warrant further investigation. Experiments must specifically be designed to address whether hypertension or ageing attenuate the capacity of IPC to evoke cardioprotection, although it is likely to be a combination of the two factors. In addition to this, it was found that a widely used ACE inhibitor captopril did not induce cardioprotection in ageing SHR, but provided a modest limitation in ageing normotensive rats. This study has important clinical implications as both hypertension and ageing are very common occurrences (and usually occur simultaneously) and the absence of IPC in both cases raises the primary concern as to whether IPC can induce protection in human paradigms of hypertension / ageing.

## CHAPTER SIX

### Assessment of the protective effects of bradykinin at reperfusion

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## 6.1. INTRODUCTION

Experiments described so far in this thesis have involved investigating the effects of bradykinin administered prior to ischaemia. In this chapter, the protective effects of bradykinin administered after the onset of ischaemia, just prior to the onset of reperfusion were investigated.

Reperfusion of the ischaemic myocardium is imperative to reduce the ongoing process of cell death. Reperfusion reduces infarct size and mortality in post myocardial infarction patients. Some investigators however, believe that reperfusion itself may precipitate additional cell injury and death of the previously viable myocytes, a phenomenon known as "lethal reperfusion injury" (Yellon & Baxter, 1999) (figure 6.1).

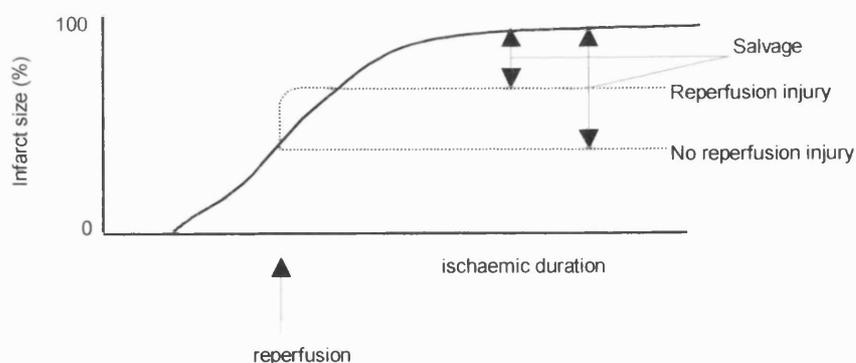


Figure 6.1. Infarct size increases progressively with the ischaemic duration. Reperfusion of the ischaemic myocardium limits infarct size. However, reperfusion itself induces cardiomyocyte death (lethal reperfusion injury).

Four types of reperfusion injury have been described; lethal reperfusion injury, myocardial stunning, reperfusion arrhythmias and vascular injury (Yellon & Baxter, 1999; Birnbaum *et al*, 1997b; Ambrosio & Tritto, 1999). Reperfusion arrhythmias frequently occur following short periods of ischaemia. Free radical scavengers limit reperfusion arrhythmias when administered at the onset of reperfusion (Hearse & Toaski, 1988; Woodward & Zakaria, 1985). Similarly, myocardial stunning also occurs following a short period of ischaemia (ie, which is insufficient to induce myocardial infarction). Free radicals are also thought to participate in inducing myocardial stunning (Bolli *et al*, 1989). Myocardial stunning is caused by reperfusion *per se* and not by ischaemia itself. Vascular injury has also been reported to occur following brief periods of ischaemia (Yoo *et al*, 1999). Nitric oxide may be scavenged by the presence of high concentrations of ROS present during reperfusion.

Lethal reperfusion injury is however the most controversial form of reperfusion injury. While there is sound evidence demonstrating that the occurrence of arrhythmias and stunning are caused by reperfusion *per se*, it is more difficult to demonstrate that lethal reperfusion injury is actually caused by reperfusion and not ischaemia itself. Some investigators believe that lethal reperfusion injury does not cause cell death. They postulate that reperfusion merely accelerates the process of death in cells that were "condemned to die" during the long episode of ischaemia (Yellon & Baxter, 1999). However, lethal reperfusion injury is very difficult to study experimentally, as it is impossible to demonstrate reperfusion injury without a preceding period of ischaemia. Thus, it is difficult to dissociate the effects of ischaemia and reperfusion. Indeed, it is very difficult to measure tissue necrosis using the traditional method of tetrazolium staining, which requires periods of reperfusion following ischaemia. The duration of reperfusion is likely to affect the extent of lethal reperfusion injury. In the dog, lethal reperfusion injury could not be demonstrated five minutes (Ganz *et al*, 1990) and three hours (Zahger *et al*, 1995) following reperfusion. Nevertheless, cellular injury induced

by reperfusion has been demonstrated in several experimental models. Farb *et al* demonstrated the existence of reperfusion injury in the *in vivo* rabbit model of coronary artery occlusion. These investigators found that following 30 minutes of ischaemia, infarct size, assessed by horseradish peroxidase and electron microscopy was  $45.3\pm 2.8\%$ , however, after 180 minutes of reperfusion, infarct size, assessed by tetrazolium staining was significantly increased to  $59.8\pm 3.3\%$  (Farb *et al*, 1993). Two studies in dogs have also demonstrated the occurrence of irreversible myocardial injury during reperfusion (Frame *et al*, 1983; Matsumara *et al*, 1998). It is important to emphasise that these studies demonstrated the extension of the infarct zone during reperfusion, ie, myocytes that were viable at the onset of reperfusion, subsequently became irreversibly damaged during reperfusion. Finally, the existence of reperfusion injury has been demonstrated in isolated myocytes subjected to simulated reperfusion (Vanden Hoek *et al*, 1996). Therefore, reperfusion can truly be regarded as a double-edged sword.

For decades calcium antagonists have been shown to limit reperfusion injury in several animal models (Garcia-Dorado *et al*, 1987; Hatori *et al*, 1993; Massoudy *et al*, 1995; Herzog *et al*, 1997). The exact mechanism by which calcium antagonists exert protective effects is not fully understood. Increased coronary flow, reduction of ATP consumption, reduction of oxygen demand and direct free radical scavenging effects may be mechanisms involved in the protective effects of calcium antagonists. More importantly, intracellular calcium concentration increases (calcium overload) at reperfusion, therefore, calcium antagonists may well induce protection by inhibiting increases in intracellular calcium.

Studies have also demonstrated that adenosine administered at reperfusion exerts protective effects (Norton *et al*, 1991; Olafsson *et al*, 1987; Zhao *et al*, 1999; Mahaffey *et al*, 1999). Further, adenosine administered at reperfusion reduced infarct size in humans

(Mahaffey *et al*, 1999). In contrast, some studies disagree with these findings and report that adenosine is not protective when given at reperfusion (Goto *et al*, 1991; Vander Heide & Reimer, 1996) .

#### **6.1.1. Growth factors and reperfusion injury**

Various growth factors have been demonstrated to exert anti-reperfusion injury effects (figure 6.2). Transforming growth factor  $\beta_1$  (TGF-  $\beta_1$ ) has been shown to limit infarct size in rat heart when given at reperfusion (Baxter *et al*, 1999). Insulin administered at reperfusion in rat heart also limited infarct size (Jonassen *et al*, in press). The effects of insulin like growth factor-1 (IGF-1), which is closely related to insulin, has also been investigated during reperfusion. Studies have demonstrated that IGF-1 exerts cardioprotective actions in rat heart when given at reperfusion (Otani *et al*, 2000; Yamamura *et al*, 2001). Cai *et al* reported that cardiotrophin-1 (CT-1) limited infarct size in rat heart when administered at reperfusion (Cai *et al*, 1999). A peptide related to CT-1, namely urocortin, also exerts cardioprotective effects in cardiac myocytes (Brar *et al*, 2000) and intact rat heart (Schulman *et al*, 2000). Finally, Cuevas *et al* showed that fibroblast growth factor-1 (FGF-1) given at reperfusion in rat, reduced infarct size and improved contractile recovery (Cuevas *et al*, 1999).

These growth factors have been shown to activate a reperfusion injury salvage kinase (RISK) pathway, involving the activation of phosphatidylinositol 3'-OH kinase (PI3 kinase), Akt/protein kinase B (PKB) and p42/p44 MAPK. Activation of the survival signal pathways may ultimately attenuate apoptosis and thereby reduce cellular injury. The cardioprotective actions of insulin (Jonassen *et al*, in press) and IGF-1 (Otani *et al*, 2000) appear to be dependent upon activation of the PI3 kinase pathway. Jonassen *et al* demonstrated that the anti-infarct effect of insulin given at reperfusion was abrogated with the selective

PI3 kinase inhibitor, wortmannin (Jonassen *et al*, in press). Similarly, Otani *et al* demonstrated that the protective effects of IGF-1 at reperfusion were abrogated with wortmannin, again implying an imperative role for the PI3 kinase pathway (Otani *et al*, 2000). Akt is thought to exert anti-apoptotic effects by phosphorylating Bad (a proapoptotic protein), and thereby inactivating the protein. Additionally, Akt has been shown to inhibit activity of caspase-9, which is pro-apoptotic (Cardone *et al*, 1998).

Figure 6.2 highlights some of the signalling pathways involved in attenuating reperfusion injury.

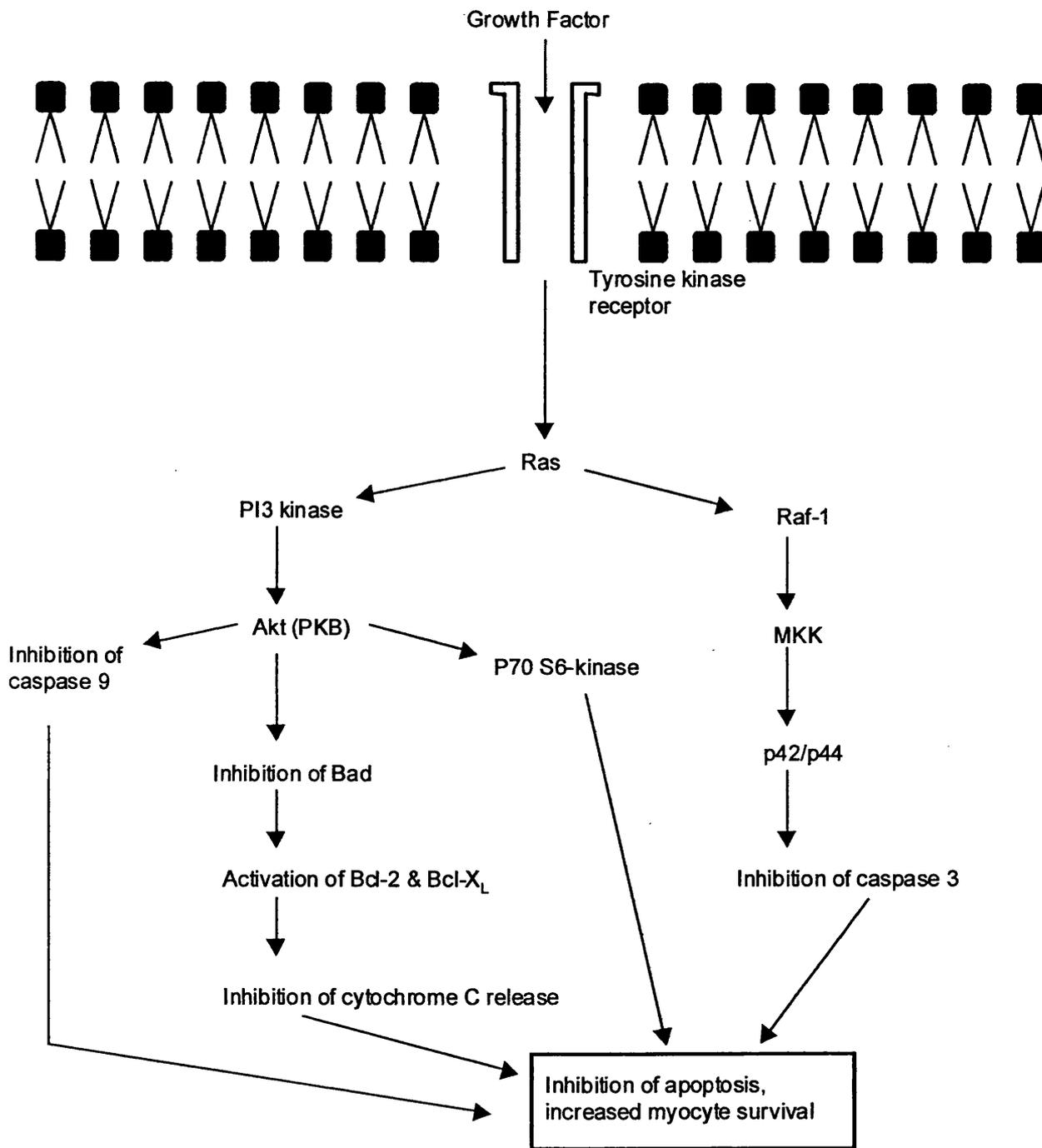


Figure 6.2. Signal cascades activated by growth factors, which may attenuate apoptosis and thereby increase myocyte survival.

Abbreviations used in figure: Ras - a monomeric GTPase (p21); Raf-1 - a mitogen activated protein kinase kinase kinase; PI3 kinase - phosphatidyl inositide 3'-OH kinase; MKK - mitogen activated protein kinase kinase; p42/p44 - mitogen activated kinase; Akt - also known as protein kinase B (PKB); Bad - Bcl-2X<sub>L</sub>/Bcl-2 associated death promoter, Bcl-2 - B cell lymphoma 2 gene

### **6.1.2. Aims of present study**

The majority of work undertaken in the field of reperfusion injury has focused upon growth factors. However, with the possible exception of insulin, such agents cannot be readily administered to man. Ritchie *et al* demonstrated that bradykinin can activate PI3 kinase and p70 S6 kinase and both kinases have shown to be implicated in attenuating reperfusion injury (Ritchie *et al*, 1999; Jonassen *et al*, in press; Otani *et al*, 2000). Thus in view of this work, it was hypothesised that bradykinin given at reperfusion attenuates infarct size via activation of PI3 kinase. Accordingly, the primary aim of this study was to investigate if bradykinin given at reperfusion limits infarct size and secondly, to determine if the PI3 kinase pathway is involved in any protection observed.

### **6.1.3. Part A Assessment of the protective effects of bradykinin at reperfusion**

## **6.2. METHODS**

Following stabilisation (15-20 minutes), hearts were assigned to one of the following five treatment groups illustrated in figure 6.3. The concentration of wortmannin was determined from previous studies which reported that wortmannin at a dose of 100 nM inhibited PI3 kinase activity successfully (Pan *et al*, 1999; Graness *et al*, 1998). The concentration of bradykinin was based upon results obtained in chapter three. Both 0.1  $\mu$ M and 0.2  $\mu$ M bradykinin induced protection in hearts (chapter three).

### **6.2.1. Treatment protocols**

Group 1: Control. Hearts underwent 35 minutes regional ischaemia and 120 minutes reperfusion.

Group 2: Bradykinin (0.1  $\mu\text{M}$ ). Hearts were perfused with bradykinin (0.1  $\mu\text{M}$ ), for 65 minutes, commencing 5 minutes before reperfusion, and continuing for the first 60 minutes of reperfusion.

Group 3: Bradykinin (0.2  $\mu\text{M}$ ). Hearts were perfused with bradykinin (0.2  $\mu\text{M}$ ), for 65 minutes, commencing 5 minutes before reperfusion, and continuing for the first 60 minutes of reperfusion.

Group 4: Bradykinin (0.2  $\mu\text{M}$ ) and wortmannin (100 nM). Hearts were perfused with both bradykinin (0.2  $\mu\text{M}$ ) and wortmannin (100 nM) simultaneously for 65 minutes, commencing 5 minutes before reperfusion, and continuing for the first 60 minutes of reperfusion.

Group 5: Wortmannin (100 nM) alone. Hearts were perfused with wortmannin (100 nM) for 65 minutes, commencing 5 minutes before reperfusion, and continuing for the first 60 minutes of reperfusion.

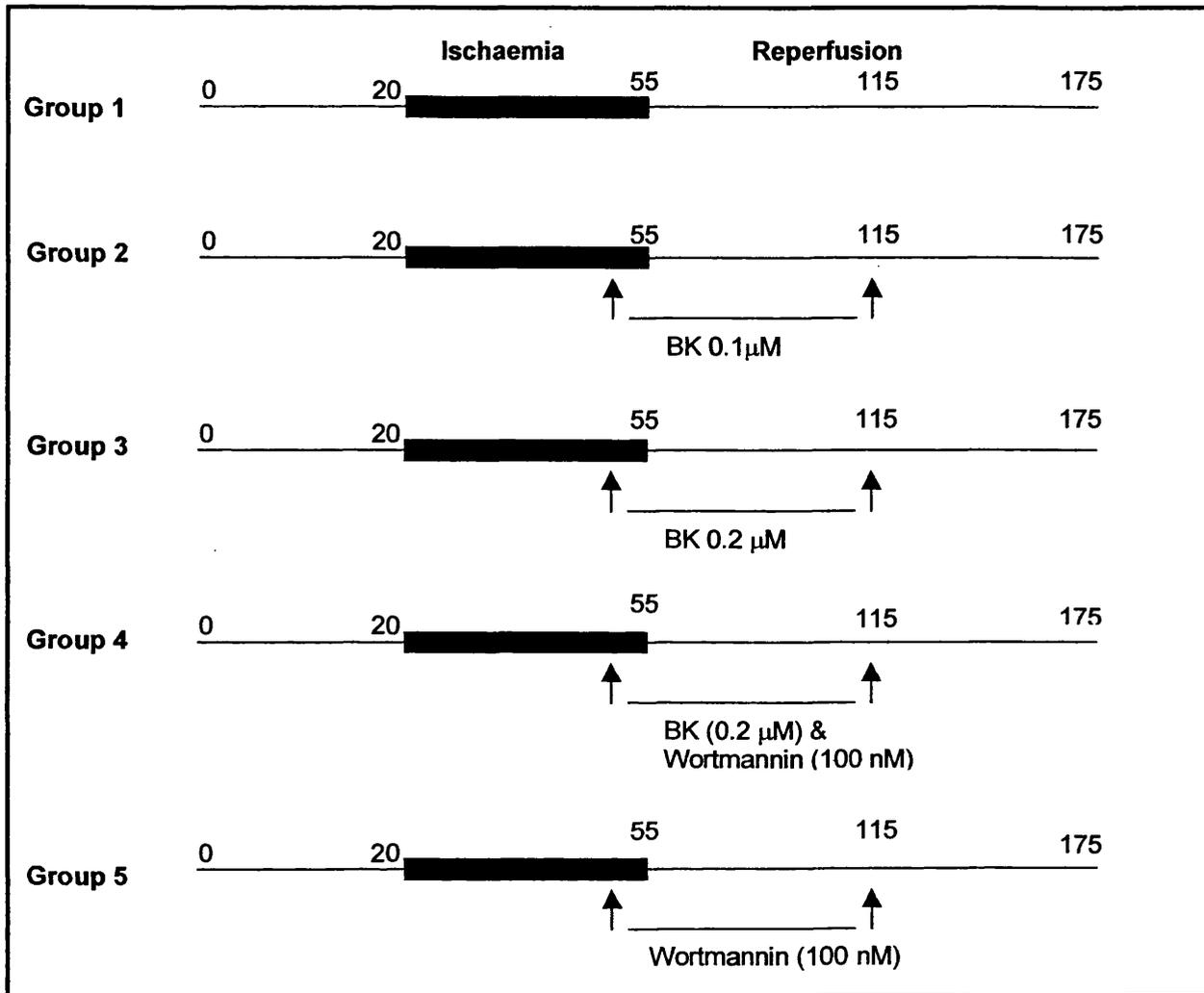


Figure 6.3. Experimental treatment protocols. Hearts were stabilised for 20 minutes, following which ischaemia was induced. Control hearts (group 1) received no drug intervention; Bradykinin (0.1 μM) perfusion commenced in the last five minutes of ischaemia and continued for the first 60 minutes of reperfusion (group 2). Similarly, in group 3, bradykinin at a concentration of 0.2 μM was used. Bradykinin 0.2 μM was perfused with wortmannin (100 nM) (group 4), in the same way; and finally wortmannin was given alone (group 5).

## 6.3. RESULTS

### 6.3.1. Exclusions

A total of 44 animals were used in this study. Two hearts were excluded due to inadequate delineation of risk zone. Therefore, data are reported for 42 successfully conducted experiments.

### 6.3.2. Body weight, risk zone volume

As highlighted in table 6.1, there were no significant differences in rat body weight and risk zone volume among the groups. Wet heart weight was also comparable among groups.

Table 6.1. Summary of rat body weight, wet heart weight and risk zone volume

Group	n	Body weight (g)	Wet heart weight (g)	Risk zone volume (cm <sup>3</sup> )
Control	14	380±7	1.5±0.03	0.47±0.02
BK (0.1 µM)	6	392±10	1.5±0.06	0.53±0.04
BK (0.2 µM)	11	380±6	1.5±0.05	0.51±0.03
BK (0.2 µM) + Wort	5	393±5	1.6±0.04	0.50±0.05
Wort	6	402±2	1.6±0.07	0.48±0.03

BK = Bradykinin

Wort = Wortmannin

### 6.3.3. Infarct limiting effects of bradykinin

Figure 6.4. summarises infarct size data, normalised as a percentage of the risk zone. Control (group 1) infarct size was  $55.5 \pm 3.0\%$ . Bradykinin ( $0.1 \mu\text{M}$ ) did not cause a significant reduction in infarct size. ( $46.1 \pm 3.0\%$ ,  $P = \text{non-significant versus control}$ ) (group 2). Bradykinin ( $0.2 \mu\text{M}$ ) at reperfusion, however, caused a significant reduction of infarct size ( $30.7 \pm 4.2\%$ ,  $P < 0.01$ ) (group 3), an effect abrogated by wortmannin ( $55.6 \pm 4.6\%$ ,  $P > 0.05$ ) (group 4). Wortmannin administered alone did not influence infarct size ( $59.4 \pm 4.9\%$ ,  $P > 0.05$ ) (group 5).

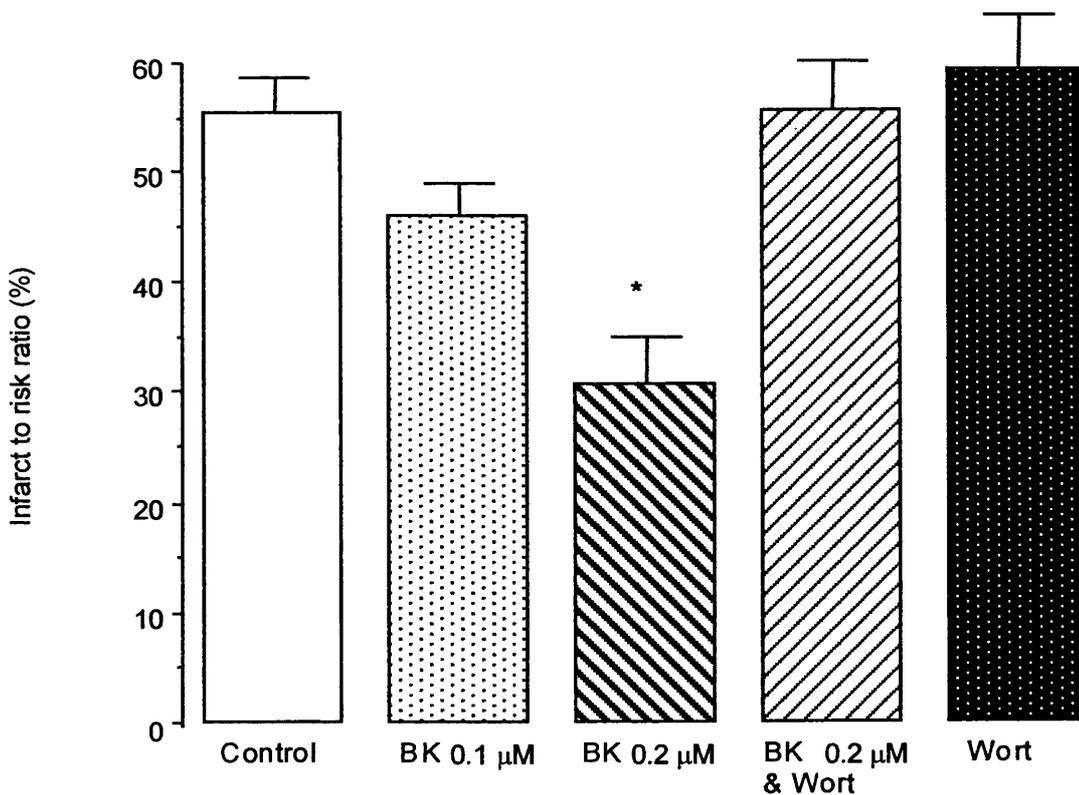


Figure 6.4. Infarct size data represented as infarct to risk ratio. BK ( $0.2 \mu\text{M}$ ) caused a significant limitation of infarct size, an effect abrogated by wortmannin. \* =  $P < 0.01$  versus control (one way ANOVA) (BK - bradykinin; Wort - wortmannin)

#### **6.3.4. Coronary flow and contractility data**

CFR and RPP are presented in tables 6.2 and 6.3 respectively. There were no significant differences in CFR during stabilisation. Immediately following coronary occlusion flow declined considerably in all groups. Administration of bradykinin increased flow immediately, both bradykinin groups had significantly higher flows compared to the control group. The augmentation of flow induced by bradykinin was not abrogated by wortmannin. RPP was comparable among groups during stabilisation. Following, ischaemia, RPP declined markedly in all groups. There were no statistically significant differences in RPP among the groups throughout the experimental procedure.

Table 6.2. Summary of CFR (ml/min/g)

Group	Basal	Pre- ischaemia	-----Ischaemia-----			-----reperfusion-----		
			5 min	30 min	(+BK) 33min	5 min	60 min	120 min
Control	10.4±0.4	9.7±0.4	5.8±0.4	5.6±0.3	5.6±0.3	8.8±0.5	6.0±0.4	5.0±0.5
BK (0.1 µM)	11.4±0.8	11.2±0.7	6.4±0.5	6.3±0.8	9.0±1.0 *	10.4±0.8	7.5±0.9	5.2±0.7
BK (0.2 µM)	10.2±0.3	9.9±0.4	5.4±0.3	5.3±0.4	8.1±0.6 *	9.8±0.7	7.1±0.7	5.6±0.8
BK (0.2 µM) + Wort	10.1±0.9	10.2±0.9	6.1±0.8	5.4±0.9	7.6±1.1 *	8.6±1.0	6.7±0.8	5.0±0.9
Wort	10.0±0.6	10.1±0.7	6.1±0.5	6.4±0.7	6.3±0.7	8.8±0.9	7.0±0.9	3.8±0.3

Table 6.3. Summary of RPP (mmHg/min X 10<sup>3</sup>)

Group	Basal	Pre- ischaemia	-----Ischaemia-----			-----reperfusion-----		
			5 min	30 min	(+BK) 33min	5 min	60 min	120 min
Control	48.5±2.8	44.7±2.4	22.1±2.4	26.1±1.8	26.0±1.8	27.5±2.0	23.3±1.9	18.0±1.6
BK (0.1 µM)	51.8±3.4	48.6±3.0	19.6±2.5	27.6±1.9	27.6±4.3	26.4±2.0	18.9±2.4	16.0±1.3
BK (0.2 µM)	48.5±3.0	47.1±2.7	21.0±2.1	25.1±2.2	23.2±1.5	30.0±3.7	22.5±2.6	18.8±3.1
BK (0.2 µM) + Wort	50.0±2.1	47.6±1.7	23.4±2.6	27.3±2.0	25.0±5.7	26.7±3.4	30.0±3.7	19.6±2.0
Wort	53.2±2.9	48.9±3.0	21.0±2.8	26.4±1.4	23.9±1.0	20.1±2.5	19.7±2.4	15.0±2.1

BK - Bradykinin; Wort - wortmannin.

\* = P <0.05 versus control group. (Repeated measures ANOVA)

## **6.4. Part B**

### **Assessment of the protective effects of bradykinin at reperfusion in the chronically hypertensive myocardium**

#### **6.4.1. INTRODUCTION**

In chapter five, it was reported that neither the ageing (11-13 month old) SHR or WKY rat hearts were amenable to IPC. Additionally, bradykinin given just prior to the onset of reperfusion limits infarct size (part A). Accordingly, this study examined whether reperfusion injury can be attenuated in these animals using bradykinin, as presumably, mechanisms of IPC and reperfusion injury are divergent.

#### **6.5. METHODS**

Hearts were subjected to bradykinin (0.2  $\mu$ M), in the same way as described in part A. It is important to note that as supply of ageing SHR/WKY animals was very limited, control experiments were randomised as adequately as possible between the study performed in chapter five and the present study.

#### **6.6. RESULTS**

An additional 12 animals were used for this part of the study. Results of the control experiments are the same as that presented in chapter five.

##### **6.6.1. Blood pressure data**

Systolic and diastolic blood pressure are depicted in table 6.4. The SHR had significantly higher systolic and diastolic blood pressure compared to WKY rats.

Table 6.4. Summary of systolic and diastolic blood pressure

Group	n	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)
SHR Cont	5	224±22 *	193±19 *
SHR BK	4	190±5 *	178±5 *
WKY Cont	3	120±11	100±16
WKY BK	3	107±2	100±16

Cont = control; BK = bradykinin. \* = P <0.05 versus corresponding WKY group (one way ANOVA)

### 6.6.2. Body weight, risk zone volume and hypertrophy index

Rat body weights are shown in table 6.5, there were no significant differences among the groups.

Myocardial risk zone volume was significantly larger in the SHR compared with WKY groups, however, when normalised to the volume of the left ventricle, there was no significant difference among the groups.

Hypertrophy index in the SHRs was 27% (consistent with results presented in chapter five).

Significant RVH had also taken place in the SHR groups.

Table 6.5. Rat body weight, risk zone volume, risk zone volume normalised to LV volume, LV/body weight ratios and RV/body weight ratios.

Group	n	rat body weight (g)	risk zone volume (cm <sup>3</sup> )	risk zone / LV volume(%)	LV/ body weight (mg/g)	RV/body weight (mg/g)
SHR Cont	8	434±16.7	1.01±0.05 *	55.0±1.7	5.0±0.14 *	0.50±0.60 *
SHR BK	6	430±15.1	0.98±0.07 *	59.2±2.1	4.9±0.15 *	0.47±0.03 *
WKY Cont	7	434±18.2	0.67±0.06	53.5±2.0	3.5±0.08	0.32±0.02
WKY BK	6	423±7.6	0.58±0.50	56.7±3.3	3.7±0.06	0.36±0.02

Cont - Control; BK - Bradykinin

\* = P < 0.05 versus corresponding WKY group

### 6.6.3. Effects of bradykinin on infarct size

Figure 6.5 summarises infarct size data, expressed as a percentage of the risk zone. SHR control infarct size was  $58.7 \pm 4.6\%$ , bradykinin ( $0.2 \mu\text{M}$ ) at reperfusion produced a non-significant reduction in infarct size to  $55.0 \pm 3.4\%$  ( $P > 0.05$ ). WKY control infarct size was  $51.8 \pm 3.2\%$ , and was significantly reduced by bradykinin treatment at reperfusion to  $28.9 \pm 6.3\%$ ,  $P < 0.05$ .

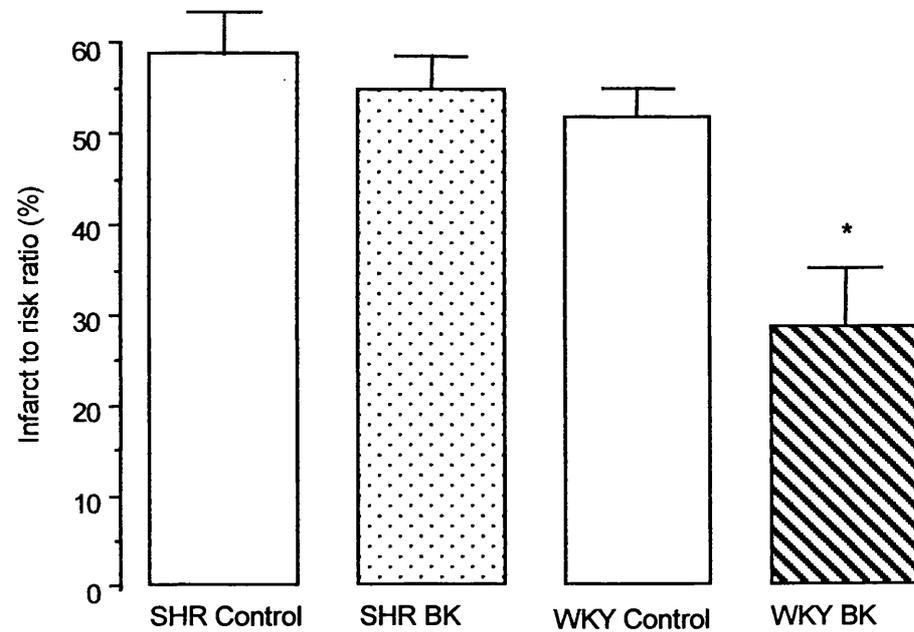


Figure 6.5. Infarct size data represented as infarct to risk ratio. BK at reperfusion did not exert a protective effect in the ageing SHR, however, in the age matched WKY rats, BK given just prior to the onset of reperfusion produced a significant limitation of infarct size. \* =  $P < 0.01$  versus WKY control (one way ANOVA) (BK = bradykinin)

#### **6.6.4. Coronary flow and contractility data**

CFR and RPP are summarised in tables 6.6 and 6.7 respectively. There were no significant differences between CFR among the groups at stabilisation. CFR was slightly higher in the WKY groups during ischaemia. Following bradykinin treatment, CFR in the WKY increased dramatically, however, bradykinin had no vasodilatory effect in the SHR. CFR increased upon reperfusion in all groups, with WKY groups displaying slightly higher values compared with SHR groups, a pattern which continues until the end of the reperfusion period.

Contractility was similar among groups during stabilisation. Ischaemia caused a similar reduction in RPP in all groups. Following bradykinin treatment, the WKY group exhibited a higher RPP compared to the WKY control group, which continued until the end of the experiment.

Table 6.6. Summary of CFR (ml/min/g)

Group	Basal	Pre- ischaemia	-----Ischaemia-----			-----reperfusion-----		
			5 min	30 min	(+BK) 33min	5 min	60 min	120 min
SHR Cont	9.1±0.2	8.1±0.3	3.9±0.2	3.7±0.4	3.7±0.4	6.3±0.3	4.5±0.3	4.0±0.3
SHR BK	9.3±0.4	8.9±0.4	4.2±0.2	3.9±0.1	4.0±0.3	6.6±0.2	4.7±0.4	3.3±0.2
WKY Cont	9.5±0.9	8.6±1.0	5.3±0.7	4.8±0.6	4.8±0.6	7.9±0.8	6.7±0.8	5.3±0.9
WKY BK	9.6±0.4	9.3±0.4	5.3±0.3	5.4±0.3	8.2±0.9 *	9.4±1.0	7.2±1.0	6.5±0.8

Table 6.7. Summary of RPP (mmHg/min X 10<sup>3</sup>)

Group	Basal	Pre- ischaemia	-----Ischaemia-----			-----reperfusion-----		
			5 min	30 min	(+BK) 33min	5 min	60 min	120 min
SHR Cont	33.2±3.6	33.6±4.6	17.3±3.0	22.1±2.8	22.1±2.8	20.1±4.0	15.4±3.0	11.0±1.5
SHR BK	39.0±3.3	38.0±3.7	19.0±2.2	25.1±2.9	23.0±2.9	23.0±3.0	17.1±2.0	10.5±1.3
WKY Cont	30.7±6.0	25.0±5.0	15.4±2.4	17.2±3.8	17.2±3.8	17.2±3.1	19.2±3.0	11.3±1.4
WKY BK	32.1±3.5	32.1±4.4	18.9±3.0	20.1±3.1	20.7±3.3	29.1±3.7 *	26.5±3.5	14.6±2.0

Cont - Control; BK - Bradykinin;

\* = P<0.05 versus WKY control group (Repeated measures ANOVA)

## **6.7. DISCUSSION**

This study demonstrated that bradykinin given just prior to the onset of reperfusion, attenuated infarct size. This protective effect was abrogated by wortmannin indicating that the PI3 kinase pathway is involved in the cardioprotection observed. Bradykinin at reperfusion did not however reduce infarct size in the ageing SHR myocardium. In contrast, a significant reduction of infarct size was observed when bradykinin was given just prior to reperfusion in the normotensive, age matched WKY rat hearts.

### **6.7.1. Bradykinin and limitation of reperfusion injury**

The first study to demonstrate bradykinin limits reperfusion injury was performed by Massoudy *et al*. These investigators demonstrated that bradykinin (at a concentration of 0.1 nM / 1 nM) or the ACE inhibitor ramiprilat given at reperfusion led to an improvement of contractile recovery following ischaemia-reperfusion in the isolated guinea pig heart (Massoudy *et al*, 1994). Similarly, Dogan *et al* demonstrated that enalapril maleate administered at reperfusion improved myocardial contractile recovery following cardioplegic arrest (Dogan *et al*, 1998a). Similar results were obtained with captopril (Dogan *et al*, 1998b). Although these studies do not provide direct evidence for the involvement of bradykinin, increased bradykinin levels as a consequence of ACE inhibition are likely to be implicated in the cardioprotective effects of enalapril maleate/captopril at reperfusion. In the studies conducted by Yang *et al* and Schrieffer *et al*, ACE / NEP inhibitors or dual ACE/NEP inhibitors administered at reperfusion or just prior to the onset of reperfusion attenuated infarct size (Yang *et al*, 1997a; Schrieffer *et al*, 1996). Furthermore, these investigators reported that cardioprotection was abolished with Hoe 140, implying that the protection was mediated by bradykinin. Thus, various studies have indicated that agents that augment bradykinin levels may attenuate reperfusion injury via a bradykinin mechanism. The study conducted by Massoudy *et al* is the only study which provides direct evidence that

bradykinin itself limits reperfusion injury (ie, as opposed to ACE / NEP inhibitors). The study presented here demonstrates that direct administration of bradykinin prior to reperfusion limits infarct size.

### **6.7.2. PI3 kinase and reperfusion injury**

Numerous studies have provided evidence that bradykinin activates PI3 kinase. In human epithelial cell lines, bradykinin was shown to increase PI3 kinase activity, an effect which was blocked by two different PI3 kinase inhibitors, wortmannin and LY294002 (Pan *et al*, 1999). Graness *et al* demonstrated in the human colon carcinoma cell line SW-480 that bradykinin induced the formation of lipid PI3 kinase lipid products, an effect abrogated by wortmannin (Graness *et al*, 1998). Jonassen *et al* recently demonstrated that the protective effects of insulin at reperfusion were abrogated by wortmannin, indicating that PI3 kinase is involved in the protective effects of insulin (Jonassen *et al*, in press). Additionally, these investigators also found an imperative role for p70 S6 kinase in the protection observed, another kinase activated by bradykinin. Similarly, we found that bradykinin induced limitation of infarct size was dependent on the activation of PI3 kinase. Consistent with our findings, Bell *et al* also found that bradykinin administered at reperfusion limited infarct size in the isolated mouse heart, an effect also abrogated by wortmannin (unpublished observation). Exactly how the activation of PI3 kinase leads to a cardioprotective effect is not known. Activation of PI3 kinase leads to the activation of another kinase - Akt (PKB) which is subsequently believed to inhibit apoptosis. Akt inhibits apoptosis by inhibiting pro-apoptotic substances for example BAD (Yellon & Baxter, 1999). The results of this study hint that bradykinin may limit reperfusion injury by attenuating apoptosis. However, apoptosis was not measured. Wang *et al* recently demonstrated that ACE inhibition attenuated apoptosis following ischaemia-reperfusion (Wang *et al*, 2001). This effect was abrogated in the presence of Hoe 140, implying that bradykinin is responsible for the anti-apoptotic

effects of ACE inhibition (Wang *et al*, 2001). Future studies should measure apoptosis following bradykinin administration at reperfusion.

### **6.7.3. Involvement of eNOS**

Two independent studies have provided evidence that eNOS is required for the cardioprotective effect of bradykinin at reperfusion. In eNOS knock out mice, the cardioprotective effect of ramiprilat given at reperfusion was absent (Yang *et al*, 1999). Similarly, Bell *et al* reported that the protective effects of bradykinin at reperfusion were lacking in eNOS deficient mice (unpublished observation). These data suggest that bradykinin given at reperfusion activates eNOS which subsequently limits infarct size. Furthermore, when Bell *et al* administered nitric oxide (ie, a nitric oxide donor, SNAP) at reperfusion in place of bradykinin, a similar reduction in infarct size was observed. Thus, a role for nitric oxide in the limitation of reperfusion injury is likely. Although higher concentrations of nitric oxide have been shown to precipitate cell death, lower concentrations may actually limit cell death (Taimor *et al*, 2000). Dimmeler and co-workers have demonstrated that activation of Akt/PKB leads to the production of nitric oxide via eNOS (Dimmeler *et al*, 1999). Thus, bradykinin mediated upregulation of Akt may lead to the activation of eNOS. Exactly how nitric oxide limits reperfusion injury is not known, although low concentrations of nitric oxide are thought to exert anti-apoptotic effects, by inhibiting mitochondrial release of cytochrome C (Brookes *et al*, 2000) and by inhibiting caspase 3 activity (Rossig L *et al*, 1999). The obligatory role of nitric oxide in mediating bradykinin induced protection should be further investigated by co-administration of a NOS antagonist (eg, L-NAME) and bradykinin at reperfusion. In contrast, the cardioprotection induced by pre-ischaemic bradykinin treatment is nitric oxide independent (when infarct size is used as the end point). However, cardioprotective actions of bradykinin at reperfusion and the delayed protective effects of bradykinin appear to be dependent upon nitric oxide

(see chapter seven), possibly implying that these two cardioprotective actions of this peptide share common signalling pathways.

#### **6.7.4. Effect of bradykinin at reperfusion in the SHR**

The present study has demonstrated that administration of bradykinin at reperfusion in ageing SHR did not exert a protective effect. However, when bradykinin was given at reperfusion to age matched WKY rats, a cardioprotective effect was observed similar to that produced when bradykinin was given to young animals (part A).

To our knowledge, previous studies have not examined reperfusion injury in the hypertensive or ageing animal. Previous studies have implied an imperative role for nitric oxide generated by eNOS in the protection mediated by bradykinin / ramiprilat at reperfusion (Yang *et al*, 1999). Putative signalling mechanisms involved in the protective effects of bradykinin at reperfusion are highlighted in figure 6.6. However, activity of eNOS may be impaired in hypertension. Indeed, activity and expression of eNOS is diminished in the SHR (Chou *et al*, 1998; Crabos *et al*, 1997; Bauersachs *et al*, 1998) . It is not known whether impaired activity of eNOS accounts for the lack of protective effect of bradykinin at reperfusion, although it is a possibility. Alternatively, it was demonstrated that activation of PI3 kinase was required for the protective effects of bradykinin at reperfusion, as treatment with wortmannin abrogated the protective effects of bradykinin (part A). It is not known if activity PI3 kinase is impaired in hypertension, however, activity of this kinase was upregulated in protein synthesis induced by AT-II and  $\alpha$  adrenoceptor stimulation (Rabkin *et al*, 1997; Schluter *et al*, 1999). Whether PI3 kinase activity is downregulated during chronic cardiac hypertrophy (ie, due to repeated stimulation during the initial stages of hypertrophy) and is resistant to further stimulation by bradykinin remains to be investigated.

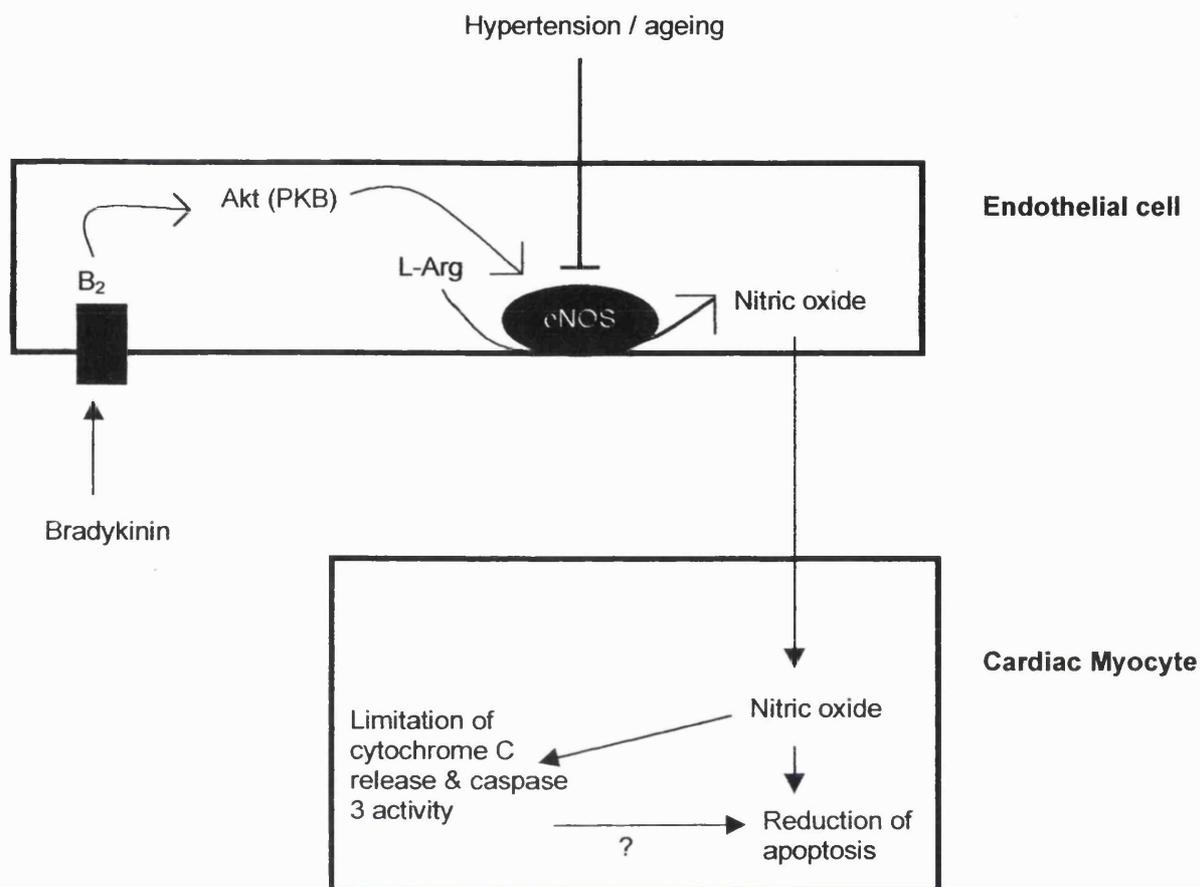


Figure 6.6. Bradykinin leads to the activation of PI3 kinase, which subsequently activates Akt. Akt leads to the phosphorylation and activation of eNOS, which may attenuate cell death by limiting apoptosis. Activity of eNOS is however impaired in aged SHR, and bradykinin at reperfusion did not attenuate infarct size in the ageing SHR. I speculate this failure of protection is related to impaired activity / expression of eNOS.

Another interesting finding was the observation that bradykinin did not enhance CFR in the SHR, whilst CFR increased markedly following bradykinin treatment in the age matched WKY rat. A similar augmentation of flow was observed when bradykinin was administered to young rats (see table 6.2, part A). Reasons for the lack of vasodilatory effect of bradykinin in the SHR are not known, although, as previously mentioned, endothelium dependent relaxations

are impaired in hypertension, due to reduced availability of nitric oxide. Consistent with this observation, Bauersachs et al reported that bradykinin induced vasodilatation was reduced in SHR hearts compared to WKY. Indeed, the authors also demonstrated a parallel reduction in eNOS expression in endothelial cells of SHR (Bauersachs *et al*, 1998).

## **6.8. CONCLUSION**

In the initial part of this study (part A) bradykinin was found to exert a cardioprotective effect when given at reperfusion. This protective action of bradykinin appears to be mediated via activation of PI3 kinase. Results imply that administration of ACE or NEP inhibitors at the time of thrombolysis in man may exert cardioprotective effects. However, ACE or NEP inhibitors at reperfusion would be expected to cause severe hypotension, which may not be desired in a post myocardial infarction patient.

In the second part of this study (part B), it was found that bradykinin administered at reperfusion did not exert a protective effect in the ageing SHR. Protection was however apparent in age matched WKY rats. Reasons for the lack of protective effect of bradykinin in the SHR remain elusive, although eNOS may be implicated.

## CHAPTER SEVEN

### Bradykinin and delayed cardioprotection

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## 7.1. INTRODUCTION

Thus far in this thesis, cardioprotective effects of bradykinin administered acutely have been investigated. In this final chapter the effects of bradykinin administered 24 hours prior to infarction are determined - completely novel effects of this peptide are reported here.

Delayed preconditioning, like classical preconditioning is dependent on the generation of various mediators during the period of antecedent ischaemia (see chapter one). Adenosine A<sub>1</sub> receptor activation and nitric oxide have both been shown to contribute independently as co-triggers for the acquisition of delayed protection (Bolli, 1997a, 2000; Baxter & Yellon, 1997b; Baxter *et al*, 1994; Qui *et al*, 1997). Baxter *et al* showed that adenosine receptor blockade during the preconditioning period abrogated the protection seen 24 hours later in rabbit heart (Baxter *et al*, 1994). Similarly, Bolli *et al* demonstrated that inhibition of NOS during preconditioning also abrogated delayed protection (Bolli *et al*, 1997a). Conversely, administration of either adenosine A<sub>1</sub> receptor agonists or nitric oxide donors could substitute for preconditioning ischaemia and induce protection 24 hours later (Baxter *et al*, 1994; Shinmura *et al*, 1999). Despite extensive literature providing evidence for the role of bradykinin in early preconditioning, it is not known whether bradykinin triggers *delayed* protection. Accordingly, the principal aim of this study was to investigate if pre-treatment with bradykinin elicits a delayed phase of myocardial protection. Nitric oxide has been demonstrated convincingly to act as a co-trigger of delayed preconditioning. Furthermore, nitric oxide has been previously implicated in some studies (though not all) as a mediator of the early cardioprotective actions of bradykinin. Therefore, it was hypothesised that bradykinin triggers delayed protection in the heart through a mechanism involving the generation of nitric oxide. In this study, rats were treated with exogenously administered bradykinin and responses to ischaemia-reperfusion were assessed 24 hours later. HSP 72 has previously been shown to be upregulated following ischaemia and heat stress induced

delayed cardioprotection (Marber *et al*, 1993). Thus, protein levels of HSP 72 were examined 24 hours following bradykinin treatment. Bolli's group have demonstrated that nitric oxide (originating from eNOS) is a trigger of delayed ischaemic preconditioning. However, it is not known what actually triggers the release of nitric oxide. Thus, it was hypothesised that bradykinin is the proximal trigger released during the preconditioning ischaemia that leads to nitric oxide generation via eNOS. In this study, it was therefore investigated whether exogenous bradykinin induces delayed preconditioning by augmenting the activity of eNOS. Accordingly, expression of various NOS isoforms were assessed 24 hours following bradykinin infusion.

## **7.2. METHODS**

### **7.2.1. Treatment Protocols**

The experimental treatment protocols are illustrated in figure 7.1. Twenty-four hours prior to infarct induction, hearts were randomly assigned to one to of the following four treatment groups.

#### Group 1: saline + saline (control)

Rats received saline 0.5 ml by dorsal tail vein injection (i.v) preceded 15-20 minutes earlier by saline 0.5 ml i.p. Animals were returned to their cages for 24 hours prior to the infarct protocol described in section 2.3.2.

#### Group 2: saline + bradykinin

Rats received bradykinin 40 µg/kg i.v., preceded 15-20 minutes earlier by saline 0.5 ml i.p. Animals were left for 24 hours as above.

### Group 3: L-NAME + bradykinin

Rats received bradykinin 40 µg/kg i.v., preceded 15-20 minutes earlier by L-NAME 10 mg/kg i.p. Animals were left for 24 hours as above.

### Group 4: L-NAME +saline

Rats received saline 0.5 ml i.v., preceded 15-20 minutes earlier by L-NAME 10 mg/kg i.p. Animals were left for 24 hours as above.

The dose of bradykinin was selected from previously published work performed by Hoagland et al who characterised effects of i.v bradykinin in dose ranges 5-80 µg/kg (Hoagland *et al*, 1999). In preliminary experiments doses of 20 µg/kg and 40 µg/kg were compared (see infarct size results). The haemodynamic response to 40 µg/kg dose was judged to be at the limit of what was tolerable. L-NAME 2.5-30 mg/kg i.p. has previously been shown to produce rapid inhibition of NOS isoforms lasting approximately six hours (Conner *et al*, 2000; Ulugol *et al*, 2000; Izzo *et al*, 1996).

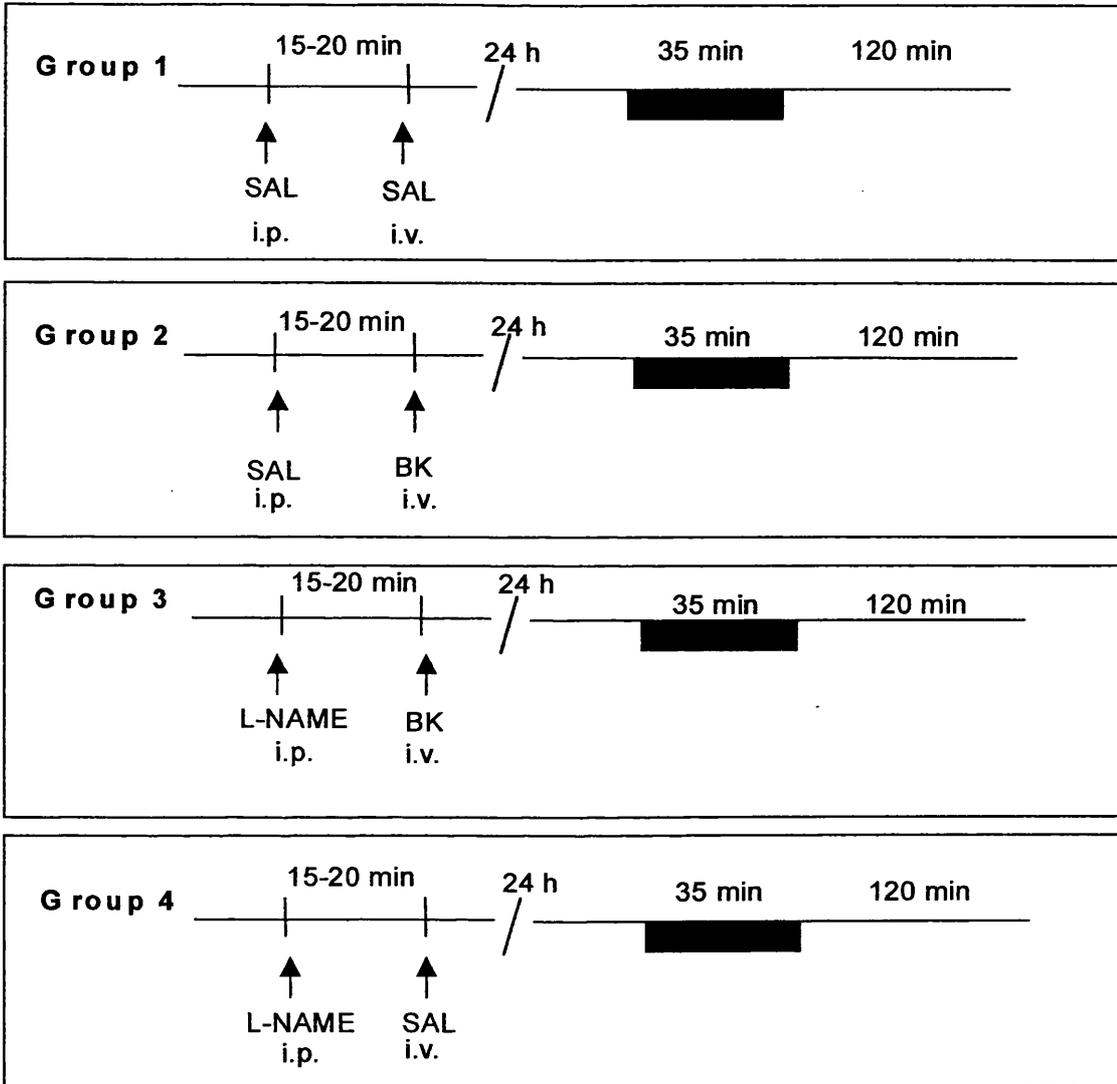


Figure 7.1. Experimental protocol. Animals were pre-treated 24 hours prior to excision of the heart and myocardial ischaemia-reperfusion sequence (SAL - saline, BK - bradykinin)

### **7.2.2. Haemodynamic effects of bradykinin pre-treatment.**

Haemodynamic effects of the agents administered to rats on day one (bradykinin, L-NAME) were investigated in a separate cohort of anaesthetised animals. As described in section 2.2, rats were anaesthetised and intubated with room air supplemented with oxygen at 70 to 75 breaths per minute (tidal volume 3-4 ml). Following the cannulation of the right carotid artery for measurement of blood pressure, the right jugular vein was also cannulated for bradykinin/saline infusion. Body temperature, arterial pH, and pCO<sub>2</sub> / pO<sub>2</sub> were monitored as described earlier (section 2.2). All rats were stabilised for a period of 10-15 minutes prior to the administration of substances. L-NAME 10 mg/kg or saline was given i.p. 15-20 minutes prior to bradykinin 40 µg/kg or saline i.v.

### **7.2.3. Detection of HSP 72 and NOS isoforms**

Twenty – four hours following the various drug treatment protocols described in section 7.2.1, hearts were excised and immediately freeze clamped in liquid nitrogen and stored at -80°C (note that the hearts were not subjected to ischaemia-reperfusion). Western blots were performed according to the technique described in section 2.7. When detecting levels of eNOS and iNOS, 60 µg of protein was separated on a 8% SDS-PAGE gel. Primary rabbit eNOS and iNOS antibodies (Santa Cruz Biotechnology) at 1:500 dilution, followed by anti rabbit horseradish peroxidase linked antibody (1:2500 dilution) were used. A 12.5% gel was prepared when analysing HSP 72 - as previously described in chapter three.

## **7.3. RESULTS**

### **7.3.1. Experimental Exclusions**

A total of 66 animals were used in this study. Thirty four animals were used for the infarct study. All animals survived the pre-treatment phase; during Langendorff perfusion, one heart was excluded due to prolonged bradycardia during the reperfusion period. Therefore,

the data for 33 successfully completed infarct size experiments are reported. Twelve animals were pre-treated but hearts were excised 24 hours later for biochemical assessment. A further 20 animals were used for acute haemodynamic measurements.

### 7.3.2. Infarct size data

Body weights and heart weights were comparable among the experimental groups (table 7.1). There were no significant differences in myocardial risk zones among the groups.

Table 7.1. Summary of body weight, wet heart weight and myocardial risk zone volume.

GROUP	n	Body weight (g)	Heart weight (g)	Risk zone Volume (cm <sup>3</sup> )
Saline + saline (control)	9	415±18	1.43±0.03	0.54±0.04
Saline + bradykinin	8	396±10	1.51±0.06	0.58±0.04
L-NAME + bradykinin	6	405±6	1.51±0.06	0.60±0.05
L-NAME + saline	7	411±8	1.52±0.03	0.54±0.04

Infarct sizes, normalised as a percentage of risk volume are shown in figure 7.2 and 7.3. Hearts from saline treated control rats exhibited an infarct size (expressed as a percentage of the risk zone) of 53.5±3.2%. Preliminary experiments were performed using a bradykinin dose of 20 µg/kg. Even though a modest reduction in infarct size was observed using 20 µg/kg bradykinin, it was not statistically significant from the saline control treated group (39.5±3.0% [n=3], P>0.05). Thus, the dose of bradykinin was doubled to 40 µg/kg, in order

to determine if a larger dose was cardioprotective. When administered to the animals, it was well tolerated like the 20  $\mu\text{g}/\text{kg}$  dose. Hence, a dose of 40  $\mu\text{g}/\text{kg}$  was used throughout the experiments, all references made to bradykinin refer to the dose of 40  $\mu\text{g}/\text{kg}$ , unless stated otherwise.

Treatment with bradykinin 40  $\mu\text{g}/\text{kg}$  24 hours prior to infarction caused a prominent reduction in infarct size to  $29.1 \pm 4.7\%$ ,  $P < 0.01$ . This protective effect was completely abrogated with the prior administration of L-NAME ( $52.3 \pm 5.0\%$ ,  $P > 0.05$ ). Finally, treatment with L-NAME and i.v saline did not influence infarct size ( $53.5 \pm 4.8\%$ ,  $P > 0.05$ ).

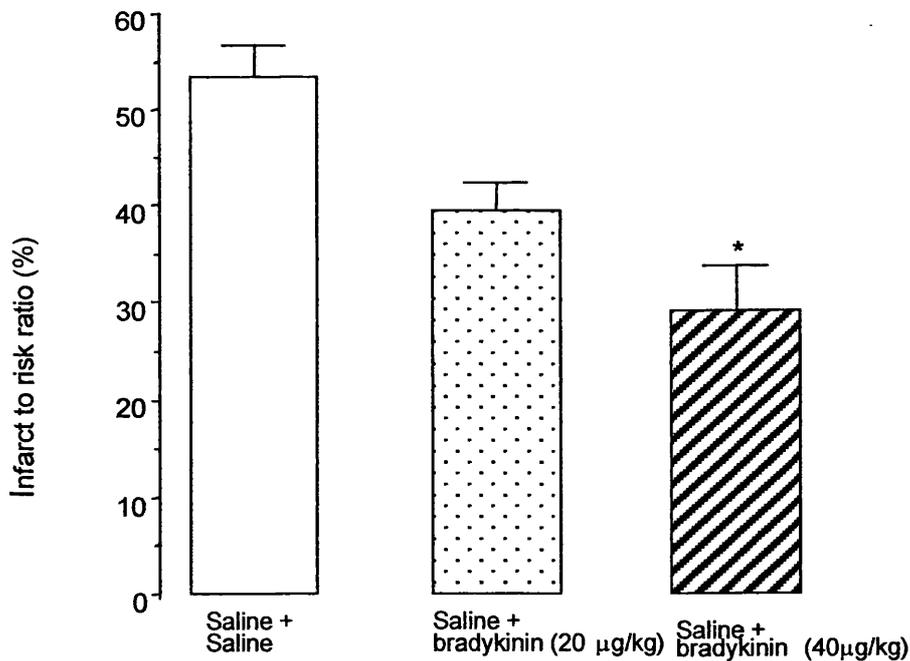


Figure 7.2. Preliminary results. \* =  $P < 0.01$  versus saline control (One way ANOVA).

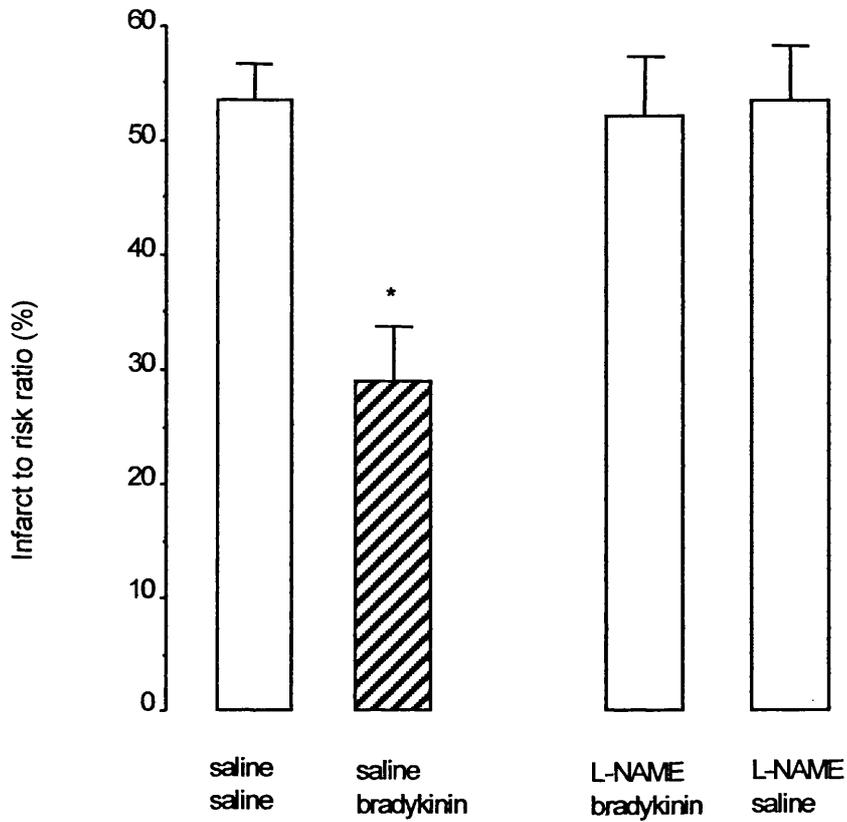


Figure 7.3. Infarct size data represented as infarct-to-risk ratio. Pre-treatment with bradykinin 24 hours prior to infarction resulted in significant limitation of relative infarct size. This delayed protective effect of bradykinin was abrogated by prior administration of L-NAME. \*  $P < 0.01$  versus saline control group (one way ANOVA)

### **7.3.3. Haemodynamic data during infarct protocol**

CFR and RPP data throughout the experimental procedure are depicted in tables 7.2 and 7.3 respectively. In the group treated with bradykinin alone (no L-NAME), CFR at baseline was significantly greater in comparison to the other experimental groups. This effect on CFR, implying a reduction of coronary vascular tone, was not seen in hearts pre-treated with L-NAME and bradykinin (group 3). During ischaemia and the remainder of the experimental protocol however, there were no significant differences among the groups although during reperfusion there was a tendency towards higher flow rates in the bradykinin treated group. RPP during pre-ischaemic stabilisation and throughout the course of the experiments did not differ significantly among the groups.

Table 7.2. Summary of CFR (ml/min)

Group	Basal	Pre-Ischaemia	-----Ischaemia-----		-----reperfusion-----		
			5 min	30 min	5 min	60 min	120 min
Saline + saline (control)	16.6±0.9	15.8±1.0	9.8±0.9	9.6±1.1	14.1±1.3	11.6±1.3	8.2±1.2
Saline + bradykinin	19.9±1.3 *	18.8±1.3 †	9.3±0.8	8.9±0.6	15.9±0.9	12.6±0.8	9.4±0.8
L-NAME + bradykinin	15.0±1.1	13.9±1.0	8.2±0.8	7.4±0.7	13.1±0.9	10.3±0.8	7.0±0.7
L-NAME + saline	15.8±1.4	15.3±1.4	9.1±1.2	8.8±1.1	13.7±1.4	11.2±1.3	8.3±1.5

Table 7.3. Summary of rate pressure product (mmHg/min X 10<sup>3</sup>)

Group	Basal	Pre-Ischaemia	-----Ischaemia-----		-----reperfusion-----		
			5 min	30 min	5 min	60 min	120 min
Saline + saline (control)	45.6±3.6	41.8±3.6	18.0±2.6	25.0±3.3	21.8±3.0	20.8±3.3	15.0±3.7
Saline + bradykinin	46.7±4.6	43.6±3.7	21.4±3.1	24.3±1.7	27.7±3.4	22.5±3.3	18.5±3.4
L-NAME + bradykinin	46.9±5.2	41.3±3.7	23.4±2.3	23.0±2.9	23.9±2.7	18.7±2.5	14.0±1.7
L-NAME + saline	40.3±2.8	35.2±3.3	18.5±3.4	22.3±2.3	19.2±2.3	17.1±2.0	12.0±2.0

\* = P < 0.05 versus all experimental groups. † = P < 0.05 versus L-NAME + bradykinin group (Repeated measures ANOVA).

#### **7.3.4. Haemodynamic effects of bradykinin *in vivo***

Variations in mean arterial pressure (MAP) induced by bradykinin (20 and 40  $\mu\text{g}/\text{kg}$ ), L-NAME (10  $\text{mg}/\text{kg}$ ) or saline pre-treatment are illustrated in figure 7.4. Following the administration of bradykinin (both doses), an immediate fall in MAP was observed. Bradykinin at a dose of 20  $\mu\text{g}/\text{kg}$  produced a smaller reduction in MAP compared with the 40  $\mu\text{g}/\text{kg}$  dose. Approximately ten seconds following the administration of bradykinin at a dose of 40  $\mu\text{g}/\text{kg}$ , there was a reduction in MAP, by approximately 40%. This hypotensive action was rapidly reversed and the MAP returned to near baseline levels within two minutes. Application of bradykinin (40  $\mu\text{g}/\text{kg}$ ) following L-NAME administration produced a similar effect. The reduction in MAP induced by bradykinin (40  $\mu\text{g}/\text{kg}$ ) was slightly attenuated by prior treatment with L-NAME suggesting that the systemic hypotensive effect of bradykinin was only partially nitric oxide-dependent. L-NAME alone did not significantly alter MAP. Heart rate (data not shown) was also monitored throughout the course of drug administration but no significant alterations were observed.

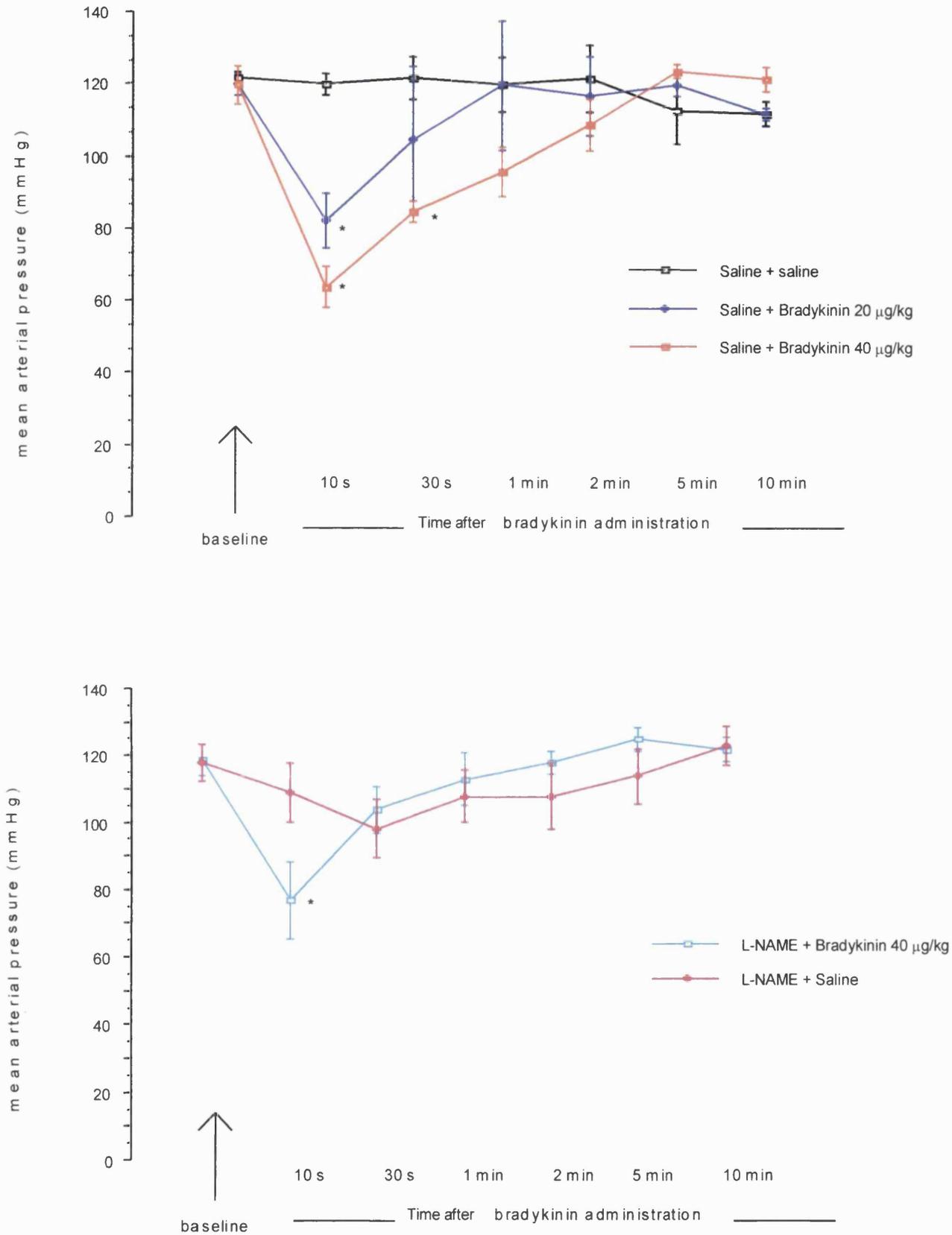


Figure 7.4. Effects of various doses of bradykinin and L-NAME on mean arterial pressure in pentobarbital anaesthetised rats. Baseline values were obtained immediately prior to i.v injection of bradykinin or saline. n = 3-5 animals per group. \* = P < 0.05 versus saline + saline group

### 7.3.5. Expression of NOS following bradykinin treatment

Expression of eNOS and iNOS were investigated 24 hours following bradykinin treatment. Figures 7.5 and 7.6 demonstrate that there was no significant induction of these proteins 24 hours following the administration of a bradykinin bolus.

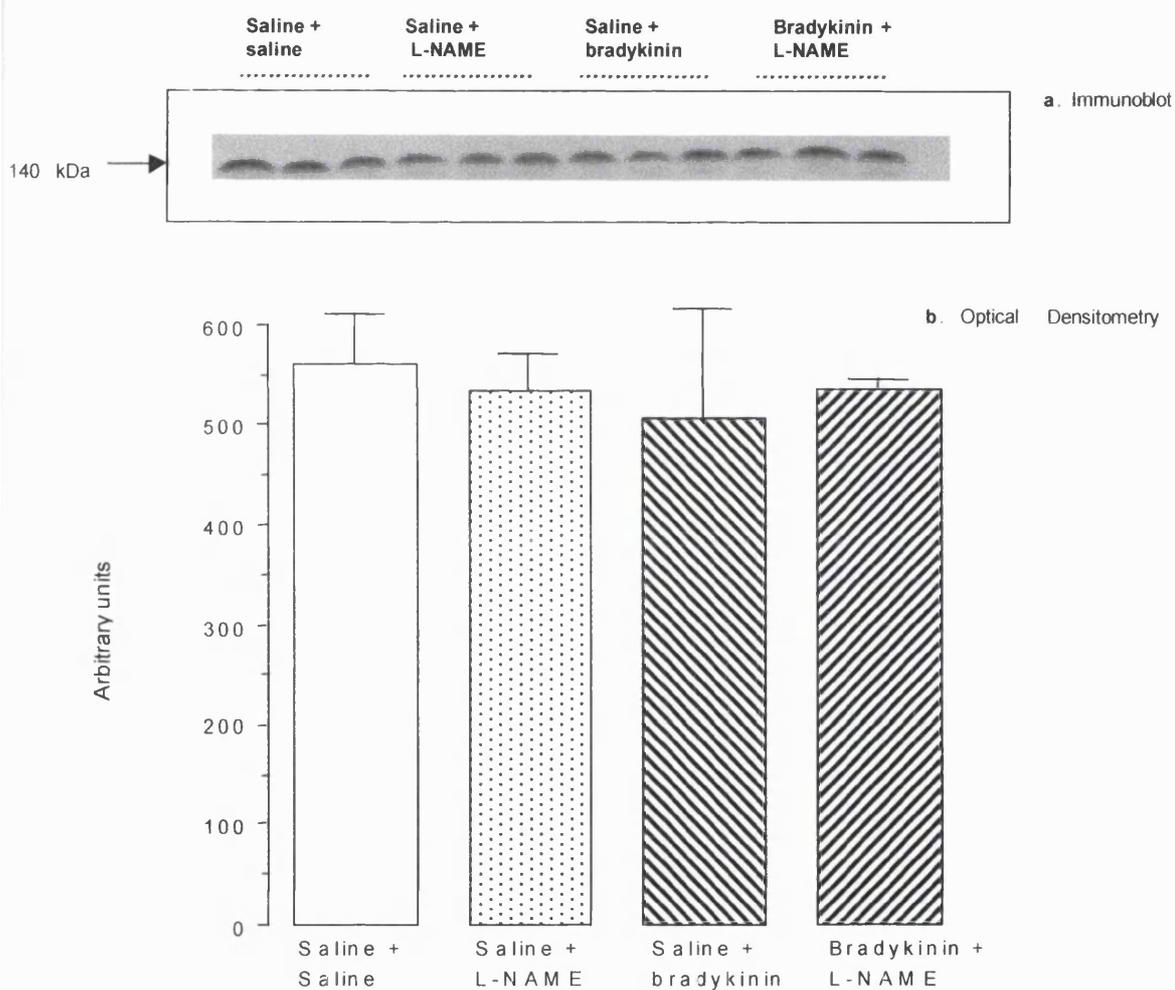


Figure 7.5.

**Panel a** - Western blot demonstrating expression of eNOS. Lanes 1-3 represent samples subjected to saline treatment (group 1). Lanes 4-6 represent samples subjected to saline and L-NAME treatment (group 4). Lanes 7-9 represent samples subjected to bradykinin treatment (group 2). Lanes 10-12 correspond to samples subjected to bradykinin and L-NAME treatment (group 3). Equal loading was ensured by Ponceau staining of membrane.

**Panel b** - Expression of eNOS in arbitrary units. No significant differences in eNOS expression were observed.

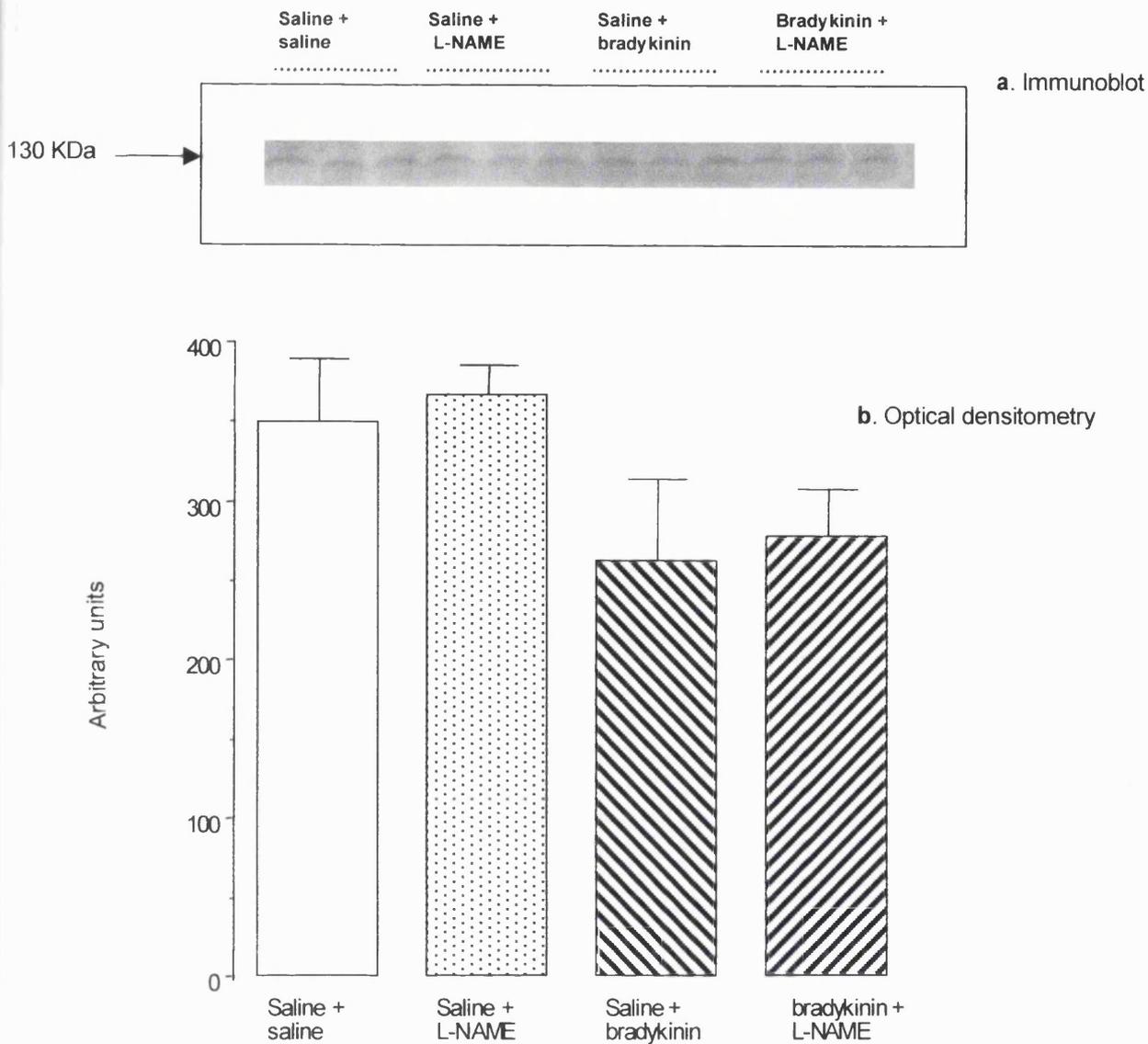


Figure 7.6.

**Panel a** - Western blot demonstrating expression of iNOS. Lanes 1-3 represent samples subjected to saline treatment (group 1). Lanes 4-6 represent samples subjected to saline and L-NAME treatment (group 4). Lanes 7-9 represent samples subjected to bradykinin treatment (group 2). Lanes 10-12 correspond to samples subjected to bradykinin and L-NAME treatment (group 3). Equal loading was ensured by Ponceau staining of membrane.

**Panel b** - Expression of iNOS in arbitrary units. No significant differences in iNOS expression were observed.

### 7.3.6. Expression of HSP 72 following bradykinin treatment

Expression of HSP 72 was investigated 24 hours following bradykinin treatment. There was no significant induction of this protein 24 hours following bradykinin treatment (figure 7.7).

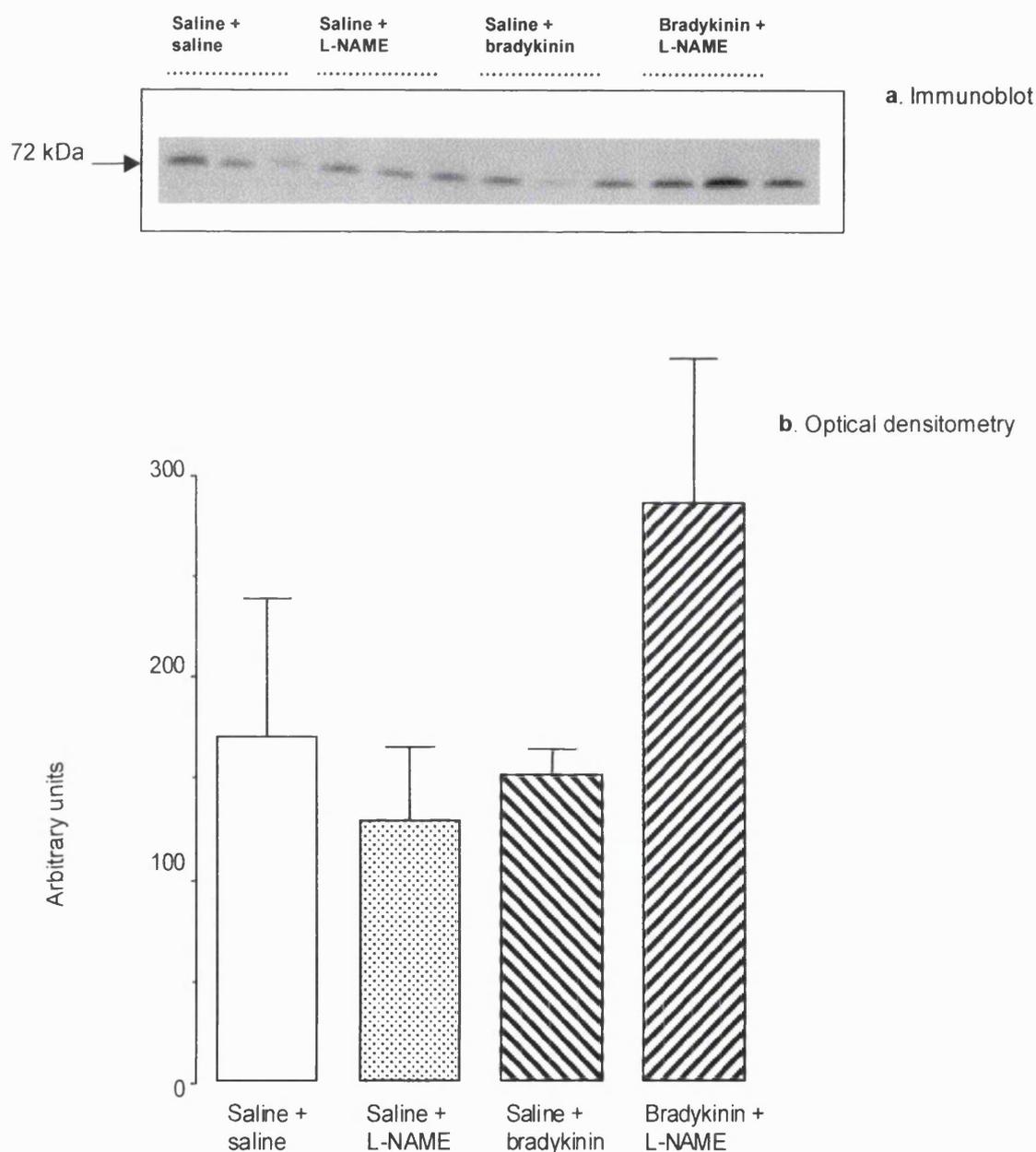


Figure 7.7.

**Panel a** - Western blot demonstrating expression of HSP 72. Lanes 1-3 represent samples subjected saline treatment (group 1). Lanes 4-6 represent samples subjected to saline and L-NAME treatment (group 4). Lanes 7-9 represent samples subjected to bradykinin treatment (group 2). Lanes 10-12 correspond to samples subjected to bradykinin and L-NAME treatment (group 3). Equal loading was ensured by Ponceau staining of membrane.

**Panel b** - Expression of HSP 72 in arbitrary units. No significant differences in HSP 72 expression were observed.

## 7.4. DISCUSSION

The major findings of the present study can be summarised as follows; (1) Systemic treatment with bradykinin evoked a **delayed** cardioprotective response 24 hours later. (2) Non-selective inhibition of NOS at the time of bradykinin administration abolished the delayed protection afforded by bradykinin. (3) The protective response was independent of the acute haemodynamic actions of bradykinin since L-NAME blocked the cardioprotective effect of bradykinin, but did not abrogate the acute hypotensive actions of the peptide. This is the first study that demonstrates bradykinin induces a delayed preconditioning like effect.

### 7.4.1. Bradykinin and delayed preconditioning

A current paradigm for delayed preconditioning invokes a primary role of several diffusible mediators generated in the myocardium during the preconditioning ischaemia. Various agents have been reported to induce delayed preconditioning. Adenosine, a key trigger of classical preconditioning has also been investigated in delayed preconditioning. Baxter et al showed that pre-treatment with CCPA, a selective adenosine A<sub>1</sub> receptor agonist caused a prominent reduction in infarct size, in the *in vivo* rabbit coronary artery ligation model (Baxter et al, 1994). Fryer et al established the ability of TAN-67, a  $\delta_1$ -opioid receptor agonist to elicit delayed myocardial protection in the *in vivo* rat model of coronary artery occlusion (Fryer et al, 1999). Indeed, opioids also have a well established role in classical preconditioning (Schultz et al, 1997). Pre-treatment with MLA (Tosaki et al, 1998), lipopolysaccharide (Zacharowski et al, 1999), lipoteichoic acid (Zacharowski et al, 2000) diacylglycerol (Baxter et al, 1997a), diazoxide (Takashi et al, 1999), nitric oxide donors (Shinmura et al, 1999), reactive oxygen species-generating solution (Takano et al, 1997), catecholamines (Meng et al, 1996a) and prostanoids (Szekeres, 1996) have been demonstrated to induce a delayed form of cardioprotection. In addition, heat stress 24 hours

prior to infarct induction is a well documented stimulator of delayed preconditioning (Marber *et al*, 1993; Cornelussen *et al*, 1998).

Despite comprehensive investigation of the various triggers of delayed preconditioning, the role of bradykinin, has not been directly examined in this phenomenon. Studies have clearly demonstrated a role for bradykinin in triggering classical preconditioning. However, it is not known if bradykinin elicits delayed preconditioning. Jaberansari *et al* recently documented that pre-treatment with an ACE inhibitor perindoprilat potentiated a subthreshold preconditioning stimulus sufficiently to induce delayed preconditioning in pig myocardium (Jaberansari *et al*, 1999). Although that study does not provide direct evidence for the involvement of bradykinin in delayed ischaemic preconditioning, the result is compatible with the hypothesis that bradykinin (or other peptides catalytically inactivated by ACE) might be implicated in triggering the delayed phase of preconditioning. Very recently however, Kukreja's group showed that delayed ischaemic preconditioning was abrogated with Hoe 140 in rabbit heart using infarct size as the experimental end point. This study which is exactly contemporaneous confirms the involvement of the bradykinin B<sub>2</sub> receptor in delayed ischaemic preconditioning (Kositprapa *et al*, 2001). Similar to the findings of this study, these authors also demonstrated that bradykinin pre-treatment 24 hours prior to infarction limited infarct size, suggesting that bradykinin can trigger delayed preconditioning. With relation to classical preconditioning, Yang *et al* has demonstrated that IPC did not protect bradykinin B<sub>2</sub> receptor knock out mice (Yang *et al*, 1997c). The present study provides confirmation that bradykinin is capable of eliciting a delayed cardioprotective response.

#### **7.4.2. Nitric oxide and delayed preconditioning**

Bolli *et al* have put forward the "Nitric oxide hypothesis" of delayed preconditioning which postulates that nitric oxide acts as both a trigger and a distal mediator of delayed myocardial

protection (Bolli, 2000). They have demonstrated that delayed preconditioning against myocardial infarction and stunning was abrogated by treatment with a NOS inhibitor during the preconditioning phase in rabbit myocardium (Qui *et al*, 1997; Bolli *et al*, 1997a). In an attempt to provide further evidence for the involvement of nitric oxide in delayed preconditioning, Bolli's group investigated if direct application of nitric oxide donors mimic delayed preconditioning. Consistent with their hypothesis, they found that pre-treatment with two structurally dissimilar nitric oxide donors, diethylenetriamine-nitric oxide and S-nitro N-pencillamine induced a delayed protective effect against both myocardial infarction and stunning 24 hours later (Qui *et al*, 1997; Shinmura *et al*, 1999). In addition, they have also reported that delayed ischaemic preconditioning was not demonstrable in iNOS knock out mice, providing strong evidence for the further involvement of nitric oxide as a distal mediator in delayed preconditioning (Guo *et al*, 1999). Further, Das's group demonstrated that pharmacological preconditioning with MLA was abolished with simultaneous treatment with L-NAME in rat heart (Tosaki *et al*, 1998). In contrast to these findings, Bell *et al* reported that CCPA induced delayed preconditioning was not abrogated in iNOS deficient mice although it was slightly attenuated (Bell *et al*, 1999). Additionally, Dana *et al* reported that CCPA induced delayed protection was not abolished with prior treatment with L-NAME (Dana *et al*, 2001). These data suggest that nitric oxide is not involved either in triggering or mediating CCPA induced delayed protection the in mouse and rabbit heart. Hence, there is controversy surrounding the role of nitric oxide in CCPA induced delayed preconditioning.

The findings of this study that bradykinin induces delayed protection through a NOS - dependent mechanism are compliant with the prevailing mechanistic view of delayed preconditioning. Figure 7.8 presents a schematic proposal for the delayed protection stimulated by bradykinin. It can be proposed that bradykinin B<sub>2</sub> receptor activation stimulates eNOS to produce nitric oxide. L-NAME inhibits the enzyme and thereby blocks

production of nitric oxide. A non selective NOS inhibitor, L-NAME was used at a dose (10 mg/kg) that has previously been reported to reliably inhibit all three NOS isoforms (eNOS, nNOS [constitutive isoforms], iNOS [inducible isoform]). Although, at present it is not certain which isoform is the source of nitric oxide in the delayed cardioprotection, it is likely to be eNOS rather than nNOS. It can be hypothesised that the nitric oxide generated as a result of bradykinin B<sub>2</sub> receptor activation could stimulate cardiac myocyte adaptation through a cascade of intracellular events similar to those invoked for delayed ischaemic preconditioning (summarised in figure 1.4, chapter one). It remains to be fully determined if nitric oxide generation via iNOS, a key mechanism in ischaemia-induced delayed preconditioning, is an additional *distal* mechanism in bradykinin-induced delayed protection.

Molecular mechanisms involved in nitric oxide induced delayed protection remain to be fully established. Bolli's group have postulated that ischaemic stress (ie, the preconditioning stimulus) leads to the generation of nitric oxide (via eNOS) and  $\cdot\text{O}_2^-$ , which react to form ONOO<sup>-</sup>, which in turn activates PKC. A complex signalling cascade then leads to the activation of other kinases and the transcription factor NF $\kappa$ B. This leads to an increase in the transcription of the iNOS gene and thereby increased activity of NOS. The increase in nitric oxide levels at this stage are responsible for mediating delayed protection, whereas, raised levels immediately following the preconditioning ischaemia are likely to be involved in triggering the delayed protection (Bolli *et al*, 1998). How an increase in iNOS expression leads to delayed cardioprotection remains elusive.

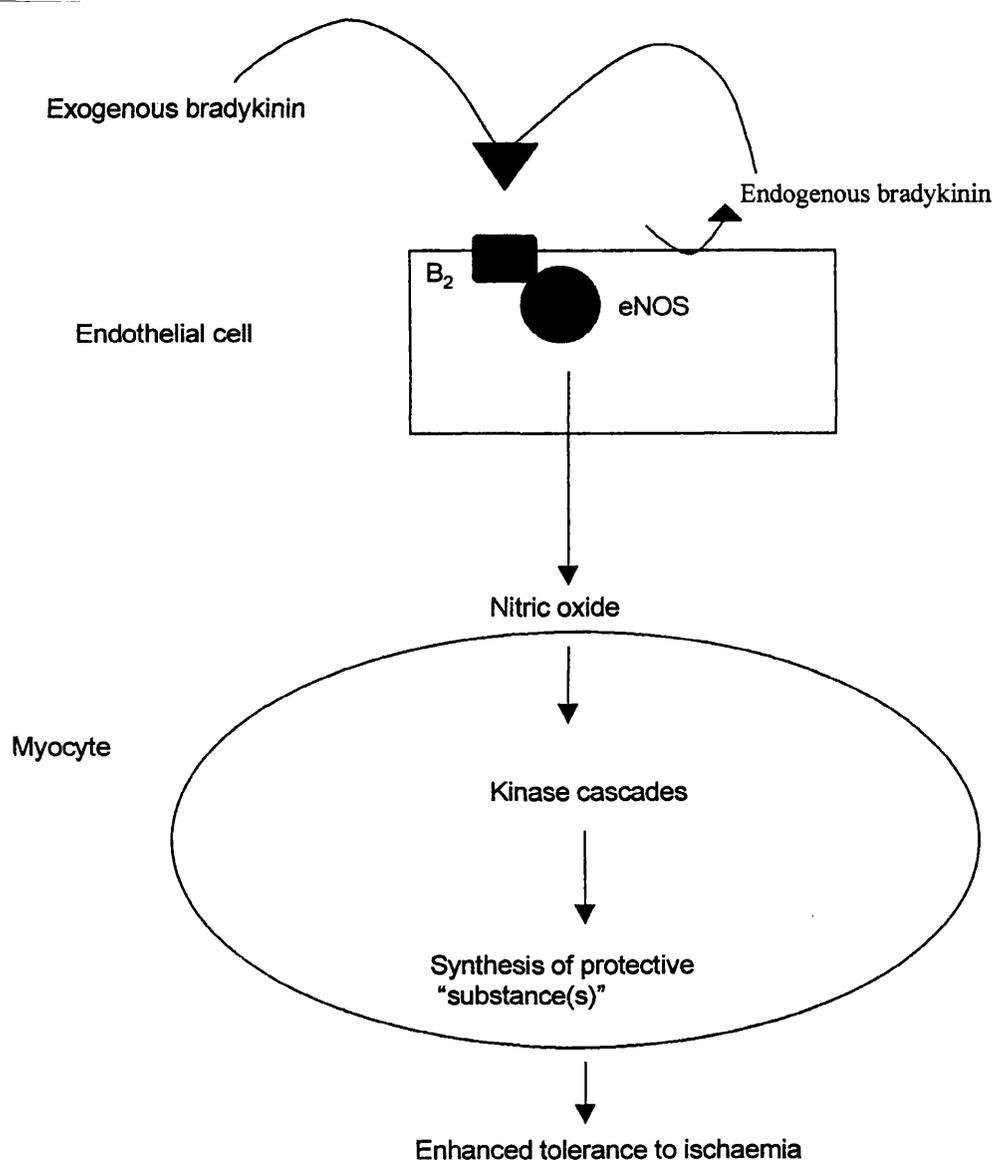


Figure 7.8. Putative mechanisms involved in the protective effects of bradykinin. Bradykinin leads to the stimulation of eNOS, which subsequently catalyses the production of nitric oxide. Nitric oxide activates kinases cascades which lead to the activation of transcription factors that enhance synthesis of "substance(s)" that protect the myocyte.

More recently, the role of COX-2 in delayed preconditioning has been the focus of attention (Shinmura *et al*, 2000; Baxter & Ferdinandy, 2001; Guo *et al*, 2000). Bolli's group demonstrated that COX-2 is involved in mediating delayed preconditioning in rabbit (Shinmura *et al*, 2000) and mouse heart (Guo *et al*, 2000). These authors subsequently demonstrated that inhibition of iNOS abrogated the increased COX-2 activity seen in delayed ischaemic preconditioning (unpublished finding), suggesting that nitric oxide (originating from iNOS) regulates the activity of COX-2.

#### **7.4.3. Role of nitric oxide in the acute versus delayed cardioprotective effects of bradykinin**

Although these data suggest a key role for nitric oxide generation in triggering bradykinin-induced delayed cardioprotection, there is no consensus that nitric oxide is involved in bradykinin-induced *acute* cardioprotection. For example, Vegh *et al* showed that the anti-arrhythmic actions of bradykinin treatment in canine heart was abolished by L-NAME (Vegh *et al*, 1993). Schoelkens and Linz showed that the functional and metabolic effects of bradykinin in the isolated rat heart were abolished by L-NAME (Schoelkens & Linz, 1992). Similarly, Feng *et al* reported that bradykinin pre-treatment improved recovery of ventricular and coronary vascular function by a mechanism that was blocked by L-NAME in rabbit heart (Feng *et al*, 2000). However, in contrast, Goto *et al* found that bradykinin-induced acute infarct limiting effect in rabbit heart was not abrogated by L-NAME (Goto *et al*, 1995). Similarly, Bugge and Ytrehus found that bradykinin-induced acute cardioprotection in rat heart was not modified by NORAG (N<sup>G</sup>-nitro-L-arginine), a NOS inhibitor with similar pharmacological profile to L-NAME (Bugge & Ytrehus, 1996). Thus, there may well be important species and end point variations in the involvement of nitric oxide in the acute cardioprotective actions of bradykinin, with some models showing nitric oxide dependency. These observations may point to critical divergences in the mechanisms involved in mediating acute and delayed actions of bradykinin.

#### **7.4.4. Vascular effects of bradykinin treatment**

The immediate systemic depressor action of bradykinin (40 µg/kg) lasted around one-two minutes and was not significantly attenuated by L-NAME. Although bradykinin-induced vasodilation may be endothelium-dependent, the contribution of nitric oxide-mediated responses may vary among vascular beds. Our observation that the systemic hypotensive effect of bradykinin was not abrogated by L-NAME is consistent with previous observations in conscious rats (Gardiner *et al*, 1990; Hoagland *et al*, 1999).

Investigators have demonstrated that the vasodilatory effect of bradykinin is only abolished with co-administration of N<sup>o</sup> methyl-L-arginine, a NOS inhibitor and the cyclooxygenase inhibitor, indomethacin in guinea pig hearts. The administration of either NMA or indomethacin alone did not prevent the effects of bradykinin. Hence, both nitric oxide and PGI<sub>2</sub> are likely to mediate the vasodilatory effect of bradykinin (Hatta *et al*, 1997).

It was observed that basal CFR was increased in hearts from bradykinin pre-treated animals. Hearts from animals that received co-treatment with L-NAME did not exhibit this basal reduction in coronary vascular tone. Previous studies of delayed cardioprotection elicited by various trigger stimuli do not reveal robust patterns of altered coronary flow. For example, Cornelussen *et al* reported that heat stress pre-treatment enhanced baseline CFR 24 hours later in the isolated working heart model (Cornelussen *et al*, 1998). Baxter *et al* reported that the Gram-negative bacterial endotoxin derivative MLA augmented coronary flow in rabbit heart 24 hours later (Baxter *et al*, 1996). Vatner's group reported that transient ischaemia augmented coronary endothelium-dependent responses 24 hours later in canine heart (Kim *et al*, 1997). On the other hand, Dana *et al* and Baxter *et al* did not find that A<sub>1</sub> agonist treatment caused an increase in CFR in either rat or rabbit heart respectively (Dana *et al*, 2000a; Baxter *et al*, 1997c). Tosaki *et al* did not observe an enhancement of coronary flow

as a consequence of pre-treatment with MLA (Tosaki *et al*, 1998). The relationship between this bradykinin-induced decrease in coronary tone and tissue injury during ischaemia-reperfusion is not known. During coronary occlusion, CFR decreased similarly in all groups and, while during reperfusion there was a tendency towards higher flow rates in the bradykinin pre-treated group, this was not statistically significant. Thus, the biological significance of alterations in coronary vessel tone and reactivity brought about by various triggers of delayed cardioprotection, and the molecular mechanisms underlying these changes is unclear at present and warrants further investigation in more appropriate and sensitive models.

#### **7.4.5. Bradykinin and NOS expression**

The eNOS is activated in endothelial cells by a variety of agents including bradykinin, acetylcholine, endothelin-1 and AT-II (ie, G-protein linked receptor agonists). Furthermore, Ju and co-workers have demonstrated that the bradykinin B<sub>2</sub> receptor is physically associated with eNOS (Ju *et al*, 1998). Marrero *et al* showed that bradykinin leads to the phosphorylation of the B<sub>2</sub> receptor and the dissociation of eNOS from the receptor, with the concomitant production of nitric oxide (Marrero *et al*, 1999). Hence, the activity of eNOS is regulated by the phosphorylation of various G-protein linked receptors, like the B<sub>2</sub> receptor. Therefore, in order to determine if eNOS was involved in the delayed cardioprotective effects of bradykinin, its expression 24 hours after the bradykinin bolus was investigated. However, no induction of eNOS was apparent 24 hours following bradykinin treatment. Thus, although eNOS was not activated 24 hours following bradykinin treatment, this does not rule out the possible involvement of eNOS in the triggering phase of bradykinin-induced delayed preconditioning. Bolli *et al* have proposed that eNOS is most likely source of nitric oxide in the trigger phase of delayed preconditioning and did not observe an increase in eNOS levels 24 hours following the preconditioning stimulus (Bolli *et al*, 1998). Hence, bradykinin

treatment may immediately lead to the upregulation eNOS as opposed to 24 hours later. In order to determine this, hearts would have to be excised immediately following bradykinin treatment and investigated for eNOS levels.

Bolli's group have also demonstrated an upregulation of iNOS 24 hours following ischaemia induced delayed preconditioning in mice (Guo *et al*, 1999). In addition, they have demonstrated that pharmacological blockade of iNOS prior to infarct induction abolishes the cardioprotective effect of delayed preconditioning (Bolli *et al*, 1997b; Takano *et al*, 1998; Guo *et al*, 1998). These findings were confirmed by an independent group; Imagawa *et al* who also reported the abolition of ischaemia induced delayed preconditioning using both dexamethasone and aminoguanidine (Imagawa *et al*, 1999). In addition to this, Das's group demonstrated that four-eight hours following MLA treatment, an induction of iNOS mRNA was detected (Tosaki *et al*, 1998). Hence, it was investigated whether iNOS mediates bradykinin-induced delayed cardioprotection. Unfortunately, many problems were encountered when detecting iNOS levels; the Western blot was repeated several times, however, only very faint protein bands could be visualised. The primary antibody was even sourced from two different laboratory suppliers, however, the quality of the bands obtained were poor (figure 7.6). This could imply two things. Firstly, the induction of iNOS may be too weak to detect under basal conditions and during the various treatments and secondly, iNOS may not be involved in mediating bradykinin-induced delayed preconditioning.

#### **7.4.6. Bradykinin and HSP 72 expression**

Studies have demonstrated an upregulation of HSP 72 content following delayed preconditioning induced by ischaemia (Marber *et al*, 1993) and heat stress induced delayed protection (Marber *et al*, 1993; Joyeux *et al*, 1998a). Additionally, Meng *et al* demonstrated an increase in HSP 72 expression following noradrenaline induced delayed preconditioning

(Meng *et al*, 1996a). No significant changes in the expression of HSP 72 were apparent 24 hours following bradykinin treatment, suggesting that this HSP is not implicated in bradykinin induced delayed preconditioning. This result is consistent with other studies investigating HSP 70 involvement in pharmacologically induced delayed preconditioning. In our laboratory, Baxter and Yellon did not observe an increase in HSP 72 levels following CCPA treatment in rabbit heart (Baxter & Yellon, 1997b). Similarly, Yoshida *et al* demonstrated that HSP 72 was not involved in MLA induced delayed protection in rabbit heart (Yoshida *et al*, 1996).

## **7.5. CONCLUSION**

In conclusion, this study demonstrates that bradykinin can elicit a delayed preconditioning-like effect in the myocardium, an action that appears to be dependent on the early generation of nitric oxide. The delayed cardioprotective effect instigated by a single bolus of bradykinin points to a novel physiological action of this peptide. Future studies could investigate whether bradykinin can induce a delayed preconditioning like effect in chronic cardiac hypertrophy.

**PART THREE**

**CONCLUSION**

## **CHAPTER EIGHT**

### **General conclusion**

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## **8.1. Summary of the work and its implications**

A substantial number of studies have demonstrated the cardioprotective properties of pre-ischaemic bradykinin treatment in a variety of animal models including human heart. The work described in this thesis demonstrated that bradykinin administered pre-ischaemically, just prior to reperfusion and 24 hours prior to infarction, reduced infarct size. Thus, bradykinin not only mimics IPC, acts as a reperfusion salvage agent but additionally mimics delayed preconditioning. Not many cardioprotective agents, if any, exert such beneficial effects.

Unfortunately, due to its potent hypotensive and pro-inflammatory effects, bradykinin cannot be administered directly to patients. However, agents that inhibit the breakdown of bradykinin, for example ACE inhibitors can be used therapeutically. Indeed, ACE inhibitors are widely used in the treatment of hypertension and heart failure. Several multi-centre clinical trials have demonstrated the efficacy of ACE inhibitors (The SOLVD investigators, 1992; The AIRE investigators, 1993; The TRACE investigators, 1995). The HOPE trial confirmed that ACE inhibitors exert effects beyond blood pressure reduction. In the HOPE trial, ramipril was found to reduce the risk of cardiovascular death, non-fatal myocardial infarction and strokes in patients with coronary heart disease, but without previous heart failure or LV dysfunction. In this trial, the mean blood pressure at entry was 139/79 mmHg. Ramipril decreased blood pressure only modestly (mean of 3/2 mmHg, systolic / diastolic, non-significant). Therefore, the blood pressure lowering effects of ramipril are not likely to be the sole mechanism for reducing ischaemic events in these patients. Additionally, benefit was seen in both patients with high blood pressure as well as those with a blood pressure of 120/70 mmHg (The HOPE investigators, 2000). These data strongly imply that ACE inhibition exerts beneficial effects, over and above blood pressure lowering.

The work described in this thesis confirms the cardioprotective properties of ACE inhibitors. It was found that captopril potentiated subthreshold preconditioning, an effect abrogated with Hoe 140, implying a role for the bradykinin B<sub>2</sub> receptor. However, when captopril was given alone (ie, in the absence of preconditioning ischaemia), no reduction in infarct size was observed. Much controversy surrounds this area of ACE inhibitor research. Some investigators report ACE inhibitors given alone are cardioprotective, while others report that direct administration of ACE inhibitors is not protective. It has been proposed that the presence of sulfhydryl groups determines whether ACE inhibitors in this setting are protective, however, ACE inhibitors devoid of a sulfhydryl moiety have been shown to induce protection, excluding this possibility. Inadvertent preconditioning could explain these discordant findings. Investigators that report ACE inhibitors alone to be protective may have accidentally subjected the myocardium to a subthreshold preconditioning episode. This is speculative and the controversy surrounding the protective effects of direct ACE inhibition is unresolved.

The work described in this thesis, reported that a dual ACE and NEP inhibitor, omapatrilat, also potentiated a subthreshold preconditioning response. However, when omapatrilat was administered alone (ie, in the absence of the preconditioning ischaemia), a reduction in infarct size was also observed. It is not known why captopril was not protective in this experimental setting, but why inhibition of both ACE and NEP, using omapatrilat produced such protective effects. However, inhibition of both ACE and NEP is expected to augment bradykinin levels to a greater extent than ACE inhibition alone. Additionally, NEP inhibition would be expected to increase levels of natriuretic peptides, which may be involved in the cardioprotective effects of omapatrilat.

Agents that mimic IPC have to be administered prior to the ischaemic event in order to limit injury. However, this poses a major problem as it is impossible to forecast when a patient will

experience a myocardial infarction. Greatest benefit in the clinic would be observed if agents could be administered after coronary occlusion or at reperfusion and therefore, limit reperfusion injury. In chapter six, it was demonstrated that bradykinin given at reperfusion, produced a substantial reduction in infarct size. This observation may have potential benefits in the clinic. However, once again, as bradykinin cannot be administered directly to patients, ACE inhibitors could be administered at the time of reperfusion instead. The protective effect of ACE inhibitors at reperfusion is largely under-investigated, thus, future studies could determine whether ACE inhibitors administered at reperfusion reduce infarct size.

Unfortunately, the protective actions of bradykinin, and captopril that were observed in the normal, healthy myocardium, could not be reproduced in the hypertrophied myocardium. Additionally, IPC was absent in 11-13 month old SHR and age matched normotensive animals. It is not known why IPC, bradykinin and captopril did not limit infarct size in chronic hypertrophy. However, defects in the kallikrein-kinin system, and impaired signalling pathways in hypertension / hypertrophy and ageing may interfere with the protective signal transduction pathways of these cardioprotectants. Future studies are warranted to investigate why these cardioprotective strategies are not effective in chronic hypertrophy (see section 8.2 below).

## **8.2. Limitations of study and possible future studies**

The present work was performed in the *in vitro* experimental setting. This can be regarded as a disadvantage as obviously, *in vivo* experiments mimic the clinical setting more closely. Thus, future work could concentrate on examining ischaemia-reperfusion and cardioprotection in models of hypertrophy in the *in vivo* setting. Experiments involving ischaemia-reperfusion in models of chronic hypertrophy are scarcely performed *in vivo*. Additionally, in this work infarction was induced by ligating the coronary artery for 35 minutes.

This does not parallel the situation in man, in which the process of coronary artery disease occurs over years.

Experiments performed here very much relied upon a pharmacological approach (ie, the use of drugs), hence, future studies described below could be performed maximising the potential of sophisticated molecular biological techniques that are available today. In chapter four, I demonstrated that cardioprotective effects of omapatrilat were dependent upon bradykinin B<sub>2</sub> receptor activation. Although it is thought that Hoe 140 is a highly selective and specific antagonist of the B<sub>2</sub> receptor, it is not known if it exerts any effects on natriuretic peptide receptors. Therefore, to confirm our findings, experiments involving omapatrilat could be repeated using bradykinin B<sub>2</sub> receptor knockout mice. Additionally, even though bradykinin levels were measured using radio-immunoassay in this study, rather puzzling results were obtained with omapatrilat. Future studies could identify reasons as to why omapatrilat did not augment bradykinin levels.

I have reported that IPC did not protect the ageing SHR or WKY rat hearts. Reasons for this were not investigated therefore, further studies are required to investigate the signalling pathways that are perturbed during the process of chronic hypertrophy. As mentioned previously, PKC is thought to be implicated in mediating both IPC and hypertrophy. Hence, PKC may be downregulated in hypertrophy and resistant to further activation by IPC. Basal PKC expression (and following IPC) at the protein and mRNA level could be examined using Western blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) respectively.

Furthermore, effects of chronic hypertrophy and ageing must be dissociated, so that one can establish whether ageing or long standing hypertension or a combination of the two factors

interfere with IPC. Captopril (or any other ACE inhibitor) should be given to SHRs on a daily basis in drinking water. In this way hypertension will be controlled and the effects of IPC can be investigated solely on the ageing rat.

I hypothesised that bradykinin levels may be diminished in hypertension which could account for the lack of protection observed with IPC and captopril. However, bradykinin levels were not measured in the SHRs, therefore, future studies could determine levels of bradykinin in hypertrophy. In addition to this, defects in the bradykinin B<sub>2</sub> receptor may exist in hypertrophy, this could be investigated using RT-PCR.

Finally, the work performed in this thesis examined classical preconditioning in hypertrophy. Even though classical IPC was not protective in chronic hypertrophy, this does not exclude the possibility that delayed preconditioning would not evoke protection in this pathology. Due to its longer duration of protection and thus clinical applicability, future work could investigate *delayed preconditioning* in models of chronic hypertrophy.

### **8.3. Clinical implications**

As mentioned previously, despite its potent cardioprotective properties, bradykinin cannot be administered to patients. However, instead, agents that inhibit the catalytic breakdown of bradykinin can be used therapeutically. Indeed, ACE inhibitors are very beneficial in the treatment of hypertension, heart failure, and in coronary heart disease patients. Additionally, as described here, omapatrilat provided significant protection against ischaemia-reperfusion injury. Indeed if these findings could be validated in man, omapatrilat could prove to be very beneficial therapeutically. However, large, multi-centre clinical trials must be performed in order to assess the real therapeutic effectiveness of dual ACE and NEP inhibitors.

Myocardial hypertrophy induced by hypertension is associated with high rates of mortality. The results obtained in this thesis imply that, the phenomenon of IPC, which has received much attention from the cardiovascular community for the last 15 years, may not protect the chronically hypertensive / elderly patient. Additionally, captopril, a widely used ACE inhibitor, was not protective in the ageing SHR, implying that ACE inhibitors may not be as cardioprotective in the ageing hypertensive patient. Clearly, further studies are required to address the role of ACE inhibitor therapy in the chronic hypertension.

Mortality as a consequence of heart disease still remains exceptionally high, however, it is hoped that basic scientific research together with clinical research will not only increase our knowledge of heart disease but also ultimately provide benefit for the patient.

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