Corrigenda

Additional References

p43 para 1 (Yellon 1994) after the sentence ending "in animal studies".

p40 middle para (Welch 1986) after the sentence ending "nucleotides like ATP".

p181 (Latchman 1997 personal communication) after the sentence ending "central nervous system".

Additional Figure: 4.6 b

Original data of force measurements

Human atrium as a model compared to ventricle:

To be inserted at the end of section 8.1 on 174.

There are differences between atrium and ventricle as discussed above. However specimens of human ventricle are difficult to obtain and where they are obtainable, e.g. children undergoing surgery for Fallots tetralogy, they are abnormal. Human atrium was used in preference to animal ventricle for the following reasons:

Mechanisms of preconditioning vary from species to species (Baxter 1996), and since it is human heart disease we are ultimately interested in treating then human tissue must be examined.

Expression of stress proteins is also different, in that hsx70 is uniquely present in the human (Pelham 1986), and hence the relevance of animal models is limited.

Myocardial protection: from cell culture to human in vitro models

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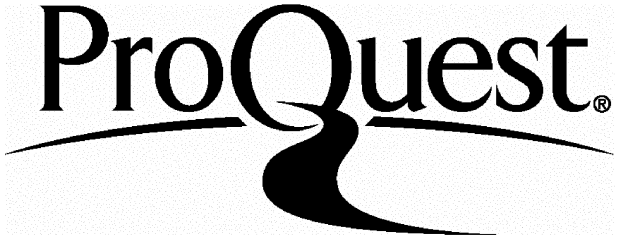

Abstract

Background: In the Western world acute myocardial infarction remains one of the most important causes of mortality in adults. Although advances such as thrombolysis and primary angioplasty have revolutionised treatment, myocardial infarction remains a major challenge. This is in part because benefits diminish as treatment is delayed. Hence the potential for exploiting the endogenous protective mechanisms within the myocardium is of prime clinical importance. Any potential agent which induces a state of enhanced resistance to ischaemia warrants investigation as it may potentially lead to the development of novel therapeutic approaches to cardioprotection in clinical practise.

Aim: The broad aim of this thesis is to investigate possible pharmacological therapeutic approaches to cardioprotection.

Methods: Two routes to myocardial protection were studied:

- The potential role for up regulating stress proteins in order to protect the myocardium was examined in an isolated rat neonatal cardiomyocyte model. A tyrosine kinase inhibitor known to induce stress proteins in non cardiac cells was studied in detail.

- The cardioprotective effects of angiotensin converting enzyme inhibitors were studied in a superfused isometrically contracting human atrial trabeculae model.

Results: It is possible to specifically up regulate 70 kDa heat stress proteins and induce a state of enhanced resistance to myocardial injury. Furthermore, in a human atrial model of ischaemia, angiotensin converting enzyme inhibitors exert cardioprotective effects, possibly via B_2 bradykinin receptors.

Conclusions: These results demonstrate that the endogenous protective mechanisms within the myocardium may be amenable to therapeutic manipulation. In the future both routes to protecting the myocardium could be of use in a clinical setting. For example a prolonged state of protection via induction of stress proteins could be superimposed with a shorter classical preconditioning type of protection when needed, for example prior to high risk coronary angioplasty or coronary artery bypass surgery.

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Abbreviations

ACE	Angiotensin converting enzyme inhibitor
ANOVA	Analysis of variance
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BK	Bradykinin
CK	Creatinine kinase
CAT	Chloramphenicol acetyl transferase
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
grp	Glucose regulated protein
HSE	Heat shock element
HSF	Heat shock transcription factor
hsp72	Inducible member of the 70kDa heat stress protein family
hsp73	Constitutive member of the 70kDa heat stress protein family
hsp90	The 90 kDa heat stress protein
hsp60	The 60kDa heat stress protein
hsp25	The 25 kDa heat stress protein
hoe 140	Icatibant
IgG	Immunoglobulin class G
ILGF-1	Insulin-like growth factor
kDa	kiloDalton
kPa	Kilo Pascal
LDH	Lactate dehydrogenase
mRNA	Messenger RNA
NO	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PGI_2	Prostacyclin
pO2	Partial pressure of oxygen
RNA	Ribonucleic acid
RSV	Rous sarcoma virus
SDS	Sodium dodecyl sulphate
SH	Sulphydryl group
SOD	Superoxide dismutase
TEMED	NNNN-tetraethylethalinediamine
TLC	Thin layer chromatography
TRIS	Tris(hydroxymethyl)methylamine

Acknowledgements

I would like to express my gratitude to the British Heart Foundation for providing the junior BHF Fellowship which enabled me to carry out this work. I am very grateful to my supervisor, Professor Derek Yellon for his guidance and support over the past two years. I would also like to thank the staff at the Hatter Institute for their help and advice which was greatly appreciated during the course of this thesis.

Chapter 1

Introduction: Ischaemia and Ischaemic Preconditioning

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1 Myocardial Ischaemia and infarction

In the Western world acute myocardial infarction remains one of the most important causes of mortality in adults. Although advances such as thrombolysis and primary angioplasty have revolutionised treatment, myocardial infarction remains a major challenge despite effective reperfusion of jeopardised myocardium. This is in part because benefits diminish as treatment is delayed. The longer the duration of ischaemia, the more complete the necrosis, and the less the salvage on reperfusion. Hence any intervention that could reduce the rate of necrosis prior to thrombolysis would preserve left ventricular function and reduce mortality. The rate of myocardial necrosis is determined by the collateral blood supply to the ischaemic zone and the inherent resistance of the myocardium to ischaemia (Reimer and Jennings 1979). Following occlusion of an infarct related artery distal pre-formed collateral's are likely to be operating maximally. The need is therefore to understand the processes that determine the inherent myocardial resistance to infarction.

It is now known that the resistance of the myocardium to ischaemia can be enhanced by both classical preconditioning (short periods of ischaemia with intermittent reperfusion) and the upregulation of cytoprotective proteins. These will be discussed in more detail later.

1.1 Pathophysiology of myocardial ischaemia

Myocardial ischaemia can be defined as a reduction in coronary flow such that the supply of substrate and the washout of metabolites is insufficient to meet the metabolic demands of the myocardium. This results initially in a reduction in contractility with progression to cell death if ischaemia is severe and prolonged. When coronary flow is reduced, in the first seconds of ischaemia, contractile failure occurs as a result of the stretching effect that an "inflated" coronary

vasculature has on the myocyte (Koretsune et al 1991), known as the garden-hose effect.

After these first few seconds of ischaemia contractility is reduced by several metabolic and ionic changes occurring within the myocyte (Poole-Wilson 1992). Reduced tissue pO_2, cessation of aerobic (mitochondrial) ATP production, and accumulation of inorganic phosphate (initially derived from the hydrolysis of creatinine phosphate) and magnesium. The accumulation of inorganic phosphate has a direct inhibitory action on the contractile proteins and reduces the free energy available from ATP hydrolysis, whilst magnesium probably competes with calcium for the calcium specific binding sites on troponin C. These processes account for the early contractile failure occurring during the first 1-3 minutes of ischaemia (Allen et al 1987).

During the next few minutes glycolysis is initially stimulated, largely as a result of AMP and inorganic phosphate increasing the activity of phosphofructokinase, the rate limiting control enzyme of the glycolytic pathway (Opie1992a and b). However the reduced tissue pO_2 inhibits mitochondrial metabolism, the end products of glycolysis are not converted to CO_2, resulting in accumulation of protons and lactate. Lack of metabolite washout results in proton and lactate build up and inhibition of glycolysis by its end products. Proton accumulation also results in reduced affinity of troponin C for calcium (Blanchard and Solaro 1984) causing further loss in contractile function.

1.2 The onset of contracture

After 10-15 minutes of ischaemia, ATP levels have fallen sufficiently to prevent the detachment of myosin crossbridges by myosin ATPase (Katz 1992). Furthermore, co-operativity of crossbridge formation occurs at low ATP levels (Ford 1990), so that for a given intracellular calcium concentration, crossbridge attachment rates are increased. This leads to rigor crossbridge formation

causing tonic myocyte shortening, manifest as an increase in resting tension in isometric isolated muscle. The fall in ATP effects other ATP dependant processes, most notably ionic gradients become disturbed as Na/K ATPase, sarcolemmal Ca^{2+}ATPase and sarcoplasmic Ca^{2+}ATPase activities fall. The failure of these ionic pumps results in intracellular accumulation of calcium and sodium (Allen and Orchard 1987). This calcium accumulation further increases rigor crossbridge formation and may additionally compromise mitochondrial function, since mitochondria are freely permeable to calcium which flows down its electrochemical gradient (Crompton et al 1988), predominantly when the mitochondrial proton gradient is recharged upon reperfusion.

1.3 Irreversible ischaemic injury and reperfusion injury

There is a critical time point beyond which ischaemic injury becomes irreversible. The critical changes that occur within a myocyte to determine its survival upon reperfusion are unknown. Reperfusion itself may exacerbate preceding ischaemic injury and further contribute to cell death - this deleterious effect is known as reperfusion injury (Yellon and Downey 1990). We do know that the re admission of oxygen results in the formation of free radicals, (Kukreja and Hess 1993) which may be important in the genesis of myocardial stunning (Bolli 1990) and reperfusion arrhythmias (Hearse and Tosaki 1987). Myocardial calcium overload is also associated with reperfusion (Opie 1989), largely as a result of intracellular hydrogen and sodium accumulation during ischaemia. On reperfusion, Na^{2+} accumulation leads to Na^{2+}/Ca^{2+} exchange, and proton accumulation leads to Na^{2+}/H^{+} exchange - thus maintaining inappropriately high intracellular Na^{2+} levels despite extrusion by the Na^{2+}/K^{+} ATPase which becomes active during reperfusion (Allen and Orchard 1987). Therefore, H^{+} accumulation during ischaemia indirectly worsens calcium overload upon reperfusion (Lazdunski et al 1985).

Hence the processes accompanying ischaemia/reperfusion injury are extremely complex, and prevention of this damage remains a major challenge for cardiologists. However, the myocardium does have an endogenous protective mechanism known as ischaemic preconditioning:

2 Ischaemic preconditioning

2.1 Definition of ischaemic preconditioning

A brief period of ischaemia followed by reperfusion renders the heart more resistant to injury from a subsequent longer ischaemic insult. This rapid, adaptive and highly protective response of the heart is known as ischaemic preconditioning.

2.2 Classical preconditioning

2.2.1 Historical perspective

Murry, Jennings and Reimer first described ischaemic preconditioning of the canine heart in 1986 (Murry et al 1986). Their study was designed to examine the effect of brief episodes of ischaemia on energy metabolism. They demonstrated that when the myocardium was pre-treated with four, 5 minute coronary artery occlusions, each separated by 5 minutes of reperfusion, the amount of myocardial necrosis after a subsequent 40 minute coronary occlusion was reduced by almost 75%. Subsequently this phenomena has been observed in many laboratories both in vivo and in isolated hearts using a variety of endpoints of ischaemic injury, including tachyarryhthmias (Osada et al 1991), enzyme efflux, and post ischaemic recovery of myocardial contractile function. This classical form of preconditioning is critically time-dependent, and

protection against all endpoints of injury is lost if the reperfusion period between preconditioning and the long ischaemic insult is extended beyond one or two hours (Van Winkle et al 1991).

Critical features of preconditioning defined in early studies are the rapidity of onset and the brevity of the protective effect. Although protection was observed almost immediately, if the interval between preconditioning ischaemia and the longer ischaemic insult was extended beyond 1-2 hours, protection was lost (Jenkins et al 1995a). However subsequent animal studies have shown that a biphasic pattern of protection is induced in preconditioned myocardium, occurring about 24 hours later and lasting much longer than the classic effect (see below). These two forms of adaptation are almost certainly mechanistically distinct but share a common trigger - i.e. transient ischaemic stress.

2.2.2 Temporal limitations to ischaemic preconditioning

There are three important temporal limitations that are characteristic of the protection afforded by classical ischaemic preconditioning. Firstly, the preconditioning ischaemia must be followed rapidly by the sustained occlusion, for example Li et al (Li et al 1994) found that in a rat model protection was lost if a delay of 1 hour was imposed between preconditioning ischaemia and the sustained occlusion. However the protection could be re-established if, after the 1 hour delay, rats received a further preconditioning ischaemic stimulus. In addition, a second window of protection occurs after 24 hours (see below).

Secondly, the heart becomes tolerant to numerous repeated preconditioning stimuli. For example Cohen (Cohen et al 1994) showed that one brief episode of ischaemia preconditioned the rabbit heart, protection was attenuated with 40-65 repeated 5 minute occlusions over 3-4 days before the sustained occlusion.

However this tolerance could be reversed by 3 days without intervention, such that protection could be re-established with a further brief occlusion prior to the sustained ischaemia.

Thirdly, protection is lost with increasing duration of sustained coronary artery occlusion (Murry 1986). Hence, preconditioning limits infarct size by delaying myocardial cell death - but does not in itself prevent necrosis.

2.2.3 The widening concept of preconditioning

Ischaemic preconditioning not only protects against necrosis, but also against other consequences of ischaemia/reperfusion injury - such as arrhythmias and post ischaemic contractile dysfunction. Unlike the delaying effect which preconditioning has on myocardial necrosis, it does not seem to alter the "bell-shaped" temporal relationship between the severity of reperfusion induced arrhythmias and the preceding duration of ischaemia (Lawson and Hearse 1992). The protective effect appears to be due to an absolute reduction in arrhythmia severity.

Protection against contractile dysfunction was first demonstrated in rabbits by Cohen et al (1991) who demonstrated improved recovery in segmental shortening, and in 1993 Przyklenk et al demonstrated a correlation between improved recovery of systolic shortening and reduced infarct size in a model of regional ischaemia. A study by Cave et al (1993) showed a reduction in enzyme leakage in association with enhanced functional recovery in a preconditioned group of isolated rat hearts subjected to global ischaemia. Hence the enhanced recovery of function due to preconditioning results from a reduction in necrosis, is proportional to infarct volume and is not due to a reduction in stunning.

Hypoxia appears to be as effective as ischaemia in inducing the protection of preconditioning, with evidence in animal models of regional and global hypoxia as well as in cell culture. For example Lasley et al (1993) found no difference in improvement in post ischaemic functional recovery in isolated perfused rat hearts pre-treated with either ischaemia or hypoxia prior to the long global ischaemic insult. This implies that accumulation of anaerobic metabolites is unlikely to be an important mechanism in preconditioning. Other studies demonstrating the effectiveness of hypoxia in inducing protection are discussed in chapter 4.

2.2.4 Clinical correlates of preconditioning

From a clinical perspective evidence for preconditioning in human myocardium is still relatively limited although experiments on human trabeculae by Walker et al (Walker et al 1994) and Speechly-dick et al (1995) confirm that human cardiac tissue can be preconditioned. These experiments have confirmed that ischaemic preconditioning is effective in an in vitro human model, and furthermore that this preparation was able to demonstrate characteristics of the preconditioning phenomenon, that is, it responded to adenosine receptor and K_{ATP} channel activation, as well as being mediated via the protein kinase C signalling system. Further evidence comes from studies on ventricular myocytes by Ikonomidis et al (Ikonomidis et al 1993) who demonstrated that preconditioning can occur in cardiomyocytes independent of interactions with other cell types. Furthermore he showed that exposure of non preconditioned cells to supernatant from preconditioned cells (exposed to 20 minutes of ischaemia) could confer protection against prolonged ischaemic conditions. Addition of the adenosine receptor antagonist 8-p-sulphophenyl theophylline (SPT) to the supernatant abolished the protection. In addition, adenosine

mediated protection of human ventricular cardiomyocytes was also dependent upon PKC.

There is also some evidence that the myocardium might be preconditioned in the clinical setting of unstable angina prior to myocardial infarction. For example Ottani and colleagues reported that infarct size was smaller in a group of patients who had angina in the 24 hours preceding the onset of myocardial infarction (Ottani et al 1993). Protection occurring 24 hours later is more consistent with the delayed phase (second window) of protection and is discussed in further detail in section 3..

During percutaneous transluminal angioplasty, Deutsch et al (1990) found that during the second inflation patients experienced less anginal pain, and there was less lactate production and less ST segment deviation on the ECG in comparison with the first inflation. There was no increase in great cardiac vein flow during the second inflation compared to the first, suggesting that the beneficial effects were not due to acute recruitment of extensive collateral flow (Deutsch et al 1990). Cribier et al (1992) studied patients undergoing five sequential angioplasty balloon inflations and also described less chest pain and ST segment shift with subsequent inflations compared to the first inflation. Although in 10 of the 17 patients there was angiographic evidence of collateral recruitment; in the remaining 7 there was not - yet even these patients experienced less anginal pain and ST segment shift. These latter patients may well have experienced true ischaemic preconditioning unrelated to recruitment of collateral vessels. More recently Tomai et al (1994a) observed that glibenclamide (a blocker of the K_{ATP} channel) prevented the beneficial effects of a second balloon inflation on ST segment shift. Furthermore, two adenosine receptor antagonists aminophylline (Claeys et al 1994) and bamiphylline (Tomai et al 1994b) were also shown to block the beneficial effects of repetitive angioplasty.

A practical consideration of these observations is that "high risk" angioplasties in which the occluded vessel supplies a large "area at risk" might benefit from a staged procedure whereby the balloon is initially inflated for 60-90 seconds, reperfusion allowed for several minutes, and then second, third and fourth inflations are produced. The "ischaemic burden" during the subsequent inflation compared to the first should be lessened by preconditioning, and / or recruitment of collateral vessels. Angioplasty might also serve as a useful tool in testing "preconditioning mimetic agents" - those agents which can stimulate the pathways that lead to preconditioning without inducing ischaemia.

Most recently Bolli's group have shown that adenosine preconditions human myocardium against ischaemia in selected patients undergoing percutaneous transluminal coronary angioplasty (PTCA) even more effectively than ischaemic preconditioning itself (Leesar 1997). In this study intracoronary infusion of adenosine prior to PTCA rendered the myocardium remarkably resistant to subsequent ischaemia as shown by ST segment shift and measurements of chest pain scores.

2.3 Mechanisms of Classical "early" preconditioning

Despite intense research the exact mechanism underlying the protection has yet to be elucidated. The most widely currently held hypothesis is that an endogenous ligand, such as adenosine, on binding to its receptor on the cardiocyte surface, activates phospholipase C (PLC) via a G protein; activated PLC causes the breakdown of phosphatidylinositol 4,5 biphosphonate and phosphatidyl choline to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate; increased DAG then activates protein kinase C (PKC) in the membrane. Downey and colleagues (Lawson and Downey 1993) (Downey and Cohen 1996) have developed the hypothesis that stimulation of a variety of G protein-coupled receptors (such as adenosine A_1, alpha 1 adrenergic,

muscarinic, bradykinin and endothelin-1 receptors) results in activation of PKC. This leads to translocation of PKC from the cytoplasm to the sarcolemma, where it phophorylates a substrate protein, possibly the ATP sensitive potassium (K_{ATP}) channel, resulting in resistance to ischaemia. There is a considerable degree of support for this hypothesis from studies with activators or inhibitors of PKC and its translocation process, with receptor agonists and antagonists, and with agents that interfere with the signalling pathway to PKC. However there is significant controversy surrounding this "Downey" hypothesis (Brooks and Hearse 1996). PKC has at least 11 isoforms, its biochemistry is complicated and not completely understood. Hence, there are many potential pitfalls in experiments investigating these pathways which play a role in this controversy.

Support for the "Downey" hypothesis comes from papers recently published by Light et al (Light et al 1996) who showed that K_{ATP} channels were activated at physiological levels of ATP by the application of purified PKC to inside-out isolated rabbit myocyte membrane patches. In fact the probability of the K_{ATP} channel opening was increased three fold and the response could be blocked completely with the specific peptide inhibitor of PKC. These authors suggest that PKC catalyses the phosphorylation of the K_{ATP} channel thus resulting in preconditioning.

However, several groups have had conflicting results. For example Przyklenk et al (Przyklenk et al 1995) showed that PKC inhibition resulted conversely in additional protection in the preconditioned canine heart. They were unable to show any difference in total amount or subcellular distribution of PKC during ischaemic preconditioning. Furthermore, using a direct measure of PKC activation they demonstrated that preconditioning did not result in activation of PKC. Even more recently, Valhaus et al (Valhaus et al 1996) were unable to block preconditioning in pigs with staurosporine, a potent PKC inhibitor.

Similar negative studies with regard to the role of PKC come from studies in other models, such as the rat (Galinanes et al 1996, and Moolman et al 1996) and the rabbit (Simkhovich et al 1995).

Unfortunately the choice of agent used in preconditioning experiments varies widely and this is compounded with the fact that many of these agents will have secondary non specific pharmacological actions. For example vascular spasm, leukocyte activation and cardiac depression may occur at pharmacologically active doses of these agents.

Hence, evidence is mounting that PKC may not be an obligatory mediator. It is known that cross talk can occur between signalling pathways, so that for example when ischaemia or alpha$_1$-adrenergic agonists precondition the heart multifactorial signalling cascades are activated and PKC may be stimulated as a secondary response. Endogenous mediators such as bradykinin, which lead to NO release and subsequent activation of guanylate cyclase, can protect the heart via a PKC independent pathway (Parratt 1994a) is further evidence against PKC as a common mechanism. Other possibilities include the mitogen-activated protein kinase (MAPK) which is activated in response to stress and hypertrophic agents (Gillespie-Brown et al 1995). MAPK can be stimulated by G_1 - coupled transmembrane receptors. A possible explanation as to why PKC activation occurs in some studies and not others is that raf-1, which acts upstream from MAPK, can be activated by both PKC dependent and independent pathways. Further elucidation of these possible pathways awaits development of selective inhibitors and activators of MAPK.

2.4 Endogenous mediators of Preconditioning

2.4.1 Role of Adenosine

There is a great deal of evidence in support of a role of adenosine as an endogenous mediator of protection. Adenosine is produced during ischaemia as a consequence of the action of intracellular 5' nucleotidase on adenosine monophosphate (AMP). Some adenosine is degraded intracellularly to inosine, but a large proportion enters the extracellular space where it is degraded by adenosine deaminase in the capillary endothelium. Adenosine is a powerful agonist and exerts its effects by binding to A_1, A_2 and A_3 receptors on various myocardial cells including myocytes, vascular smooth muscle cells and sympathetic neurones. Stimulation of the A_2 receptor, which is abundant in blood vessels, results in vasodilation. A_1 and A_3 receptors are present on myocytes, A_1 receptor stimulation resulting in activation of G_i protein, inhibition of adenyl cyclase and activation of PKC.

Liu et al (Liu et al 1991) provided the early experimental evidence of the role or adenosine as a mediator of preconditioning in the rabbit. They showed that activation of the A1 receptor with adenosine and the selective A_1 agonist R-PIA (N^6-(2-phenyl-isopropyl)adenosine) could mimic preconditioning, and that blockade of the A_1 receptor during preconditioning with SPT (8-p-sulphophenyl theophylline) and PD115,199 could prevent protection. These findings were confirmed in large animal hearts by Grover's group (Grover et al 1992) who showed that intracoronary R-PIA could successfully precondition the heart, and the beneficial effect of this agent as well as ischaemic preconditioning could be blocked by SPT.

There is both direct and indirect evidence that sufficient adenosine is released during the first episode of ischaemia to precondition the heart. In fact the development of the preconditioned state can be speeded up by dipyrimadole, an

agent that increases the concentration of adenosine in the extracellular space (Miura et al 1992). However, much less adenosine is available to bind to the A1 receptor during a second 5 minute episode of ischaemia, and hence adenosine is less certain as the agonist during preconditioning induced by repetitive 5 minute cycles of ischaemia and reperfusion. Perhaps additive effects induced by other mediators released during ischaemia, such as bradykinin, may be involved.

2.4.2 Bradykinin and other endogenous mediators of preconditioning

Intracardiac production of bradykinin has been shown to be increased in myocardial ischaemia (Noda et al 1993; Goto et al 1994; Baumgarten et al 1993) and furthermore recent reports suggest that infarct size limitation in the rabbit is blocked by the B_2 receptor antagonist Hoe140, and mimicked by kinin administration (Wall et al 1994).

It has also been reported that direct intracoronary infusion of bradykinin can reduce infarct size in dogs (Martorana et al 1990), and also mimic preconditioning by reducing the severity of ischaemia-induced arrhythmias (Parratt 1994b). These effects have been proposed to be mediated through prostacyclin and /or nitric oxide and increasing cGMP. Vegh et al (Vegh et al 1992a and b) carried out a series of experiments to determine whether inhibition of constitutive nitric oxide synthase (with various analogues of L-arginine), or inhibition of guanylyl cyclase, the target for nitric oxide in cardiac myocytes, could modify the protective anti-arrhythmic effects of ischaemic preconditioning. In these experiments prevention of nitric oxide generation, or inhibition of its effects on guanylyl cyclase, attenuated the protective effects of

ischaemic preconditioning. However, this evidence is indirect, relying upon the use of agents that block various sites in the pathway leading to the elevation of cGMP within cardiac myocytes. Some of these drugs, such as methylene blue, are not sufficiently specific.

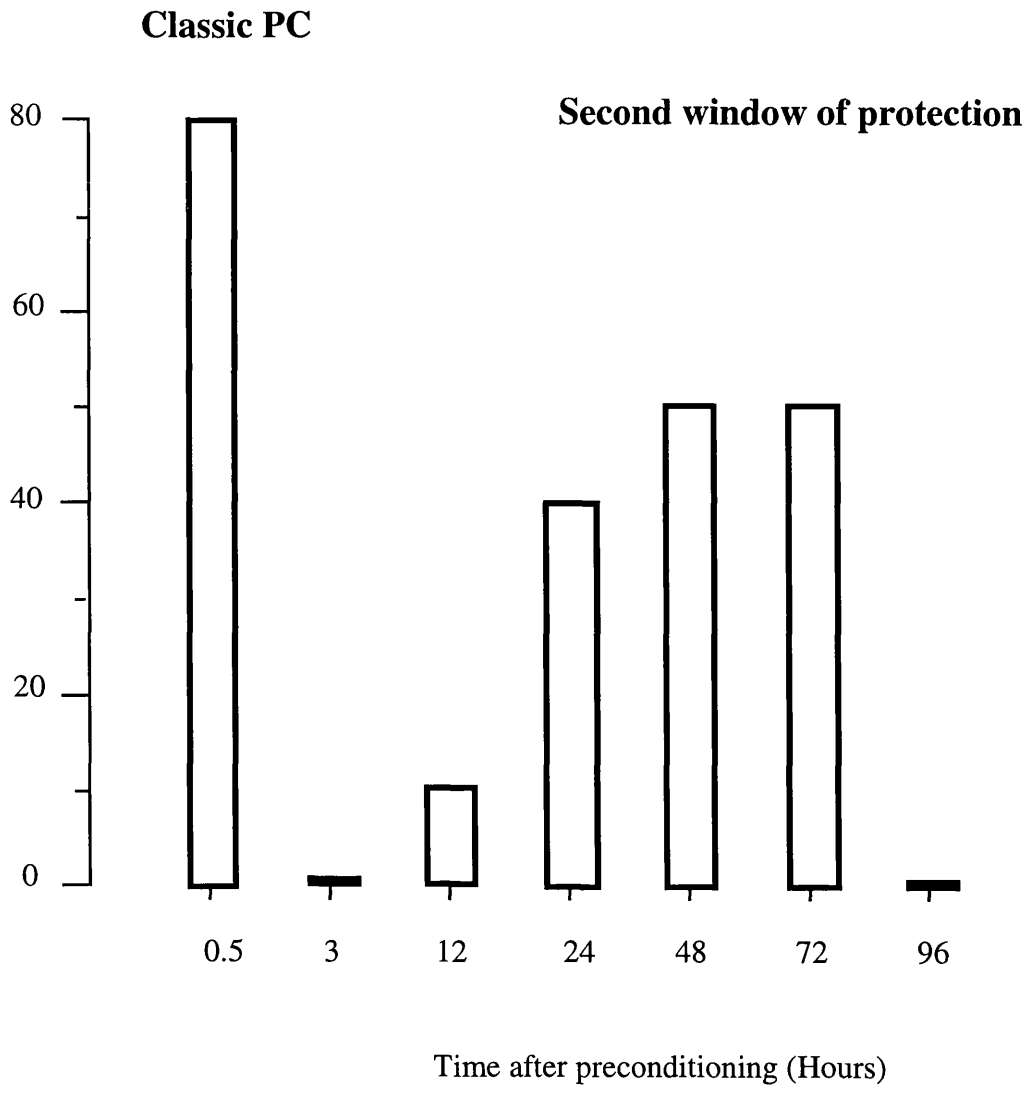
Recently Goto et al (1995) have clarified the role of bradykinin in an a rabbit model of myocardial ischaemia. They found that Hoe140 blocked the protection from one 5 minute period of preconditioning but could not block protection when a more profound preconditioning stimulus was used (four cycles of 5 minutes ischaemia and 10 minutes reperfusion). They also showed that Hoe 140 could block protection when given prior to, but not after, ischaemic preconditioning, in contrast to adenosine whose receptors must be occupied during the long ischaemic insult in addition to the preconditioning ischaemia in order for protection to occur.

3 The "second window" or delayed phase of protection

The delayed phase or :"second window" of protection was originally described in 1993 by two groups using rabbit (Marber et al 1993) and canine (Kuzuya et al 1993) models of coronary artery occlusion. Recent work from our laboratory suggests that the delayed anti infarct effect of preconditioning in the rabbit may extend over a period of 1 to 3 days (Baxter et al 1995), with maximal protection occurring at 48-72 hours after preconditioning (see figure 1.1).

With regard to rapid pacing-induced delayed protection against arrhythmias, Vegh's group (Kaszala et al 1995) have extended the interval between the pacing stimulus and coronary occlusion up to 72 hours and found that protection against fibrillation was almost completely lost by 48 hours. There

Figure 1.1 **Composite figure derived from several experimental studies indicating the biphasic timecourse of ischaemic preconditioning against necrosis**



Adapted from GF Baxter and DM Yellon in Ischaemia: preconditioning and adaptation. Eds Marber MS and Yellon DM 1996. p119.

was evidence, however, that even 72 hours later some attenuation of parameters such as electrical inhomogeneity was present (Kaszala et al 1995)
There is also evidence for a late phase of protection against myocardial stunning, with brief episodes of ischaemia conferring resistance to post ischaemic dysfunction 24 hours later in a conscious pig model (Sun et al 1995).

Associations between enhanced myocardial tolerance and stress induced cytoprotective protein activity, the gradual onset of the second window of protection (Kuzuya et al 1993) and the prolonged period of protection (Baxter et al 1995) suggest that the phenomenon is related to changes in patterns of protein activity in the preconditioned myocardium. Certain proteins have been proposed as putative mediators of delayed protection, and changes in the content or activity of some of these proteins, notably heat shock proteins (hsps) and antioxidant proteins, have been recently documented with respect to late preconditioning.

3.1 Endogenous anti-oxidants

Endogenous antioxidants such as catalase and superoxide dismutase (SOD) play a fundamental role in maintaining homeostasis in the face of intracellular reactive oxygen species production. The heat stress response in the myocardium following whole body hyperthermia, which confers increased tolerance to ischaemia-reperfusion 24 hours later in both isolated and in vivo hearts, has been associated in some studies with increases in myocardial catalase activity (Karmazyn et al 1990; Steare and Yellon 1993). Specifically with respect to brief ischaemia, there is some evidence from in vivo animal studies that SOD activity may be elevated 24 hours following ischaemic preconditioning. Hoshida et al (1993) described the temporal dynamics of manganese SOD activity following preconditioning in canine myocardium and

described a biphasic pattern of enzyme activity over a 24 hour period. Following four 5 minute coronary occlusions in the dog, manganese SOD activity was elevated in the epicardial risk zone within 1 hour, returned to normal within 3 hours and was elevated again 24 hours later. The myocardial content of manganese SOD increased gradually in the ischaemic and non ischaemic zones over 72 hours following ischaemic preconditioning. In studies with isolated rat cardiac myocytes, Yamashita et al (1994) reported that hypoxic preconditioning resulted in increased activity of manganese-SOD 24 hours later, at which time point marked protection was observed against a prolonged hypoxic insult. Both the rise in enzyme activity after preconditioning and the acquisition of cellular tolerance to hypoxia were abolished when myocytes were treated with staurosporine, a protein kinase inhibitor, or with anitsense oligonucleotide directed against manganese SOD during preconditioning. More recent reports suggest an association between adenosine receptor activation and increased SOD activity (Baxter et al 1996) .

3.2 Stress Proteins and their role in Cardioprotection

3.2.1 Heat Stress Proteins

The stress response is a universally conserved cellular defence program consisting of the upregulation of stress proteins. The heat shock response was first observed in Drosophila Melanogaster over 30 years ago - simply raising the temperature of Drosophila above its physiologic norm resulted in the decreased expression of those genes which were active before the temperature shock and the increased expression of genes encoding the heat shock proteins (hsps). Similar changes in gene expression have been observed in cells from all organisms, and these proteins appear highly conserved with respect to their primary structure, mode of regulation and biochemical function. In addition to

heat shock treatment, many other types of metabolic insults such as metabolic poisons, heavy metal exposure, as well as a variety of relevant insults in vivo, such as ischaemia/reperfusion also elicit increased expression of the hsps. Hence the more general term stress response is often used and the proteins whose expression increases, stress proteins.

Any agent or treatment which induces the stress response will reduce injury upon exposure to a subsequent related or in some cases unrelated stressor. It is also known that most of these proteins are in fact expressed constitutively in normal "unstressed" cells. There are five major groups of hsps based on molecular size - 70, 90, 50-60, 20-30 and 100-110 kDa (Minowada and Welch 1995). The stress proteins can be divided into two groups based on their classical mode of induction: the ***heat shock proteins*** (**hsps**) and the ***glucose regulated proteins*** (**grps**), both groups of proteins are identified according to their apparent molecular mass as determined by SDS-PAGE (N.B. native molecular weight is often very different, see table 1.1. These two families of stress proteins exhibit considerable similarities with respect to both structure and function.

Members of the grp family were first observed to exhibit increased expression in cells deprived of glucose. Expression of these proteins is also increased in cells subjected to anoxic - like conditions, treatment with calcium ionophores, or reducing agents. Many of these agents which induce grp expression have an adverse effect on protein secretion - and it is known that the major grps are located within the endoplasmic reticulum and participate in various aspects of protein secretion.

Table 1.1 Stress Protein Families

Name	Size kDa	Location	Function
Ubiquitin	8	cytosol/nucleus	Involved in non lysosmal degradation pathway
Hsp10	10	Mitochondria	Cofactor for hsp60
Low molecular weight hsps	20-30	Cytosol/nucleus	Possible regulator of actin cytoskeleton and molecular chaperone
Hsp47	47	Endoplasmic reticulum	Collagen chaperone
Hsp56	56	Cytosol	Part of steroid hormone receptor complex; binds FK506
Hsp60	60	Mitochondria/chloroplast	Molecular chaperone ("chaperonin")
TCP-1	60	Cytosol/nucleus	Molecular chaperone related to hsp60
Hsp72	70	Cytosol/nucleus	Highly stress inducible
Hsp73	70	Cytosol/nucleus	Constitutively expressed molecular chaperone
Grp75	70	Mitochondria/chloroplast	Constitutively expressed molecular chaperone
Grp78(BiP)	70	Endoplasmic reticulum	Constitutively expressed molecular chaperone
Hsp90	90	Cytosol/nucleus	Part of steriod hormone receptor complex; possible chaperone for retrovirus-encoded tyrosine protein kinases
Hsp104/110	110/140	Cytosol/nucleus	Required to survive severe stress; possible molecular chaperone

There are an extensive number of proteins whose expression increases after metabolic stress and the list continues to grow. In this thesis I have therefore concentrated on those stress proteins which are best characterised within the myocardium.

Much of the work on hsps within the myocardium has focused on hsp70, as it is the most abundant and inducible stress protein. With stress hsp70 translocates to the nucleus where it associates with the nucleoli. During stress and recovery hsp70 mRNA is preferentially translated even though the RNA from stressed cells will produce a full complement of proteins in an in vitro translation system.

3.2.2 Hsp70 as a Molecular Chaperone

All members of the hsp70 family bind to ATP through a highly conserved amino-terminal nucleotide binding domain, whose overall structure appears very similar to that of two other ATP binding proteins, actin and hexokinase. In addition, hsp70 family members bind to both unfolded proteins and short polypeptides in vitro via their carboxy terminal domain. Members of the hsp70 family, within their own distinct subcellular compartments, interact with other cellular proteins undergoing synthesis on the ribosome or translocation into subcellular organelles. These observations lead to the hypothesis that these proteins function in the early stages of protein maturation by binding to and stabilising the unfolded state of a newly synthesised protein. Once synthesis or organellar translocation of the target protein has been completed the hsp70 family member is released, a process requiring ATP, the target protein commences folding and/or assembly.

The function of hsp70 gives us some clues as to the mechanisms by which these proteins may result in myocardial protection. During ischaemia the

cellular internal milieu changes profoundly with the intracellular accumulation of protons and sodium ions. These changes are compounded by the free radical stress and the marked increase in intracellular calcium associated with reperfusion. Under these circumstances the tertiary structure of proteins changes sufficiently to alter their function. In the presence of an excess of hsp70 these adverse conformational changes may be prevented or reassembly of denatured proteins may be promoted.

3.2.3 Functions of the other stress proteins

In a similar manner, members of the hsp60 family also bind to ATP, and interact transiently with unfolded polypeptides. Hsp60 members are characterised by their distinctive 7-membered ring-like structure, often being found in vivo as two rings stacked on top of one another. Members of the hsp60 family, also referred to as chaperonins, in addition to binding unfolded proteins also appear to "catalyse" protein folding and assembly. Under electron microscopy it appears that the target protein to be folded is present within the central cavity of the hsp60 "double-donut" and here it eventually acquires its final folded structure in a process fuelled by ATP hydrolysis (Georgopoulos and Welch 1993). It is becoming clear that there may be up to 8 or more hsp60 - like homolgs (known as the TCP-1 family). It is thought that molecular chaperones such as hsp60 and 70, while not conveying specific information concerning folding, participate in the process by preventing improper or non productive intra- or inter- molecular interactions that could lead to protein misfolding or aggregation. Hence they may facilitate productive folding and assembly pathways and thereby ensure high fidelity and efficiency in the protein folding and assembly process. They may even work in tandem - e.g. cytosolic hsp70 may stabilise a newly synthesised unfolded nascent chain as a new protein emerges from the ribosome. Once synthesised the polypeptide is

transferred to a member of the chaperonin family for folding or higher order assembly.

Similarly hsp90 also has a chaperone - type role. It has been shown to interact with various steroid hormone receptors, intracellular proteins which when activated by their appropriate steroid ligand now become active as a transcription factor (Smith and Toft 1993). Steroid receptors are inactive when bound to an oligomeric protein complex that includes hsp90, binding of the steroid hormone results in release of the hsp90 from the complex enabling the receptor to acquire a DNA binding conformation. Hsp90 has also been found to interact with pp60src, a virally encoded tyrosine protein kinase of the Rous sarcoma virus - in this complex the pp60src is inactive only becoming active when released and partially integrated into the plasma membrane. A similar pathway appears to occur with other retrovirus - encoded oncogenic tyrosine protein kinases. Hence hsp90 may play a role in regulating the biological activities of certain target proteins - perhaps by binding to or masking domains which are critical for their biological activation and / or function.

The lower molecular weight hsps (20-30 kDa) also exhibit molecular chaperone-like properties - but unlike hsp60 and 70 they do not appear to bind nucleotides like ATP. These hsps may play a role in regulation of the actin based cytoskeleton. For example hsp28 may act as an inhibitor of actin polymerisation and promote disassembly of already formed actin filaments in vitro. Many of the agents that induce rapid phosphorylation of hsp28 (e.g. heat shock, cytokines, mitogens) also result in rapid rearrangement of the actin cytoskeleton, particularly the cortical actin network underlying the plasma membrane, furthers the hypothesis that hsp28 is involved in regulation of the actin cytoskeleton.

Elucidating the functions of these proteins in normal cells improves our understanding of why these proteins are upregulated in conditions of metabolic

stress. Under these conditions protein folding is perturbed or pre-existing proteins begin to unfold and denature. In these conditions increased synthesis of stress proteins enables these proteins to be identified and possibly also refolded, or to bind them for targeting to a proteolytic system, and facilitate the synthesis and maturation of new proteins to replace the denatured ones.

3.2.4 HSP70 and Myocardial Protection

It is now 8 years since the association between heat stress proteins and myocardial protection was first described by Currie et al (1988). They showed that 24 hours after elevating the temperature of rats to 42°C for at least 15 minutes both cardiac hsp70i and catalase activity were increased, whilst at this time point hearts became resistant to ischaemia/reperfusion injury. In heat stress compared to control hearts post-ischaemic contractile recovery was enhanced whilst creatinine kinase efflux was reduced. These findings were subsequently confirmed by a number of other groups e.g. (Black and Luchesi 1993). Of greater pathophysiological relevance was the observation that ischaemia itself could result in hsp70i (the inducible form of hsp70) induction and cardioprotection, but increases also occurred in a 60kDa stress protein and in another myocardial antioxidant enzyme, superoxide dismutase (Marber et al 1993, Hoshida et al 1993). Thus these studies still fell short of proving a causal relationship between hsp70 induction and cardioprotection.

Compelling evidence that upregulation of hsp70, in myocardial cells, affords significant protection comes from recent genetic modification studies in which transfected myocyte lines overexpressing hsp70, but not hsp60 or 90, have enhanced resistance to hypoxic stress (Heads et al 1995), and hearts from transgenic mice overexpressing the inducible hsp70 gene have enhanced resistance to ischaemic injury (Marber et al 1995). In transgene positive compared to transgene negative hearts, the zone of infarction was reduced by

40%, contractile function at 30 minutes of reflow was doubled and efflux of CK reduced by approximately 50% (Marber et al 1995). In addition overexpression of hsp70 did not alter the macroscopic phenotype of the mouse, contractility of the heart or antioxidant protein content of the myocardium (Marber et al 1995). This study provides direct evidence that elevation of myocardial hsp70 increases the inherent resistance of the myocardium to infarction.

3.2.5 Regulation of the Stress Response

Many of the heat shock proteins are in fact expressed constitutively in normal or "unstressed" cells where they play a fundamental role in a number of important biological processes. A diverse array of metabolic insults induce a stress response - many of these agents share the common property of being "protein chaotropes," they adversely affect the proper conformation and therefore function of proteins (Pelham 1986). The heat shock factor (HSF 1) present as an inactive monomer in the normal unstressed cell, rapidly trimerizes in response to metabolic stress enabling it to bind to the heat shock element and activate transcription of the genes encoding stress proteins. Recently another related transcription factor, HSF 2, has been identified. Shown to be important in regulating the expression of hsp transcription during haemin - induced differentiation of K562 cells, HSF 2 may also be important for controlling hsp gene expression in normal or unstressed cells.

The activation of the heat shock genes within myocardium is known to occur in response to brief periods of cardiac ischaemia in vivo (Marber et al 1993). In these instances it is thought that HSF activation is a consequence of intracellular ATP depletion (Mestril and Dillman 1995).

During ischaemia, substrate deprivation and metabolite accumulation results in a significant breakdown of adenosine triphosphate (ATP) causing the

accumulation of interstitial adenosine that gives rise to the sensation of angina (Gaspardone 1995). In addition, on the basis of animal studies, the cellular ATP content remains low for many hours following a brief period of ischaemia - hence periods of ischaemia as short as 5 minutes are sufficient to trigger hsp gene activation in animal studies. The obvious question is, does protection follow episodes of angina in patients?

3.2.6 Myocardial Adaptation

It is interesting to speculate whether myocardial adaptation, perhaps by hsp 70i induction, follows an episode of sublethal ischaemia or angina in man. A history of angina for at least 7 days before an acute myocardial infarction seems to predict a less complicated in-hospital course and reduced mortality (Muller et al 1990). This observation is complicated by differences that may exist between symptomatic and non symptomatic patients particularly in terms of collateral vessel formation and concomitant medication. However a recent analysis of a large and well documented thrombolysis trial data base has controlled for these variables and reports that the protective benefits of a 48 hour history of angina prior to infarction reduces mortality independent of any of the standard predictors of outcome (Kloner et al 1995). The magnitude of the advantage associated with preinfarction angina is substantial. For example the absolute risk of death in anterior infarction without antecedent angina can be increased as much as two fold (Anzai et al 1995), with the enzyme derived myocardial damage similarly increased 1.5 - 2 fold (Kloner et al 1995 and Anzai et al 1995). In these studies the temporal relationship between angina and infarction is more consistent with protection by the upregulation of cytoprotective proteins rather than classical preconditioning.

The recent identification of a polymorphism within the heat shock element of one of the hsp70 genes may be significant (Professor Steve Humphries, Rayne Institute, UCL, personal communication). It is possible that differences may result in differing degrees of inducibility of hsp70 and hence different levels of cytoprotection for individuals depending upon their particular genotype. As yet this research is in its infancy, but if this polymorphism results in altered binding or affinity of activated HSF to the heat shock element then this could have clinical implications i.e. with individuals who have a lower affinity binding being more susceptible to ischaemic myocardial damage.

Another interesting finding is the effect of non-steroidal anti-inflammatory agents, such as salicylates and indomethacin, on the heat shock response. These agents have the ability to potentiate the stress response by inducing the DNA binding state and thereby lowering the threshold for HSF activation. For example it has recently been shown that in HeLa cells indomethacin acts synergistically with elevated temperature resulting in enhanced protection against cytotoxic conditions (Lee et al 1995). Perhaps of more relevance is the finding that sodium salicylate also partially induces the human heat shock response.

4 Cardioprotection in clinical practice

4.1 Theoretical considerations

The profound effect of preconditioning on a number of end-points with clinical relevance makes it potentially a very attractive therapeutic tool. In order to derive clinical benefit there are several important requirements. In the first instance it is necessary to show that the human myocardium can be preconditioned, limited evidence for which has been outlined above. Secondly the temporal envelope of the protection must be clinically relevant and the

degree of protection sufficiently significant. Finally, that patients proposed for such treatment are not already maximally preconditioned. Unfortunately, the fact that these criteria are not easily met probably explains the lack of any pharmacological preconditioning agent currently available for clinical practice.

Due to the temporal limitations of classical preconditioning - in which protection lasts at best for a maximum of 90 minutes, it may prove more fruitful to exploit the delayed phase "second window" of protection. There is compelling evidence (as outlined above) on the basis of animal studies that stress proteins, in particular hsp70, can increase the resistance of the myocardium to infarction. In addition, our understanding of the mechanisms regulating stress protein expression is increasing.

These factors are providing an impetus to manipulate the regulation of the genes encoding hsp70 to confer a clinical advantage. For example, agents could be developed which may be able to bypass the usual stress response and directly up regulate the 70 kDa stress proteins without concomitant cell damage, thus providing a pharmacological route to cytoprotection. In an attempt to address this issue experiments were carried out in an isolated rat neonatal cardiomyocyte model examining the role of stress proteins in protecting these cells from stress (see below).

4.2 Cardioprotective effects of ACE inhibitors

In addition, there are agents in current use which may well have myocardial protective effects partly as a consequence of the preconditioning phenomenon - for example angiotensin converting enzyme inhibitors.

The introduction of angiotensin converting enzyme (ACE) inhibitor therapy has had enormous impact on clinical practice, far beyond initial expectations when first introduced as antihypertensive agents. As well as their use in the

management of heart failure they are now being implicated as myocardial and vascular "protective" agents that not only prevent left ventricular remodelling, but also reduce myocardial ischaemic events. These anti-ischaemic and anti-remodelling effects were somewhat unexpected and the exact mechanisms involved uncertain. Large trials such as SOLVD (1992) and SAVE (1994) have shown a significant effect of ace inhibitors on the prevention of myocardial ischaemic events. More recent evidence comes from trials such as GISSI 3 (1994), in which the lisinopril treated group had a significant reduction in early mortality (6 weeks), and ISIS 4 (1995) which showed a 7% decrease in five week mortality in the captopril group.

There are probably several mechanisms by which ACE inhibitors can provide cardiac and vascular protection, possibly operating simultaneously (Lonn et al 1994). These include improved coronary flow, reduced sympathetic activity, improved loading conditions, reduced left ventricular remodelling, improved endogenous thrombolysis, and direct anti-atherogenic effects (antimigratory effect on vascular smooth muscle cells and leukocytes, and plaque stabilisation - yet to be confirmed in humans). The increased coronary flow produced by ACE inhibitors seems to be caused by a stimulation of PGI_2 and nitric oxide release from endothelial cells in the coronary bed, triggered by bradykinin (BK) and related kinins (Linz et al 1995).

There is also accumulating evidence for a directly protective role for ACE inhibitors in the ischaemic/reperfused myocardium (Parratt 1994b, Hartman et al 1993a, Wall et al 1994, Noda et al 1993), although the literature is conflicting. Experiments to date have largely demonstrated this protective effect

if the ACE inhibitor is given as pre-treatment prior to infarction. It was proposed that ACE inhibitors protect in this manner by inhibiting the breakdown of bradykinin, since Hoe 140 has been shown to reverse the protective effect (Wall et al 1994, Noda et al 1993). A recent study by Miki et al (1996), showed that captopril potentiates the infarct size-limiting effect of ischaemic preconditioning via B_2 bradykinin receptors in a rabbit model.

There is now increasing awareness that ACE inhibitor activity is mediated in part via increased local bradykinin levels, and evidence outlined above confirms a potential role for bradykinin itself as one of the endogenous mediators of preconditioning.

5 Aims and scope of the thesis

Whilst preconditioning has been studied over the past 15 years, there is no therapeutic intervention resulting from this work. As a clinician my aims in this project were to examine the possible clinical implications of preconditioning - considering both classical preconditioning and the delayed or second window of protection, and primarily to study the possible pharmacological induction of a preconditioning-like protective response - the most likely means by which such an effect could reach clinical use.

The experimental work involves the use if two models:

Isolated rat neonatal cardiomyocyte model:

In an isolated rat neonatal cardiomyocyte model experiments were carried out to examine the role of stress proteins in cytoprotection. Specifically after induction with Herbimycin-A, which has recently been shown to induce the

expression of stress proteins in non cardiac cells. Experiments to define the effects of Herbimycin-A and to elucidate the mechanisms of action of this agent were carried out. The isolated cell culture model was chosen to enable the direct study of cardiomyocytes compared to in situ or isolated working models of regional ischaemia in which one cannot delineate between effects on cardiomyocytes and other cell types.

5.1.1 Herbimycin-A

As the search for a pharmacological route to cardiac protection continues any potential agent which induces stress proteins warrants investigation as it may potentially lead to the development of novel therapeutic approaches to cardioprotection in clinical practise. Herbimycin-A, a benzoquinoid ansamycin antibiotic, was found to induce stress proteins (specifically hsp72 and 73) in A431 human epidermoid carcinoma cells (Murakami et al 1991). This compound was originally isolated as a potential herbicide and inhibits tyrosine specific protein kinases. Herbimycin-A administration has been found transiently to reverse the phenotype of cells transformed with the src family of protein kinases. Although the precise mechanism of tyrosine kinase reduction in cells is unknown it has been shown to bind to reactive -SH groups of p60 src and blocks its function in vitro (Uehara et al 1989). Furthermore Fukazawa et al (1991) found that this agent did not have any inhibitory effect on cAMP-dependent kinase or protein kinase C. Herbimycin-A has been used as a tool for studying the roles for tyrosine kinases in signal transduction involved in cell growth and differentiation. Yoneda et al (1993) studied the effects of Herbimycin-A on osteoclastic bone resorption, in various cell types such as mouse marrow cultures, fetal rat long bone cultures, and rodent osteoclasts. They found no evidence of toxicity in any of these culture systems or in mice treated with Herbimycin-A. In fact Herbimycin-A treated animals did not

manifest any marked changes in food consumption, body weight, and motional activity.

Recently Hedge et al (1995) examined the ability of Herbimycin-A to induce stress proteins in a variety of (non cardiac) cells, including rat embryo fibroblasts, HeLa cells, NIH 3T3 cells and COS cells (monkey). Following treatment of these cells with Herbimycin-A increased stress protein levels were demonstrated as well as the development of a thermotolerant phenotype. Increases in the expression of stress proteins continued for as long as the cells were exposed to Herbimycin-A and was independent of the pre-existing levels of stress proteins. This is important since patients who may ultimately be suitable for treatment with cardioprotective agents may already have increased stress protein levels, for example patients with angina may have ischaemia-induced stress protein induction. Furthermore, unlike heat shock or other metabolic stressors, they did not observe any adverse cellular effects following Herbimycin-A exposure. For example, unlike other agents that elicit the stress response, Herbimycin-A treated cells exhibited no abnormalities with respect to protein maturation, protein insolubility, the integrity of the intermediate filament cytoskeleton, or overall cell viability. Many of the agents that elicit the stress response fall under the general category of being protein denaturants. For example, heat, various metals, or amino acid analogues are known to interfere with the folding or assembly of polypeptides and are potent inducers of the stress response. Finally, at least in rodent cells, Herbimycin-A exposure did not result in any obvious activation of the heat shock transcription factor.

My aim in examining this agent in a rat neonatal cardiocyte culture system was to determine its ability to induce stress proteins in this cell type and to quantify any resultant protection from thermal and ischaemic insults. Furthermore, the potential mechanisms of action of Herbimycin-A were examined. From the evidence outlined above this agent may eventually lead to the possibility of

developing therapeutic agents to induce cardioprotection via up regulation of cytoprotective proteins, without causing cellular damage which would be unacceptable in a clinical setting.

5.2 Human cardiac tissue

In order to extrapolate any positive results to a clinical setting it is important to assess this agent in human tissue. Hence the possible hsp inducing properties of Herbimycin-A in human tissue was also investigated. Unfortunately there is a limited supply of human cardiac tissue available for investigation, however specimens of right atrial human appendage can be taken from patients undergoing coronary artery bypass grafting. Although there are differences between atrial and ventricular tissue, in terms of stress protein induction, one could reasonably extrapolate results from one to the other. Therefore, stress protein levels were measured in trabeculae obtained from these samples of right atrial trabeculae.

The results of cell culture give values for cell viability but in a clinical setting, although of course cell death is of importance, it is recovery of function following infarction that we are primarily concerned with. Furthermore, the importance of studying human cardiac tissue cannot be overemphasised. In order to measure recovery of function a model of preconditioning using isolated human atrial trabeculae was developed. Unfortunately it has proved impossible to treat trabeculae with agents such as Herbimycin-A, then to allow time for maximal stress protein induction (20-24 hours) and then measure contraction of these trabeculae following a severe ischaemic insult. Although muscle preparations with a small diameter, superfused in an organ bath containing oxygenated physiological buffer, can remain viable without arterial perfusion, this cannot be extended to over 24 hours. Preliminary experiments were carried out to determine if this approach would be viable. However, trabeculae

maintained for 24 hours did not contract sufficiently for any meaningful results to be obtained. Several approaches were attempted which will be discussed in later chapters. The development of this model proved untenable but as a clinician the need to extend this research to a more clinically relevant human model was of prime importance. The broad aim of this thesis is to investigate possible pharmacological therapeutic approaches to cardioprotection. Hence, the second part of my work has concentrated on using the human atrial trabeculae model to study the effects of agents already in widespread clinical use which may have a potential role as myocardial protective agents. In this regard the possible role of ACE inhibitors in providing protection was examined together with their possible mechanism of action. It is possible that in the future both routes to protecting the myocardium could be of use in a clinical setting. For example a prolonged state of protection via induction of stress proteins could be superimposed with a shorter classical preconditioning type of protection when needed, for example prior to high risk coronary angioplasty or coronary artery bypass surgery.

5.3 Angiotensin Converting Enzyme Inhibitors

Angiotensin converting enzyme inhibitors (ACE) inhibitors are commonly used drugs in the of a variety of cardiovascular diseases. As well as being effective antihypertensive agents, they reduce symptoms and mortality in patients with congestive heart failure.

5.3.1 Rationale for the cardioprotective effects of ACE inhibitors

The renin angiotensin aldosterone system is complex and acts as a circulating hormonal system, a local endogenous tissue hormonal system with autocrine

and paracrine effects, and a neurotransmitter and neuromodulator. Current evidence suggests that ACE inhibitors reduce the risk associated with atherosclerotic cardiovascular disease through multiple mechanisms. These can be classified into two groups: "cardioprotective" effects, i.e. benefits in overall cardiac haemodynamics, energetics, electrical stability, and the reduction in left ventricular mass; and "vasculoprotective" effects, i.e. antiproliferative, antiatherogenic, and favourable effects on thrombotic mechanisms, arterial compliance and tone. ACE inhibitors probably exert these protective effects by blocking both circulating and tissue renin-angiotensin systems.

There is also evidence for a directly protective role for ACE inhibitors on the myocardium, and that the likely mechanism is that of bradykinin potentiation, with the subsequent release of nitric oxide and prostacyclin, rather than angiotensin inhibition.

5.3.2 Reduction in myocardial ischaemic damage

One of the earliest studies on the limitation of infarct size by ACE inhibitors was carried out in dogs (Ertl et al 1982). They demonstrated that in an acute coronary artery occlusion model captopril reduced the extent of cellular necrosis, as assessed by the triphenyltetrazolium staining technique, at the end of a six hour period of occlusion. The authors ascribed this reduction in ischaemic injury to an increase in myocardial blood flow. In rats subjected to a 24 hour complete coronary artery occlusion without reperfusion enalapril has been shown to reduce myocardial infarct size and reduce creatinine kinase depletion (Hock et al 1985). Furthermore, when enalapril is given 30 minutes after the onset of ischaemia in a cat model of ischaemia, there was a reduction in plasma creatinine kinase and ST segment elevation when subjected to a five hour coronary artery occlusion (Lefer et al 1984). However, the situation is not clear cut and a reduction in ischaemic injury was not seen by all groups. For

example, Daniell et al (1984) who subjected conscious dogs to a 24 hour occlusion.

More recent studies have investigated whether infarct size reduction is independent of angiotensin II synthesis inhibition. Noda et al (1993), in an anaesthetised bilaterally nephrectomised male mongrel dog model, found that following 9 minutes of occlusion of the left anterior descending coronary artery without reperfusion, there was a reduction in infarct size when captopril was given intravenously both prior to and following coronary artery occlusion. The authors suggest that the results were due to an increased generation of bradykinin in the captopril group, measured in the local anterior interventricular vein. Hartman et al (1993), in an anaesthetised rabbit model given 30 minutes of coronary artery occlusion followed by 2 hours reperfusion, found that ramiprilat, when given intravenously just prior to reperfusion, reduced infarct size by half.

Investigations suggesting that the protection provided by local ACE inhibition is due to bradykinin came initially from Martorana et al (1990). They studied the effects of ramiprilat on mongrel dogs subjected to a six hour occlusion of the left anterior descending artery, without reperfusion. The infarct size was 53% of the area at risk, which was reduced by both bradykinin (1ng/kg/min) and Ramiprilat (40 ng/kg/min), each given into the main stem of the left coronary artery 30 minutes prior to occlusion and lasting throughout the duration of the experiment. These locally administered doses had no systemic haemodynamic effects. The reduction in infarct size was marked: 25% in the ramiprilat group and 33% in the bradykinin group. The protection was abolished by Hoe 140, a highly selective bradykinin receptor antagonist, which itself had no deleterious effect on infarct size.

Therefore, there is some evidence that ACE inhibitors reduce the severity of some indices of ischaemia, such as CK release or ST segment changes, but

there is no conclusive evidence for infarct size reduction unless the myocardium is reperfused. Even with reperfusion, some studies have not shown a reduction in infarct size by ACE inhibitors. For example, in one study infarct size is greater in dogs treated with captopril (de Logeril et al 1992), at least in those animals with a high degree of collateral flow. In this study there was a marked difference in the effects of captopril on infarct size between animals with high and those with low collateral flow. This variation has been attributed to a blood flow reduction in the ischaemic area induced by a reduction in perfusion pressure or to an increase in flow to normal areas of the left ventricular wall, resulting in a form of coronary steal. Thus the level of coronary flow could be a major factor in determining whether ACE inhibitors are cardioprotective. Other factors that should be considered include the degree of reduction in systemic blood pressure, the dose and timing of administration of the ACE inhibitor and possibly the chemical structure of the particular ACE inhibitor (presence of -SH groups).

There is evidence against the reduced synthesis of angiotensin II being an important mechanism of cardioprotection by ACE inhibitors. For example Hartmann et al (1993) were able to show that losartan, an angiotensin II receptor antagonist, had no effect. This suggests that direct angiotensin II receptor stimulation or antagonism does not alter the degree of myocardial necrosis. As already alluded to, it is more likely to be an increase in bradykinin production that is responsible for the cardioprotective effects. Evidence comes from studies that show an increase in local bradykinin levels in the local venous drainage of the ischaemic area, and by those studies in which the protective effects of ACE inhibitors are blocked by Hoe 140 a bradykinin B_2 receptor antagonist. Bradykinin production would result in the release of nitric oxide and prostacyclin from vascular endothelial cells, with a subsequent increase in GMP (cyclic guanosine monophosphate), vasodilation, inhibition of platlet

aggregation and adhesion, and increased glucose uptake. It is also possible that ACE inhibitors and bradykinin may attenuate the contribution of poymorphonuclear leukocytes to tissue injury. Felsch et al (1992) demonstrated that ramiprilat or bradykinin reduced the detrimental effects of neutrophils and platlet activating factor on reduction in left ventricular developed pressure and creatinine kinase, as well as the generation of oxygen free radicals. They suggested that protection might be mediated by enhanced nitric oxide and prostacyclin levels.

5.3.3 Antiarrhythmic effect of ACE inhibitors

There is also some evidence for an effect of ACE inhibitors on a reduction in severity of ischaemia and reperfusion induced arrhythmias (Parratt et al 1994). The most likely explanation for the antiarrhythmic effect of ACE inhibitors is the prevention of bradykinin breakdown. Kinins are generated in the presence of ischaemia, probably by acid optimum enzymes in the vessel wall. Certainly ACE inhibitors increase levels of bradykinin in blood draining the ischaemic region of the left ventricular wall after coronary occlusion up to three times (Noda et al 1993). Infusion of bradykinin into a local branch of the left anterior descending artery significantly reduces the severity of life threatening arrhythmias in anaesthetised dogs when the artery is subsequently occluded (Vegh et al 1991). It is thought that this antiarrhythmic effect of bradykinin results from a decrease in the severity of ischaemia since epicardial ST segment changes in the region supplied by the occluded vessel are reduced by bradykinin (Vegh et al 1991). A range of other studies have shown that bradykinin reduces the severity of ischaemic changes following coronary artery occlusion, such as reduced production of lactate, reduced lactate dehydrogenase release and better

maintained myocardial high energy phosphate and glycogen levels (Linz et al 1990).

Much of the antiarrhythmic effect of bradykinin is due to stimulation from vascular endothelial cells of the release of nitric oxide and prostacyclin, mediated by bradykinn B_2 receptors, resulting in an increase in cGMP. Evidence in favour of this hypothesis comes from a study showing that an inhibitor of the L-arginine nitric oxide pathway (N^G-nitro-L-arginine methyl ester, L-NAME) abolishes protection if infused locally prior to infusion of bradykinin (Vegh et al 1993). Further evidence comes from Scholken's group (Linz et al 1992) who found that bradykinin reduced reperfusion arrhythmias in rat isolated hearts but this was abolished by either L-NAME or the B_2 antagonist Hoe 140.

5.3.4 Possible mechanisms in the cardioprotective effects of ACE inhibitors

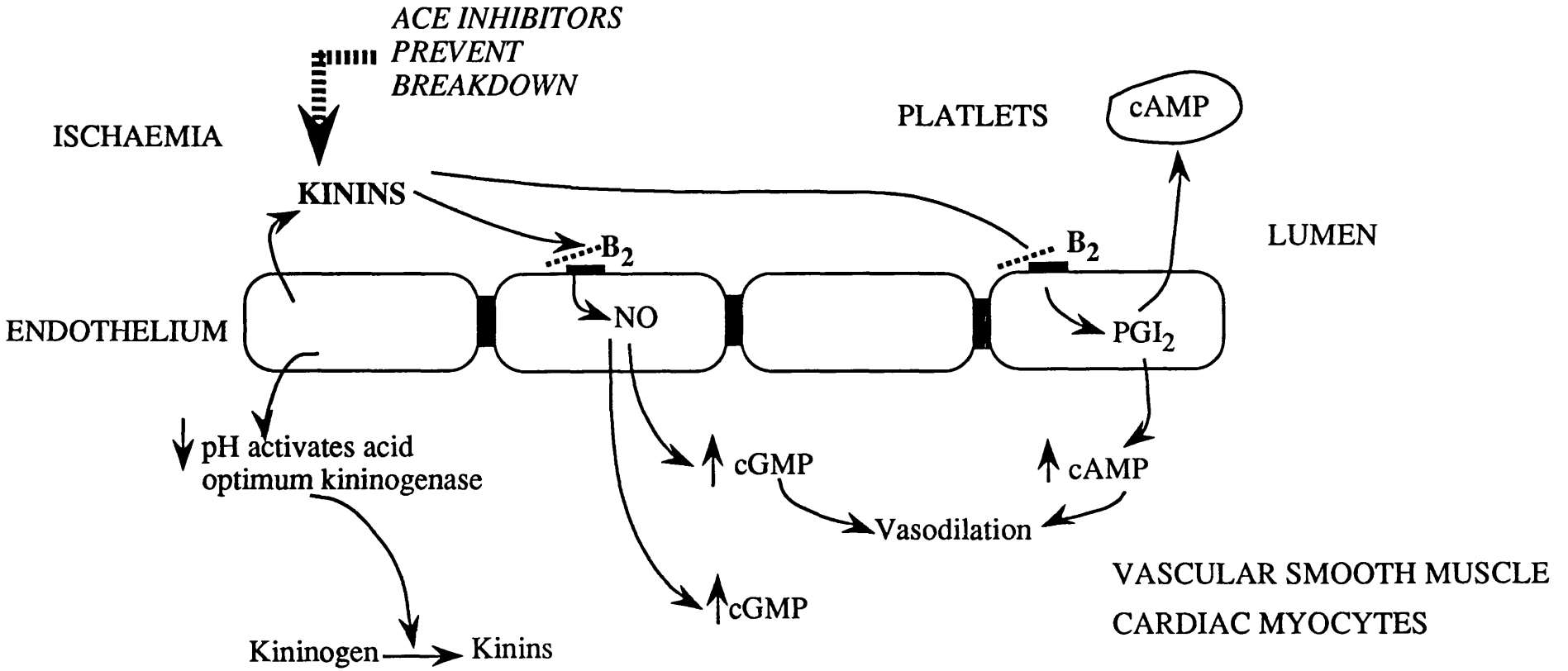
Inhibition of kinin breakdown

See Figure 1.2.

The evidence, partially outlined above, consists of three main groups of findings.

Firstly, the release of kinins into coronary venous blood soon after ischaemia is significantly greater in the presence of ACE inhibitors. For example Baumgarten et al (1993) showed that ramiprilat increased the outflow of bradykinin from isolated rat hearts following left coronary artery occlusion. This lead to the conclusion that bradykinin is formed in isolated hearts, despite the absence of blood, and that this is increased by ACE inhibition during normoxia, ischaemia and reperfusion. Both the precursor (kininogen) and enzymes responsible for the formation of bradykinin are present in isolated

Figure 1.2 **Possible mechanisms for the protective effects of kinins (and of ACE inhibitors)**



Kinins are generated from in plasma or endothelial cells by acid optimum kininogenase. This stimulates protstacyclin and nitric oxide release through an interaction with bradykinin B_2 receptors. This leads to platlet adhesion and aggregation, relaxation of vascular smooth muscle and a "cardioprotective" effect. Adapted from Parratt JR 1994 "Cardioprotection by ACE inhibitors."

KEY:

..... Hoe 140

NO: nitric oxide

hearts. Immediately after infarction in bilaterally nephrectomised, anaesthetised dogs, a significant increase in kinins was observed in the anterior interventricular vein, the effect being potentiated by captopril (Noda et al 1993). It is known that endothelial cells contain kininogen, and enzymes capable of releasing kinins from kininogen are present within the vessel wall as well as in the sarcoplasmic reticulum of cardiac myocytes (Xiong et al 1990). The presence of a local kallikrein kinin system in the heart has been demonstrated more recently by Nolly et al (1994) by measuring kallikrein in tissue and in the incubation medium of rat heart slices. They found that the mRNA coding for kallikrein was present in the atria and ventricles and in cultured primary neonatal rat atrial and ventricular cardiocytes. Kallikrein was isolated from rat heart homogenates by affinity chromatography on immobilised kallikrein antibodies and shown to be an active serine protease with kininogenase activity. This confirms the work of Xiong et al (1990). Furthermore, when epicardial slices were incubated in vitro, kallikrein was released into the medium in concentrations too high to be the result of non-specific release due to cellular damage . Kallikrein release was significantly decreased by pre treatment with the protein synthesis inhibitor puromycin - suggesting that the released kallikrein originates from a pool that requires de novo synthesis. The theory that the heart contains an independent kallikrein- kinin system is also strengthened by the fact that kininogen was found in epicardial slices and in the bathing medium. Similarly this release of kininogen was inhibited by pre treatment with puromycin, again suggesting de novo synthesis. These results suggest that cardiac tissue synthesises and releases both kallikrein and kininogen. Other investigators have reported that the mRNA for high molecular weight kininogen is present in endothelial cells (Schmaier et al 1988), and that vascular smooth muscle cells in culture release both kallikrein and kininogen (Oza et al 1990).

The second piece of evidence that bradykinin formation is an important component in the cardioprotection afforded by ACE inhibitors is the similarity in the effects of these drugs and bradykinin itself. These include increases in coronary blood flow, a reduction in the severity of ischaemia as assessed by various biochemical markers, and suppression of ischaemia/reperfusion induced arrhythmias.

The third piece of evidence comes from studies involving the selective bradykinin (B_2) receptor antagonist Hoe 140. This agent, as discussed above, abolishes the reduction in infarct size resulting from both ACE inhibitor and bradykinin administration, with similar effects on reperfusion arrhythmias. This approach has recently been used to examine whether bradykinin contributes to the antiarrhythmic effect of preconditioning (see above).

Other mechanisms involved on the cardioprotective effects of ACE inhibitors not related to bradykinin include: changes in the cardiac action potential (increase in duration found by some groups), interfering with cardiac sympathetic transmission (angiotensin II increases noradrenaline release from the adrenal medulla), prevention of the formation of angiotensin II (almost certainly less important than bradykinin accumulation), and increase in coronary blood flow (secondary, in part, to stimulation of prostacyclin and nitric oxide release from endothelial cells triggered by bradykinin) (Parratt 1994) .

5.3.5 ACE inhibitors and preconditioning

Miki et al (1996) demonstrated that captopril potentiates the protective effect of preconditioning in a rabbit model of myocardial infarction. infarct size and area at risk were determined by tetrazoleum staining and fluorescent particles respectively. Using a protocol of 30 minutes coronary occlusion and 3 hours

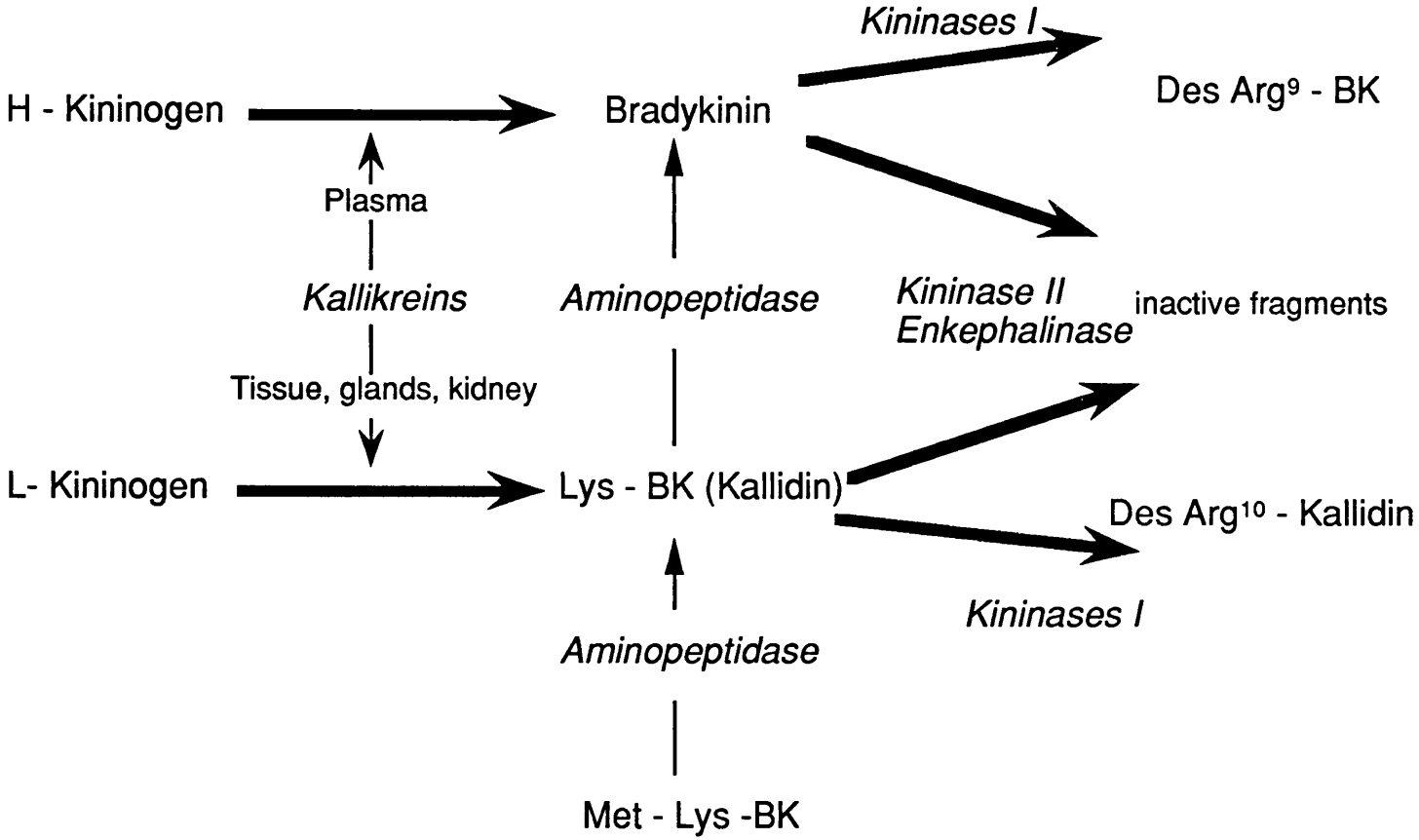
reperfusion in an in vivo model, they studied the effects of captopril (1mg/kg) given intravenously 22 minutes prior to the coronary occlusion. They found that there was a significant reduction in infarct size in the captopril plus 2 minutes preconditioning ischaemia group to 20.2 % (infarct size expressed as a percentage of area at risk), compared to either captopril alone (41.7%) or 2minutes preconditioning ischaemia alone (34.5%). The value for control groups was 42.9%. Furthermore, Hoe 140 the specific B_2 bradykinin receptor antagonist completely abolished these protective effects. Plasma kinin levels remained the same in all groups, and the authors therefore suggest a role for kinin produced endogenously within the heart in mediating the protection observed.

5.3.6 The Kallikrein Kinin system

Kinins are released from precursors kininogens, three types of which have been found so far in mammalian species. High and low molecular weight kininogen are present throughout the mammalian lineage whereas the third type (T-kininogen) is unique for the rat. From these kininogens, kinins are released by the kinin forming enzymes (kininogenases) the best known of which are plasma and glandular kallikrein (Linz et al 1995). Kinins (BK, Lys-BK [kallidin] and Met-Lys-BK) are oligopeptides containing the sequence of BK (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) in their structure and act as local hormonal factors by autocrine-paracrine mechanisms. If at all, they circulate at very low concentrations in the plasma and are rapidly degraded within 15 seconds by a group of peptidases known as kininases (see figure 1.3). Kininase II is known as angiotensin converting enzyme (ACE).

Two types of kinin receptors, B_1 and B_2, have been characterised based on their pharmacological responses to various bradykinin analogues. B_1 kinin receptors are more sensitive to the kininase I metabolites Des-Arg^9-BK and

Figure 1.3 **Formation and destruction of the kinins**



Key:

Kininases I:	Carboxypeptidase N, M (CPN, CPM)
Kininase II:	Angiotensin converting enzyme (ACE)
Enkephalinase:	Neutral endopeptidase 24. 11. (NEP)

Adapted from: contribution of kinins to the cardiovascular actions of ACE inhibitors. (Linz et al 1995)

Des-Arg^{10}-kallidin, whereas B_2 receptors are more abundant and have greater affinity for BK and kallidin (Linz et al 1995). B_1 receptors mediate contraction of the isolated rabbit aorta and rat duodenum, and relaxation of rabbit mesenteric arteries. Probably B_1 receptors are not present in normal tissues but are thought to be induced by certain pathological conditions such as tissue injury or stress. B_2 kinin receptors mediate most of the effects of bradykinin. Icatibant (Hoe 140) is the most potent and stable long lasting specific B_2 kinin receptor antagonist (Wirth et al 1991), and is a synthetic decapeptide with structural analogy to BK. It is characterised by the presence of two unnatural amin acids, D-tetrahydroisoquinolone-3-carboxylic acid and octahydroindol-2-carboxylic acid, replacing a proline residue at position 7 and a phenylalanine residue at position 8, respectively, of the authentic BK sequence. In addition, modifications are made at position 1, 3 and 5. Hoe 140 does not interact with receptors for other peptides. Hoe 140 binds tightly to the B_2 (but not B_1) receptor with a K_D of less than 0.05 nM, thereby outstripping the K_D of the natural ligand, BK.

5.4 Human in vitro model:

A model of preconditioning using isolated superfused isometrically contracting human atrial trabeculae was developed. This model has the advantage of avoiding complications of collateral flow, and the mix of necrotic and viable tissue within each specimen is comparable to the situation in evolving acute myocardial infarction. Previous studies have established that in this model the protective effects of preconditioning can be induced by activation of A_1

receptors (Walker et al 1995) and that protein kinase C and the activation of the ATP dependent potassium channels are also involved (Speechly-Dick et al 1995), confirming results obtained in animal studies.

In this human atrial model experiments were initially carried out to develop a delayed phase (second window) model, as described above and in more detail later in this thesis.

This was followed by experiments to examine the potential role for angiotensin converting enzyme (ACE) inhibitors and bradykinin in this model. The specific aims of this part of the study were to demonstrate any role for ACE inhibitors in directly protecting human cardiac tissue and further more to delineate some of the possible mechanisms involved.

Chapter 2:
Methods: Experimental Models

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1 Primary rat neonatal cardiomyocyte culture

1.1 Historical Perspective

Tremendous progress has been made since the pioneering, but long overlooked, observation of Burrows in 1912 (Burrows1912) that single beating heart cells migrated away from embryonic chick heart isolated tissue explants. He suggested that this finding supported the myogenic theory for cardiac beating activity. It was several decades later that the realisation that isolated cell culture might serve as useful tools for studying biological systems. In the 1950s these techniques were revived by Moscona (Moscona 1952), who isolated cells from embryonic tissues by proteolytic digestion. He also determined the essential materials, cofactors and vitamins necessary for maintenance of successful cell culture. In 1955, Cavanough (Cavanough 1955) isolated and maintained growing functioning heart cells from chick embryo and in1960 Harary and Farley (Harary et al 1960) prepared the first cultures of neonatal heart cells.

The first objective in culturing heart cells was to establish not only that they beat, but also that they serve as a model system reflecting the state of in vivo adult cells. Therefore many of the early studies were not only directed towards improving techniques for keeping cells in long term cultures, but to determine the ability of cultured cells to generate action potentials (Lehmkuhl et al 1963), and maintaining their specificity towards the drugs and hormones regulating beating rate (Yasumura et al 1966), as well as specific metabolic studies (Pinson et al 1974).

There is a vast and rapidly increasing number of publications concerning cultured cardiocytes and the wide variety of techniques available undoubtedly accounts for the great variation in the results in the literature. Thus it is necessary to be cautious in the interpretation of data from these studies. There are still considerable gaps in knowledge particularly with regard to the fact that

the optimal requirements for tissue disaggregation and nutrition have yet to be established.

1.2 Culturing heart cells

1.2.1 Cell Dissociation

The optimal enzyme requirements for cell dissociation vary with the type of tissue and the age of the animals used. Furthermore, it is well established that crude enzyme preparation are more efficient than purified ones. For example, highly purified collagenase is a poor dispersing agent - impurities in cruder preparations render this process more efficient (Kono 1969). Most crude enzyme preparations contain other proteolytic enzymes in varying quantities - for example crude trypsin also contains chymotrypsin and elastase (Speicher et al 1981). There are also significant variations between different batches of commercially available crude enzymes in their ability to dissociate heart cells - some of them causing severe damage. Therefore batch testing was carried out for all enzyme preparations used with minor adjustments to concentrations being made to allow for differences in proteolytic activity.

1.2.2 Media and Sera

Cell division, growth and metabolism depend on the exact composition of the medium and on the length of the lag periods between media changes when less substrate may be available to cells. In cultured cells, isolated from neurohumoral influences, cell division, differentiation and metabolism are controlled by the composition of the extracellular medium, contact between cells and the relative populations of different types of cells. The frequency of medium changes is critical in this respect. Substrates such as glucose, fatty acids and amino acids, are completely taken up by the cells within 12 hours of a

medium change (Pinson et al 1987). At this stage cells adapt metabolically either by storing excess glucose as glycogen or by exporting it from the cells as lactate - so that it is rapidly exhausted from the medium.
Serum serves as a universal substitute for interstitial fluids, contributing many biologically active substances such as nutrients, hormones, growth factors, carrier molecules and many other ill-defined components.

Plating density is a major factor affecting relative populations of different cells. Cells isolated from new-born rats consist of two populations, about 80% are cardiomyocytes and the other 20% are non- muscle cells (NMCs) consisting mostly of fibroblasts and endothelial cells. Seeding at a high density (10^6 cells/ml) assures that the culture reaches confluency and contact inhibition within 24 hours, thus minimizing cell division in the non cardiomyocyte cells and preventing them from overgrowing the cardiocytes. This has been confirmed by [3H]thymidine uptake studies (Yagev et al 1984). A low seeding density, on the other hand, allows rapid fibroblast proliferation. An alternative method is the "selective adhesion" technique, which depends on the different rates of attachment of various types of cells to the substratum.

1.2.3 Cell Division

Myocardial cells can be distinguished from other cell types in culture by their "dense" cytoplasm, well-developed mitochondria and Golgi system, the presence of myofibrils and intercalated discs, and their spontaneous contractile activity. The average mitotic cycle is 2.5-fold longer in myocardial cells than in NMCs.
The general rule that differentiated cells no longer divide has limited applicability to myocardial, since the cardiomyoblast, which can synthesise myosin and also divide, is an intermediate state between the presumptive

myoblast and the adult postmitotic cardiomyocyte. Under optimal culture conditions the most characteristic expression of differentiation, automatic synchronous beating, occurs within 24 hours. The post natal rat heart contains cells in different stages of development, from 55% dividing cardiomyoblasts in 1 day old rats to 40% by day 4. In the intact animal all cardiac muscles cells are post-mitotic by day 21.

1.2.4 Selecting cell populations

The heart consists of a mixed population of cells with only 50% cardiomyocytes. Both in vivo and in vitro, proper functioning of muscle cells requires the presence of NMCs. However, the interpretation of biochemical and pharmacological data is complicated by the presence of two types of cells in the Petri dish. NMCs proliferate more quickly and may eventually overgrow the culture.

Two techniques to overcome this problem were employed, the selective adhesion technique and the use of serum free medium. Cells of the fibroblast type become more readily attached to the substratum and then spread over the surface, while the myoblasts retain their round shape for longer, and therefore remain in suspension. Several preliminary experiments were carried out to determine the critical time point (30 minutes) for replating the cells to obtain a population consisting mainly of cardiocytes.

Techniques employed by other investigators include treatment with 7-ß-OH cholesterol or with the Ca^{2+} ionophore A23187, which lyses cardiac NMCs. Alternatively DNA synthesis inhibitors have been used. However, all these chemical methods have the disadvantage that they may damage myocardial cells. In addition, the elimination of one type of cell population from the Petri dish

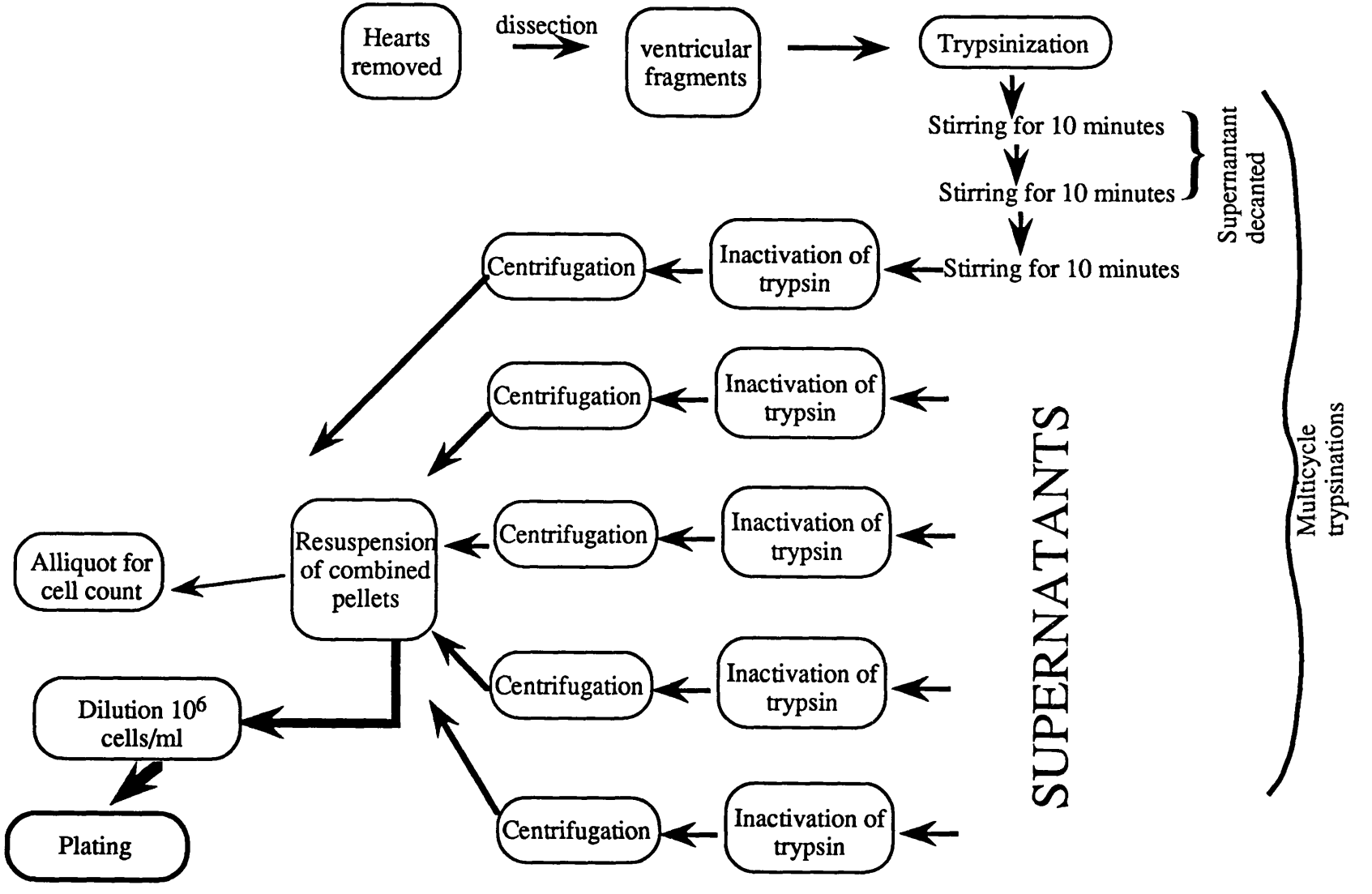
surface while the myocytes are already in a non dividing state may lead to a non confluent culture, which would give rise to significant errors in expressing data (Yagev et al 1984).

1.3 Isolating heart cells and preparing cultures

All procedures were performed in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986* published by HMSO 1986, London.

Ventricular myocytes from the hearts of neonatal Sprague Dawley rats less than 2 days old were cultured using previously described methods (Chien et al 1985, Buja et al 1988, Knowlton et al 1991). Sterile conditions were employed throughout. Rats (15 to 45 depending on availability) were killed by decapitation and allowed to bleed. The animals were immediately immersed in 70% ethanol and then held from the back in order to stretch the chest skin, thus revealing the position of the heart. The chest was then opened via a longitudinal cut with sterile scissors and the hearts removed aseptically with forceps. The ventricles were cut into 4-5 segments and placed into a Petri dish containing Ads buffer (see Appendix 1). Atria, fat and connective tissue were trimmed away from the hearts with a fine scissors and forceps. The cells were dispersed in a nominally calcium free, HEPES buffered salt solution containing pancreatin 0.6 mg/ml (Gibco-BRL) and type II collagenase 0.5 mg/ml (approximately 266 units/mg) (Worthington Biochemical Corporation), via a series of incubations at 37°C (see figure 2.1). The hearts were placed in 6ml of enzyme solution and incubated in a gentle stirrer at 37°C for 10 minutes. Fragments were allowed to settle and the supernatant was aspirated with a sterile Pasteur. This was repeated for a second time. The first two digestions, essentially containing cell debris, red blood cells, pericardial and endothelial cells, were discarded. Cells

Figure 2.1: Schematic representaion of primary cardiomyocyte culture methods



from subsequent trypsinizations were placed in a 50ml Falcon tube together with 2ml of fetal calf serum to inactivate the enzymes. These cells were centrifuged for 6 minutes at 1000 rpm. The pellet was then resuspended in 4ml of fetal calf serum and transferred to a clean Falcon tube, which was placed in an incubator at 37°C with the lid loosened to allow for gas exchange. These steps were repeated 3 or 4 times, until all fragments were dissociated. Whilst repeating these steps the six well plates were coated with 1% gelatin. Finally, the falcon containing all the pooled cells was centrifuged for 6 minutes at 1000 rpm. The supernatant was aspirated and the cells resuspended in growth medium and seeded onto 60mm diameter Petri dishes. The Petri dishes were shaken gently and linearly to achieve a more uniform distribution, and incubated for 30 minutes at 37°C to allow the fibroblasts to adhere to the plate, the myocytes remaining unattached. The myocyte enriched unattached cells were then replated on 6 well gelatine coated plates at a density of 1.5-2 million cells per well. The cardiac myocytes were cultured at 37°C, 5-7% CO_2, in 4:1 Dulbecco's modified Eagles medium / Medium 199 (Gibco-BRL) supplemented with 10% horse serum, 5% fetal calf serum and 1% penicillin / streptomycin. After 24 hours the medium was replaced with serum free medium to reduce fibroblast contamination. Cardiocyte cultures under these conditions start to beat synchronously within 72 hours, the percentage of beating cells exceeding 85% for the duration of the experiment. The cultured cardiomyocytes at 48 hours are shown in figure 2.2

Figure **2.2**

Primary rat neonatal cardiomyocytes

Rat neonatal cardiomyocytes at 48 hours after preparation.

Magnification X200

2 Myocardial Stress Protein Quantitation by SDS-PAGE and Western Blotting

2.1 Principles of SDS-PAGE and Western Blotting

Proteins are separated according to molecular mass and charge by electrophoresis through polyacrylamide gels (Laemmli 1970). The relative distance that a protein migrates is dependant on: (i) The effective pore size of the polyacrylamide gel. Pore size decreases as the concentration of acrylamide increases (Hames 1990). (ii) The native charge of the protein. (iii) The effective radius of the protein which is determined by its quaternary structure. The migration distance can become more dependant on molecular weight, and less dependant on the native charge and quaternary structure of proteins by denaturing the protein with a reducing agent in the presence of excess SDS. This acts to unfold the protein which then binds SDS in a constant weight ratio so that different proteins have essentially identical negative charge densities and hence will migrate in polyacrylamide gels of the correct porosity according to size (Hames 1990). Once proteins have been separated spatially within the gel, they are transferred to nitrocellulose by applying an electric field perpendicular to the plane of the gel. The proteins then become immobilised within the nitrocellulose matrix, whilst epitopes still remain accessible to immunoglobulins to allow immunodetection.

2.2 Preparation of Cardiomyocyte Specimens

The cells were scraped off with SDS and placed in eppendorf tubes.

In initial experiments Bradford assays were performed, however consistent yields were obtained so protein content was not assessed in later experiments.

Bradford Assay:

Bicinchoninic acid (BCA), in the form of its water - soluble sodium salt, is a sensitive, stable and highly specific reagent for the cuprous ion (Cu^{1+}). Peptide bonds and four amino acids (cysteine, cystine, tryptophan and tyrosine) have been reported to be responsible for colour formation in protein samples when assayed with BCA.

The BCA Protein Assay (Pierce, USA Product number 23225) combines the biuret reaction (protein reducing Cu^{2+} in an alkaline medium to produce Cu^{1+}) with the unique features of BCA. The purple reaction product, formed by the interaction of two molecules of BCA with one cuprous ion (Cu^{1+}), is water soluble and exhibits a strong absorbance at 562 nm. This allows the spectrophotometric quantitation of protein in aqueous solutions.

For the quantitation of protein within the samples the Bradford protein assay as described above was used according to manufacturers instructions. A microtitre plate protocol was used. A set of protein standards was prepared by diluting the stock BSA solution in the same diluent as the experimental samples. This protocol is known to be applicable for protein concentrations in the range of 10-2000 µg/ml. 10µl of each standard, blank or unknown sample was pipetted into the appropriate microtitre plate wells. 200µl of Working Reagent (Pierce) was added to each well and samples were mixed on a microtitre plate shaker. Following incubation at 37°C for 30 minutes the absorbance was read on a microtitre plate reader (Lab system multicam RC, UK) at 562 nm. A standard curve was prepared by plotting the net (blank corrected) absorbance at 562nm versus protein concentration. This standard curve was then used to determine protein concentration for each unknown protein sample.

2.3 Preparation of SDS-Page Gels

The polyacrylamide gels were prepared between glass plates separated by 1mm spacers. Two gels of differing acrylamide concentration and pH were poured

between the same glass plates, the upper "stacking" gel having wells to load the protein samples, which then migrated into the lower "resolving" gel. The stacking gel serves to concentrate proteins into a tight band before protein separation according to molecular weight in the "resolving" gel.

The lower resolving gel (pH 8.8) was made by mixing 30% acrylamide solution, tris buffered (to pH 8.8) SDS solution, and distilled water in varying proportions (see Appendix 2 Sections 2 and 3) to give either a 12.5% or 10% acrylamide gel mixture. Before pouring the gel mixture between the glass plates, ammonium persulphate (APS) and NNNN-tetraethylethalinediamine (TEMED) were added, to promote acrylamide polymerisation and crosslinking. Water saturated butanol was floated on the upper meniscus formed between the glass plates by the resolving gel solution, to prevent drying during polymerisation.

Once the lower resolving gel had polymerised, the upper stacking gel solution was poured after discarding the butanol layer. The upper stacking gel (pH 6.8) was prepared by mixing 30% acrylamide solution, TRIS buffered (to pH 6.8) SDS solution, and distilled water in proportions to give a 5% acrylamide gel mixture which was polymerised by adding APS and TEMED (see Appendix 2 Section 2 and 3). A toothed plastic well former was placed between the glass plates in the stacking gel solution, which, after polymerisation was removed to allow loading of the samples. samples were mixed 1:1 with loading buffer (see Appendix 2), boiled for 5 minutes and loaded on the gel before electrophoresis at a constant current of 40 mA.

2.4 The Loading of Samples and the One-Dimensional electrophoretic Separation of Proteins in Polyacrylamide Gels

The polymerised stacking and resolving gels sandwiched between the glass plates were placed in an electrophoresis apparatus (BRL, Maryland, USA) so that the lower and upper margins of the gel were in contact with the running buffer (see Appendix 2 Section 4) in separate lower and upper chambers. The only communication between these chambers was through the gel. The upper chamber in contact with the upper margin of the stacking gel was connected to the cathode and the lower chamber in contact with the lower margin of the resolving gel was connected to the anode. The voltage gradient across the gel therefore tended to cause the negatively charged SDS-bound proteins to migrate towards the anode at the lower margin of the resolving gel.

Samples were loaded into the wells formed in the stacking gel using a 20µl Gilson. Sample buffer has a high glycerol content, therefore samples were heavier than the running buffer in the upper reservoir and sank to the bottom of their individual wells. Once samples had been loaded, the anode and cathode were connected to their respective terminals of a power pack (BRL, Maryland, USA). A current of 40mA was delivered through each gel, initially this corresponded to a voltage of 200V, as the protein samples migrated through the polyacryalmide the resistance of the gel and therefore the voltage increased. In order to prevent gel heating, secondary to this rise in resistance, the power pack was set to deliver a constant current of 40mA until the voltage reached 400V and then to maintain a constant voltage of 400V. Under these conditions the potential difference would be applied across both 10% and 12.5% acrylamide gels until the "dye front", the fastest migrating portion of the samples, reached the bottom of the resolving gel. The gel plates were then removed from the

apparatus, glass plates separated and stacking gel discarded. The resolving gel would then be used either for western blotting or visualisation of proteins by Coomasie staining.

2.5 Coomasie Staining

Coomassie blue R-250 (BDH, Dorset, England) binds to proteins and can detect bands containing as little as 0.2-0.5μg of protein, and staining remains quantitative for up to 15μg of protein (BD 1990) per lane. The resolving gels containing the electrophoresed samples were gently agitated overnight in Coomasie blue staining solution (see Appendix 2 Section 5). The following day, the background non-specific Coomasie stain was washed out of the gel by destaining with methanol - containing de-staining solution (see Appendix 2 Section 6) and the gel preserved in 7% acetic acid.

2.6 Western Blotting

The proteins separated according to molecular weight by polyacrylamide gel electrophoresis, were transferred to nitrocellulose membranes (Hybond C, Amersham, UK) by western blotting. This was done by laying the nitrocellulose membrane "en face" over the polyacrylamide gel under blotting buffer (see Appendix 1 Section 7), taking care to exclude air bubbles between gel and membrane, followed by seven layers of 3 mm Whatman paper on both sides. The membrane, gel and Whatman paper were then sandwiched in a plastic cassette within a blotting tank (BioRad, Herts, England) containing the same blotting buffer. A current was applied orthogonal to the plane of the gel and membrane, with the anode on the nitrocellulose side of the gel, forcing the negatively charged proteins to migrate out of the gel and into the matrix of the nitrocellulose. Gels were blotted overnight using a current of 210mA. The

following day the polyacrylamide gels were discarded and the nitrocellulose membranes used to immunolocalise heat stress protein (hsp).

In initial pilot studies, the polyacrylamide gels were stained with Coomasie blue following Western blotting. Directly visualised protein transfer was shown to be complete apart from very high molecular weight proteins close to the resolving gel, stacking gel junction.

2.7 Immunoprobing Nitrocellulose Membranes

Initially, a number of concentrations of primary antibodies (binding to transferred antigen on the nitrocellulose) and secondary antibodies (binding to the primary antibody and allowing detection) were used for the detection of stress proteins before the binding conditions were optimised.

2.7.1 Detection of hsp90

The nitrocellulose membranes containing the immobilised proteins were agitated for 1 hour at room temperature in phosphate buffered saline (PBS) containing 5% (w/v) dried skimmed milk powder (Marvel) and 0.05% Tween 20 (Sigma, Mousourri, USA). This acts to block further non-specific protein binding to the nitrocellulose. Following blocking, membranes were washed for 3, 5 minute periods in PBS containing 0.05% Tween 20 and 0.1% (w/v) dried skimmed milk powder. The membrane was then incubated with a mouse IgG monoclonal antibody to hsp90 (AC88, a gift from Dr D Toft, Mayo Clinic, Rochester) at a concentration of 12.5 µg/ml in PBS/0.05% Tween 20 for 1 hour with agitation to ensure that the whole membrane surface was wetted. The membranes were then washed again for 3, 5 minute periods in PBS/0.05% Tween 20 and then incubated for 1 hour with horse radish peroxidase conjugated rabbit IgG directed against mouse IgG (Dakopatts, Glostrup, Denmark) at a concentration of 1 in 2000 in PBS/0.05% Tween 20. The non-

specifically bound horse radish peroxidase conjugated antibody was washed off the nitrocellulose membrane by three, 5 minute rinses with PBS/0.05% Tween 20. Membranes were then developed using an enhanced chemiluminesence technique (ECL, Amersham, Bucks, England) according to the manufacturers instructions. This system detects the peroxidase activity within the membrane by oxidation of the substrate luminol in the presence of hydrogen peroxide. Immediately after oxidation, luminol emits light for a short period of time which is detected using photographic film.

2.7.2 Detection of the Inducible and Constitutive Forms of hsp70 (hsp72 and 73)

The procedure to detect members of the hsp70 family was as described above. Blots were probed with monoclonal antibodies specific for the inducible isoform of hsp70 (hsp72) (C92F3A-5, Stressgen, Canada) or with monoclonal antibodies specific for both constitutive and inducible isoforms (hsp73 and 72 respectively) (SPA-820, Stressgen, Canada) at 1:1000 dilution. There is no antibody specific for hsp73 alone available.

2.7.3 Detection of Other Heat Stress Proteins

In addition, blots were probed with, monoclonal antibodies to hsp60 (clone 4B9/89, Affinity Bioreagents, USA) at 5 μg/ml, or to hsp25 (H0148 Sigma, U.K.) at a 1:500 dilution.

2.7.4 Detection of Glucose Regulated Protein 78

Certain blots were also probed with a monoclonal antibody specific for grp78 (SPA-827M, Bioquote, U. K.) at a dilution of 1:500.

2.7.5 Densitometric Quantitation of hsp90, 73, 72, 60, 25 and grp78

Densitometric assessment of specific protein bands was used to quantitate myocardial stress proteins (Norton and Latchman 1987).

Protein bands visualised by chemiluminescence, and recorded on photographic film, were scanned using a white light source and absorption data digitised and transferred to a personal computer for subsequent analysis (Biorad model 620 video densitometer, Hemel Hempstead, Herts, England). When bands were not homogeneously dense, the band was scanned at a number of points along its length to calculate a mean absorption value.

As already described above the protein content of samples was equalised on the basis of protein assessment with the Bradford assay in initial experiments and little variation was found between samples, probably because extreme care was taken to load an equal density of cells in each well during preparation of the cardiomyocyte cultures. However the Bradford assay was carried out this was done prior to boiling and final centrifugation. Therefore to control for any variation in final protein concentration of the samples identically loaded SDS-PAGE gels were also prepared for Coomasie staining to control for this variability. Samples on the gel for stress protein visualisation by western blotting and protein visualisation by Coomasie staining were loaded simultaneously and run on opposite sides of the same gel electrophoresis apparatus. The actin band on these identically loaded Coomasie stained gels was also scanned into the densitometer to allow adjustment for these variations. The absorption patterns were analysed using Biorad Analyst 2 version 3.1 software, (Biorad, Herts, England). For each lane the absorption value for the stress protein band was divided by the absorption value for the actin Coomasie band, giving a ratio independent of protein loading. It was shown that the

absorption values for both the Coomasie actin band and the stress protein bands were linear over the range of loading conditions.

3 Preparation and Transfection of Plasmids

The plasmids were prepared with a maxi plasmid preparation method using a Qiagen kit (Qiagen, UK) according to manufacturers instructions.
DNA concentration was estimated by spectrophotometry (Sambrook et al 1989). 10ug of plasmid DNA was used per well.

3.1 Calcium phosphate mediated transfection

The calciumphosphate procedure (Gorman et al 1983) is one of the most commonly used methods for transfection of plasmid DNA into cultured cells, although the mechanism remains obscure. It is thought that the DNA enters the cytoplasm of the cell by endocytosis and is transferred to the nucleus. The advantage of this method is its simplicity and relatively high efficiency, although this is dependant on the cell type being transfected.

Method (from Sambrook et al 1989)

The following solutions were prepared:

2 X HEPES buffered saline(HBS)

280mM NaCl

10mM KCl

1.5mM $Na_2HPO_4.2H_2O$

12mM dextrose

50mM HEPES

The pH was adjusted to 7.05 with 0.5 N of NaOH, and the solution sterilised by passage through a 0.22 micron filter.

2M $CaCl_2$

10.8g of $CaCl_2.6H_2O$ was dissolved in 20ml of distilled H_2O, and sterilised by passage through a 0.22 micron filter.

0.1 X TE (pH 8.0)

1mM Tris.Cl (pH 8)

0.1mM EDTA (pH8.0)

The solution was sterilised by passage through a 0.22 micron filter.

Carrier DNA

Salmon sperm DNA (Sigma D1626) was dissolved in 0.1 X TE (pH8.0) at a concentration of 40 ug/ml by overnight incubation at 37°C. Carrier DNA was sterilised before use by ethanol precipitation.

24 hours before transfection, the rat neonatal cardiocytes were replated at a density of 1 x 10^5 - 2 x 10^5 cells/cm^2 in 60 mm tissue culture dishes in serum-containing medium. The cultures were incubated for 24 hours at 37°C in a humidified incubator with 5-7% CO_2.

The **calcium phosphate DNA coprecipitate** was prepared for each 60 mm monolayer of cells:

220 ul of the carrier DNA prepared as above containing 10ug of the plasmid DNA to be transfected was mixed with 250 ul of 2 x HBS in a 15ml sterile Falcon (2509).

31 ul of 2M $CaCl_2$ was then slowly added and gently mixed for 30 seconds.

This mixture was incubated for 20-30 minutes at room temperature, to allow a fine precipitate to form. At the end of the incubation, the mixture was pipetted up and down to resuspend the precipitate. Care was taken to avoid the rapid formation of coarse precipitates that would reduce efficiency of transfection. Crucial factors include speed of mixing of the solutions, concentration and size of DNA and pH of the buffer.

The calcium phosphate-DNA suspension was transferred into the medium above the monolayer, using 0.5ml of suspension for 5ml of medium. The dish was rocked gently to mix the medium, and the transfected cells were incubated for 4 hours at 37°C in a humidified incubator at 5-7%CO_2.
The medium and precipitate was removed by aspiration, the cells washed with phosphate buffered saline, and 5ml prewarmed complete growth medium was added. The cells were returned to the incubator for 24 hours

At this stage the cells could be treated with Herbimycin -A, genistein, or heat stress as described in the protocols and reincubated for a further 24 hours depending on the experiment, and where necessary CAT activity assessed (see below).

3.2 CAT assay protocol

3.2.1 Vectors carrying reporter genes

A number of different prokaryotic genes have been used as reporters of the transcriptional activity of mammalian promoters. The following considerations are important when selecting a reporter gene (Gorman et al 1982):
- The enzymatic activity encoded by the gene must be readily distinguishable from any similar activities present in the mammalian cells prior to transfection.

- There should be no interference or competition from other enzymatic activities in the cells.

- The assay for the encoded enzymatic activity should be rapid, sensitive, reproducible and convenient.

The enzyme CAT (Chloramphenicol acetyltransferase) fulfils all of these criteria and has therefore become the most widely used reporter gene for indirect assay of promoter activity in transfected mammalian cells. The cat gene was originally derived from the transposable element Tn9 and confers resistance to chloramphenicol. The coding region is 1102 bp in length which is ordinarily flanked by two 768 bp IS elements. A plasmid, pSV2CAT, has been constructed that contains the SV40 promoter/enhancer, 29 bp of 5' untranslated sequence, the CAT coding sequence, and 8 bp of DNA 3' to the UAA stop codon. pSV2CAT cannot confer chloramphenicol resistance on bacteria because the CAT gene is not linked to a prokaryotic promoter. To assay putative promoters in mammalian cells, a derivative of spSV2CAT has been constructed (pSVOCAT; Gorman et al 1982) in which the promoter region of the SV40 is replaced by the promoter being tested - in this case the heat shock element cleaved from the hsp70 promoter. CAT modifies and inactivates chloramphenicol by mono- and diacetylation, and several assays have been developed to measure CAT activity in mammalian cells.

Incubation of extracts prepared from transfected cells with ^{14}C-labelled chloramphenicol was used in this study. The extent of modification of chloramphenicol is measured by thin-layer chromatography (TLC) on silica, which separates the mono- and diacetylated derivatives of chloramphenicol from the unmodified compound. The TLC plate is exposed to X-ray film and aligned with the resulting autoradiograph, allowing densitometric assessment of the degree of conversion ^{14}C chloramphenicol to acetylated forms.

3.2.2 Thin-layer chromatography method for chloramphenicol acetyltransferase

The method described is a modification that described by Gorman et al (1982).

1. Cells were harvested and the cell pellet from one 90mm dish was resuspended in 100 ul of 0.25 M Tris - Cl (pH7.8), vortexing vigorously to break up clumps of cells.

2. The cells were disrupted by three cycles of freezing in dry ice and ethanol and thawing at 37°C.

3. The suspension of disrupted cells was centrifuged at 12,000g for 5 minutes at 4°C in a microfuge. The supernatant was transferred to a fresh microfuge tube, 50ul was reserved for the CAT assay, the remainder being stirred at -20°C.

4. The 50 ul aliquot was incubated at 65°C for 10 minutes to inactivate deacetylases. If the extract was cloudy or opaque at this stage, the particulate material was removed by centrifugation at 12,000g for a further 2 minutes at 4 °C in a microfuge.

5. The **CAT reaction mixture** was prepared as follows:

1M Tris Cl (pH 7.8)

50ul

^{14}C-labelled chloramphenicol

(60mCi/mmole, diluted in water to 0.1mCI/ml)

10ul

acetyl coenzyme A

(freshly prepared at a concentration of 3.5 mg/ml in H_2O)

20ul

80ul of CAT reaction mixture 1 was required for each 50ul of cell extract.

6. Each of the samples to be analysed was mixed with 80 ul of CAT reaction mixture1 and incubated at 37°C for 30 minutes.

7. 1ml of ethyl acetate was added to each sample, and mixed thoroughly by vortexing for three 10 second periods. The mixtures were centrifuged at 12,000g for 5 minutes at room temperature in a microfuge. The acetylated forms of chloramphenicol partition into the organic (upper) phase; unacetylated chloramphenicol remaining n the aqueous phase.

8. 900ul of the upper phase was transferred to a fresh tube, care being taken to avoid the interface and the lower phase.

9. The ethyl acetate was evaporated under vacuum, by placing the tubes in a rotating evaporator (Speedvac) for 1 hour.

10. The reaction products were re dissolved in 25ul of ethyl acetate, carefully washing the sides of the tube.

11. 10ul of the dissolved reaction products was applied to the origin of a 25-mm silica TLC plate, the origin on the plate having been marked with a soft lead pencil (about 3cm from the bottom of the plate). 5ul was applied at a time and evaporated to dryness with a hair dryer after each application.

12. A TLC chamber was prepared containing 200ml of chloroform:methanol (95:5).

13. The TLC plate was placed in the chromatography chamber, the chamber closed, and the solvent font allowed to move approximately 75% of the distance to the top of the plate.

14. The TLC plate was removed from the tank and allowed to air dry at room temperature. Adhesive dot labels marked with radioactive ink were placed on the TLC late to align the plate with the film. The plate was then exposed to X ray film.

15. The X ray film was developed and aligned with the plate, allowing the visualisation of three spots. The spot migrating the least distance consisting of

nonacetylated chloramphenicol, the two faster migrating spots consisting of singly or doubly acetylated chloramphenicol. However, doubly acetylated chloramphenicol is only detected with high levels of CAT activity, which was not usually the case here.

16. CAT activity was quantitated using a phospho-imager (Bio-Rad Laboratories, Richmond, CA), to give percent conversion of chloramphenicol to acetylated chloramphenicol for each experiment.

3.3 Critique of methods:

In these experiments an internal control was not used. It is usual to cotransfect cells with two plasmids - one that carries the construct under investigation and another that constitutively expresses an activity that can be assayed in the same cell extracts prepared for measurement of CAT activity. This would enable differences in the level of transcription from differences in the efficiency of transfection or in the preparation of extracts to be distinguished. An enzyme frequently used for this purpose is E. coli ß-galactosidase, which is expressed in transfected mammalian cells from a promoter with a broad host range (the SV40 early promoter or the Rous sarcoma virus LTR). Extracts of most types of mammalian cells contain relatively low levels of endogenous ß-galactosidase activity, and an increase in activity of up to 100 fold can usually be detected during the course of transfection. A number of different techniques can be used to normalise CAT activity to ß-galactosidase activity, such as measurement of protein in individual extracts and the assays then carried out using standard amounts of protein for each assay. Alternatively, the ß-galactosidase activity in a constant volume of extract is measured and the CAT assay is carried out with amounts of extract that contain a defined amount of ß-galactosidase activity.

Alternatively slot blots of DNA extracts from transfected cells can be performed which are probed with, for example in this case the CAT gene, the strength of

the signal as measured by densitometry being proportional to the efficiency of transfection. This second technique was attempted for these experiments, however due to the very low transfection efficiency achieved with the cardiomyocytes, while low level but consistent CAT activity could be detected, which was not present in control cells (non-transfected, non-CAT plasmid transfected), background signals negated the use of the slot blotting technique and thus results were not corrected for plasmid uptake in these experiments.

Chapter 3

Results: Induction of Stress proteins and Cytoprotection in Rat neonatal cardiomyocytes

1 Introduction

Over the past few years investigations have shown that part of the myocardium subjected to ischaemic damage is potentially salvageable. Restoration of normal cardiac function would require removal of denatured cardiac protein and re-establishment of normal cardiac protein synthesis. The heat stress proteins, which are synthesised in all organisms in response to stresses such as heat and ischaemia, may well play a pivotal role in providing protection from a subsequent stress (Minowada and Welch 1995, and Yellon and Marber 1994). The heat stress response is known to be initiated by activation of the heat shock factor (HSF-1) which is normally present as a monomer in unstressed cells and rapidly trimerizes in response to metabolic stress. This enables HSF-1 to bind to the heat shock element located within the promoter of the genes encoding the heat shock proteins which results in high level of transcription of these genes (Minowada and Welch 1995).

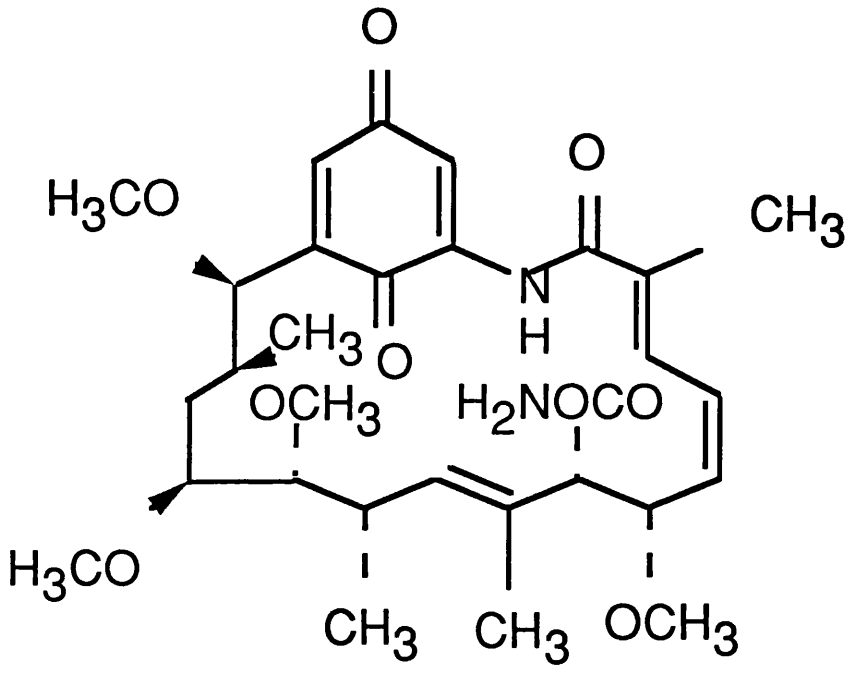
Previous investigators have found that the induction of hsps coincides with the appearance of cytoprotection. For example, exposure of cells to sub-lethal hyperthermia results in induction of hsps and the acquisition of thermotolerance (Welch and Suhan 1986). Furthermore the induction of hsps by one insult, such as toxin exposure, may confer protection from a subsequent different insult, such as heat shock challenge (Li 1983). This potentially exploitable property of cells may allow a therapeutic approach to minimizing cellular injury during myocardial ischaemia. In cardiac tissues a wide variety of insults result in the synthesis of stress proteins, including myocardial ischaemia (Dillman et al 1986, and Mehta et al 1988), trauma (Currie and White 1981), and hyperthermia (Hammed et al 1982). Further studies have shown a direct correlation between the amount of the inducible 70kD heat stress protein and the

degree of myocardial protection. For example we have examined the effects of whole body heat stress using an isolated rabbit papillary muscle model (Marber et al 1994), concluding that the degree of contractile recovery following 30 minutes of hypoxia was related to the content of hsp72. In addition Hutter et al (1994) were able to show that myocardial infarct size in the rat, 24 hours after different severities of whole body heat stress, was inversely correlated with myocardial hsp72 content. Moreover, evidence that myocardial stress proteins are directly protective is provided by the observation that transfected myocyte lines overexpressing HSP70, but not hsp90, have enhanced resistance to hypoxic stress (Mestril et al 1994, Heads et al 1994, and Williams et al 1993).

There is compelling evidence, therefore, that stress proteins may directly influence the resistance of the heart to ischaemia; and hence any pharmacological intervention that would raise stress protein levels within the cell by a direct and "non-stressful" means would have important therapeutic implications. Interestingly, Herbimycin-A, a benzoquinoid ansamycin antibiotic, which inactivates p-$60^{v\text{-}src}$ tyrosine kinase, has been shown to induce hsp72 in a range of cells, including A431 human epidermoid carcinoma cells, HeLa S3 cells, chick embryo fibroblasts and N1H3T3 cells (Murakami et al 1991). As yet the exact mechanism of action of Herbimycin-A in this regard is uncertain, but preliminary evidence suggests that hsp induction is not secondary to cellular damage (Hedge et al 1995). A more full description of Herbimycin-A is included in the introduction. The structure of Herbimycin-A is shown in figure 3.1.

Thus while the ability of Herbimycin-A to induce hsp70 has previously been shown in other cells (see above), neither the effects in cardiocytes nor effects on hsps other than hsp70 in any cell type have previously been studied. Thus the question can Herbimycin-A induce a protective response in

Figure 3.1: Structure of Herbimycin-A



Molecular weight 574.67

$C_{30}H_{42}N_2O_9$

cardiocytes, and if so, is this due to a generalised induction of the stress response or due to induction of hsp70 specifically will be answered.

We have therefore attempted to ascertain the ability of Herbimycin-A to induce hsps in primary cultured neonatal rat cardiomyocytes, using genistein, another tyrosine kinase inhibitor, for comparison. Genistein is not known to have any effect on hsp synthesis and therefore serves as a control to delineate whether any effects observed on hsps are due to the action of tyrosine kinase inhibition in general or to a different action of Herbimycin-A which is independent of its effects on tyrosine kinase inhibition. Genistein, whose structure is not related to Herbimycin-A, (see figure 3.2), was originally isolated from a culture broth of pseudomonas. This compound inhibits the activity of tyrosine kinases such as the epidermal growth factor (EGF) receptor and $pp60^{src}$, but has limited effect on serine and threonine kinases such as cAMP-dependent protein kinase. In common with Herbimycin-A, it is soluble in organic solvents, but poorly soluble in water.

Furthermore, these experiments assessed the ability of these agents to protect cardiomyocytes against "lethal" heat stress and "lethal" simulated ischaemia. In addition we compared these agents to "thermal preconditioning", which is an established method of hsp induction (Karmazyn et al 1990, and Yellon et al 1992).

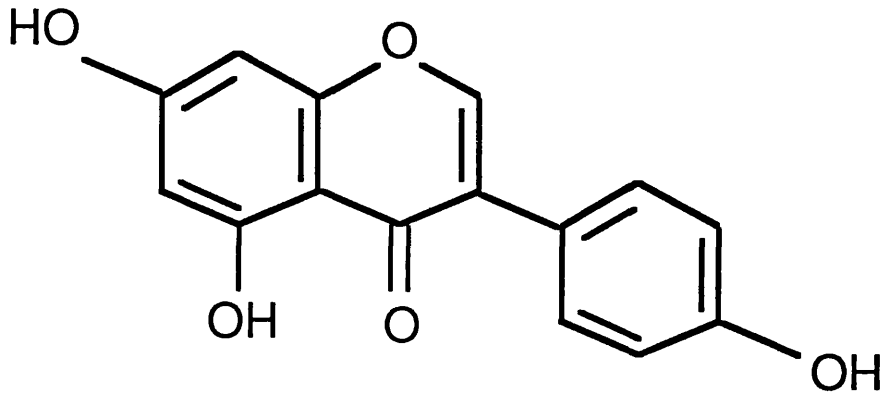
The following definitions apply in this thesis:

Non- stressful: Does not induce a wide range of stress proteins.

Mild stress: Does not cause major lethality, i. e. known to be sufficient to induce stress proteins but not significant cell death.

Severe stress: Kills a high proportion of cells.

Figure 3.2: Structure of Genistein



Geistein: 5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; 4',5,7-trihydroxyisoflavone

Molecular weight: 270.23

HSP induction and the protective effects of Herbimycin-A

2 Experimental Protocols:

In order to assess the effects of the different treatments on hsp induction and protection experiments were carried out as described below. The rat neonatal cardiocytes were prepared as described in the previous chapter. Once the cultured cardiomyocytes were beating synchronously the following protocols were carried out. Six-well plates were incubated with Herbimycin-A (1 μg/ml), or with genistein (50 μM), both agents having been dissolved in DMSO. These concentrations were chosen after thorough review of previously published work with these agents. Control plates were incubated with the vehicle DMSO with no treatment. The cells were then washed with medium and returned to the incubator at 37 °C. Experiments were also carried out in which plates of cells were "thermally preconditioned" by incubation at 43°C for 30 minutes. An initial series of experiments was carried out to determine the optimum conditions for "thermal preconditioning". This involved incubating cells at a range of different temperatures and for varying lengths of time. Incubation at 43°C for 30 minutes resulted in high levels of stress protein induction with minimal evidence of cell death determined by trypan blue (see below). The following day cells were either harvested for the detection of hsp levels by western blotting or subjected to severe heat shock or "simulated" ischaemia, to assess the degree of protection afforded by the different treatments in comparison to controls.

2.1 Polyacrylamide Gel Electrophoresis and Western Blotting

At various times after the above treatments the cells were harvested for analysis of HSP levels: approximately 2 x 10^6 cells were harvested in 100 µl of 2x concentrated SDS PAGE sample buffer and proteins separated by SDS PAGE as described in chapter 2 (Laemmli 1970). Proteins were transferred to nitro-cellulose membranes (Hybond C, Amersham) and blots were probed with monoclonal antibodies specific for the inducible isoform of hsp70 (hsp72) or with monoclonal antibodies specific for both constitutive and inducible isoforms (hsp73 and 72 respectively) In addition, blots were probed with monoclonal antibodies to hsp90, to hsp60, or to hsp25. Full experimental details are described in chapter 2. Certain blots were also probed with a monoclonal antibody specific for grp78. Grp78 is another stress protein, a member of the glucose regulated proteins (grps). These grps were first observed to exhibit increased expression in cells starved of glucose. The major grps reside within the endoplasmic reticulum and participate in various aspects of protein secretion (see chapter 1). The relative levels of hsp were determined using densitometry (Biorad) normalising to the actin band on a duplicate Coomassie Brilliant Blue R250 (BDH) stained gel (as described in chapter 2) to adjust for slight variations in protein loading between samples. Initially, in order to determine the optimum time for further experimentation, the cells were harvested at intervals between 30 minutes and 48 hours after treatment with tyrosine kinase inhibitor or "thermal preconditioning" to determine hsp levels. Maximal induction occurred at 20 hours after either treatment (see below), therefore protection and further quantification of hsp levels was sought at this time point.

2.2 Tyrosine kinase inhibition:

In order to demonstrate tyrosine kinase inhibition by Herbimycin-A and genistein, at the doses used in these experiments, the following protocol was carried out. Herbimycin-A (1μg/ml) or genistein (25, 50, 100, 150 μM) was added to wells of cardiocytes and incubated at 37°C, controls were incubated with the vehicle DMSO or were left untreated. The reason for using several different concentrations of genistein will be discussed below. Initially this experiment was done with insulin-like growth factor 1 (ILGF-1) added to each well in order to stimulate tyrosine kinase activity. ILGF-1 is a method of stimulating tyrosine kinase activity in cells, however there was sufficient intrinsic tyrosine kinase activity in control cells without growth factor for this experiment to be carried out on unstimulated cells. Samples were harvested with SDS PAGE sample buffer at the following time intervals after the addition of drug: 10, 30, 60, 120, and 240 minutes. Polyacrylamide gel electrophoresis and Western Blotting were carried out as above using a monoclonal antiphosphotyrosine antibody (Upstate Biotechnology, Inc. New York) at a dilution of 1:1000. The secondary antibody was a peroxidase conjugated rabbit anti mouse IgG antibody (DAKO), diluted to 1:2000, and enhanced chemiluminescence was used as above. Again relative levels of phosphotyrosine were determined by densitometry normalising to the actin band.

2.3 "Lethal" Heat Stress

20 hours following incubation with Herbimycin-A, genistein or DMSO; or following thermal preconditioning, the cultures were subjected to a severe thermal stress by incubating them at 47°C for 90 minutes. This protocol was determined by several initial experiments with differing temperatures and times to give optimal conditions in which there was sufficient cell death without killing all cells.

2.4 "Lethal" Simulated Ischaemia:

20 hours following incubation with Herbimycin-A, genistein, or DMSO; or following thermal preconditioning, the cardiocytes were transferred to an ischaemic buffer adapted from Esumi et al (1991) who measured NADH in adult rat myocytes during simulated ischaemia: (137mM NaCl, 3.8mM KCl, 0.49mM $MgCl_2$, 0.9mM $CaCl_2.2H_2O$, 4mM HEPES) supplemented with 10mM deoxyglucose, 0.75mM sodium dithionate, 12mM KCl and 20mM lactate (pH 6.5), and incubated for 90 minutes at 37°C. This buffer is designed to simulate the extracellular milieu of myocardial ischaemia, with the approximate concentrations of potassium, hydrogen and lactate ions occurring in vivo. As with thermal stress the development of this protocol required several initial experiments to determine the optimum conditions - i.e. sufficient cell death and giving consistent levels of cell death with repeated experiments.

2.5 Determination of Cardiocyte Viability:

2.5.1. Trypan blue exclusion:

Following lethal heat stress or simulated ischaemia the cells were washed with phosphate buffered saline (PBS), trypsinised for two minutes in 0.25mg/ml trypsin in versine (Gibco-BRL) which was then neutralised with neonatal calf serum. Cells were then centrifuged, the supernatant aspirated and the cardiocytes resuspended in 300ul of PBS. A 20µl aliquot of cell suspension was then added to an equal volume of trypan blue and the percentage of dead (blue) cells was determined using a haemocytometer. Trypan blue is a dye which is unable to permeate the plasma membrane of viable cells which therefore remain unstained when viewed under the light microscope. In order to establish that the administration of these drugs did not have a directly toxic effect upon the cardiocytes trypan blue exclusion was also performed at 4 and 24 hours following incubation with Herbimycin-A or genistein, in comparison to controls without lethal stress.

2.5.2 Lactate Dehydrogenase (LDH) Assay

Lactate dehydrogenase (LDH) catalyses the reversible inter conversion of lactate and pyruvate. It is widely distributed, with high concentrations in the heart, skeletal muscle, liver, kidney, brain and erythrocytes, and in a clinical setting measurement of plasma is used as a marker of cell damage. It is released from damaged cells and is one of the markers used to diagnose and assess damage in evolving acute myocardial infarction in the coronary care unit. LDH activity released from lethally stressed cardiomyocytes was determined using an LDH test kit (Sigma). This test relies on the fact that LDH catalyses the reduction of pyruvate to lactate resulting in an equimolar amount of NADH being oxidised to

NAD. The oxidation of NADH results in a decrease in the absorbance at 340 nm. The rate of decrease in absorbance at 340 nm is directly proportional to lactate dehydrogenase activity in the sample. Therefore using a spectrophotometer measuring absorbance at 340 nm the mean absorbance change per minute was determined in the media from each experimental group. This method of assessing cell damage was chosen as it is reliable even in the presence of ischaemic buffer, unlike creatinine kinase activity (Iwaki et al 1993). LDH values were measured at several different time points. Released LDH was assessed immediately prior to treatment with Herbimycin-A, Genistein or thermal preconditioning; and further LDH measurements were made after these pre treatments. This enabled a comparison to be made between these LDH values and those obtained following "severe stress"

2.6 Statistical Analysis

All experiments were performed in duplicate and on at least five separate preparations of cells. Values are expressed as mean +/- SEM. The unpaired Student's t test was used to identify significant differences between experimental and control groups. Statistical significance was assumed at the $p<0.05$ level.

2.7 Does Herbimycin-A mediate its effect through the heat shock element:

2.7.1 Transfection of plasmids

The following experiments were carried out to elucidate the possible mechanism by which Herbimycin-A induces hsp70, i.e. is there a direct effect on the heat shock element (HSE) within the promoters of the hsp genes or is some other

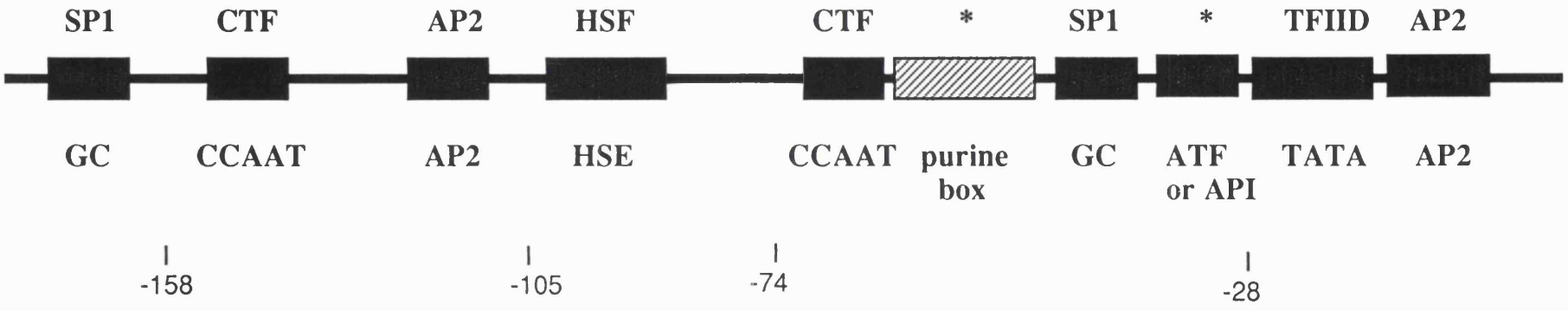
method of hsp induction involved? Hence three different plasmids were used to determine whether Herbimycin-A acts through the heat shock element to induce stress protein synthesis. Two of these plasmids were different mutated hsp70 promoter constructs - one containing a mutation in the region of the heat shock element (HSE), the other with a mutation in a region of the promoter unrelated to the HSE.

The plasmids were LSN-CAT and LSPN-CAT (obtained from Gregg T Williams, Department of biochemistry, Northwestern University, Evanston, Illanois 60208, USA) (Williams et al 1989), with RSV-CAT as a control. Transcription of the human hsp70 gene is regulated by a complex array of cis-acting promoter elements that respond to conditions that include both normal conditions of cell growth and induction following physiological stress. They found that, using a technique of promoter mutations and a transient transfection assay, stress - induced transcription (including heat shock, heavy metals and amino acid analogs) is mediated by a single heat shock element between -105 and -91 consisting of three contiguous 5 base pair units, NGAAN, on the basis of in vitro transfection data.

They found that maximal inducible expression requires a fully functional basal promoter. They also found that spacing mutations that vary the relative spacing and rotational alignment between different regions of the promoter have minimal effects on inducible expression - indicating a surprising degree of flexibility in the mechanism of heat shock promoter transcriptional regulation.

The human hsp70 promoter has a HSF binding site at -100, as well as binding sites for other transcription factors as illustrated in figure 3.3 These sites include those for CTF at -150 and -67, Sp1 at -160 and -48, AP2 at -120 and -20, and TFIID at -28. As shown in figure 3.4 of the LSN transfection vector the mutation occurs in the context of -188, whereas for the LSPN transfection vector the mutation is in the context of -100, i. e. the region of the HSF binding

Figure 3.3: schematic of human HSP70 promoter



Adapted from Williams et al 1989

Promoter sequences from -1 to -188 are represented schematically by a line. The boxes represent the approximate locations of transcription factor binding sites in the promoter. The factors that have been shown to bind to these regions are indicated above the boxes. The corresponding genetic elements located in these binding regions are indicated below the boxes.

Key:

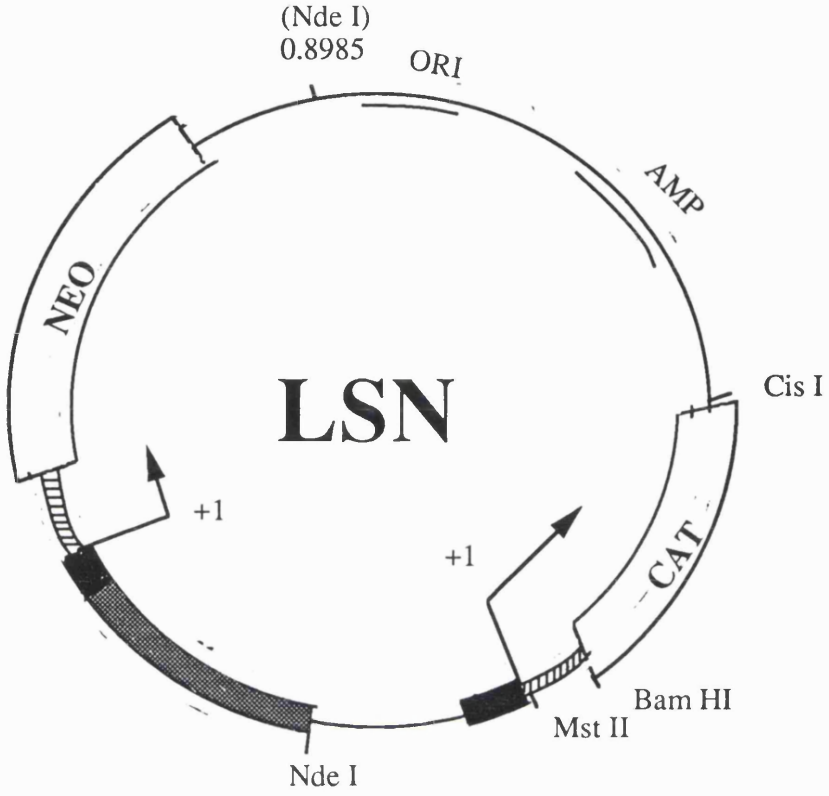
 Protein-DNA interactions characterised by DNase I footprinting

 Interaction characterized by exonuclease III footprinting

* Sites where binding activity has been detected but not identified

Sequences upsteam of the -100 LSPN boundary may contain additional transcription elements, which include a CCAAT box, a GC box, an AP2 site, and an intact heat shock element (HSE).

Figure 3.4: LSN Transfection vector



Adapted from Williams et al 1989

Key

- Human HSP 70 Promoter sequences
- Human HSP 70 untranslated leader sequences
- Bacterial genes
- Vector sequences
- λ Spacer DNA

The LSN and LSPN series contain LS mutations in the context of -188 and -100 HSP 70 promoters respectively. The linker regions in these mutations are indicated by boldface letters.

site. In Williams experiment this LSPN construct, which has completely lost its distal 5 base pair unit, was non responsive to all three forms of stress (heat shock, metals and amino acid analogs) even though the potential MRE (metal responsive element) is left intact.

2.7.2 Protocols for transfection experiments:

For each of these plasmids the following protocols were carried out:
60 mm plates containing monolayers of rat neonatal cardiocytes having were transfected by a standard calcium precipitation technique (as described in chapter 2) with one of the three different plasmids (LSN-CAT, LSPN-CAT or RSV-CAT). Following transfection the cells were incubated with Herbimycin-A (1 μg/ml), or with genistein (50 μM), both agents having been dissolved in DMSO. Control plates were incubated with the vehicle DMSO or were simply left in medium. The cells were then washed with medium and returned to the incubator at 37 °C. As a comparison in some experiments plates of cells were "thermally preconditioned" by incubation at 43°C for 30 minutes. The following day cells were harvested for CAT activity. These experiments were repeated five times for each group.

3 Results

Incubation with Herbimycin-A or genistein at all concentrations studied, or treatment by thermal preconditioning did not result in significant cell death compared to controls as assessed by trypan blue exclusion (less than 3%) and LDH assay. In addition light microscopic appearances were unchanged and the cells continued to beat synchronously.

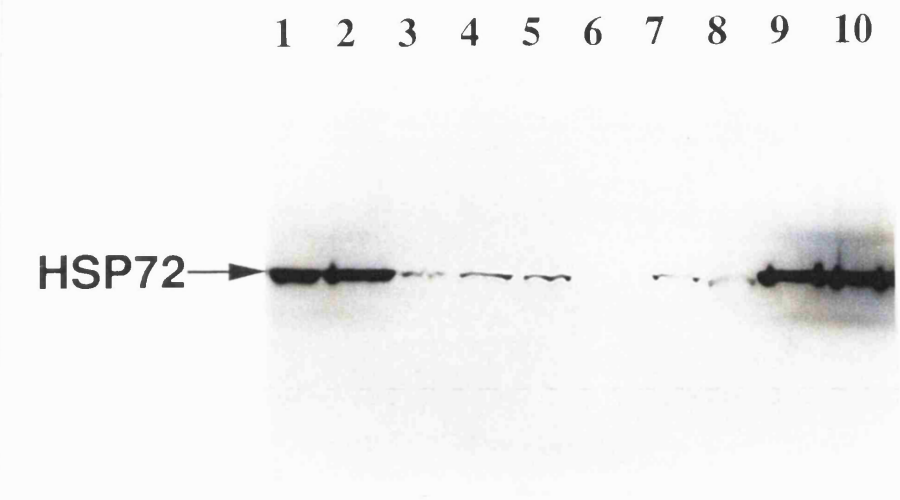
3.1 Heat Shock Protein Induction

Following four hours of incubation with Herbimycin-A there was a significant increase in hsp72 from almost negligible levels to more than 100X that found in control cells ($p<0.0001$) when samples were normalised to the actin band from a Coomassie stained gel (Figure 3.5a and 3.5b). This level of hsp72 increase was of a similar magnitude to that induced by thermal preconditioning, and there was no statistically significant difference between amounts of hsp72 induced by Herbimycin-A or thermal preconditioning. Genistein treatment at a wide range of doses did not lead to any significant hsp induction. A study of the time course of hsp72 induction over 48 hours showed maximal induction at between 19-24 hours after Herbimycin-A treatment or thermal preconditioning; but again there was no evidence of hsp induction by genistein up to 48 hours (Figure 3.6).

To examine the effect of Herbimycin-A on the constitutive 70kDa hsp (hsp73), we used an antibody which recognises both hsp72 and 73 since there is no antibody available which is specific for hsp73 alone. In this experiment Herbimycin-A induced both constitutive and inducible isoforms of hsp70 (hsp73 and 72 respectively) whereas in the thermal preconditioning group the induction, as expected, is mainly observed in the inducible hsp72 band (Figure

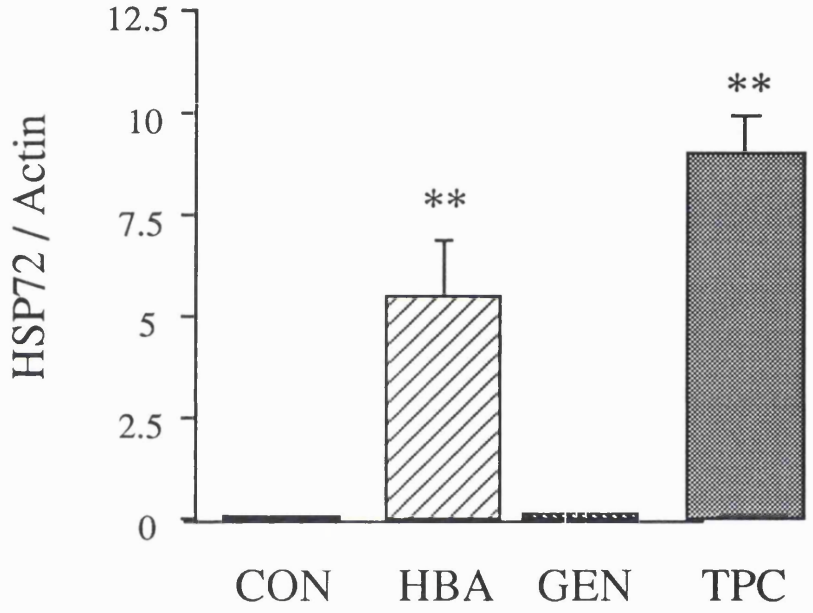
Figure 3.5a

72 kDa stress protein levels 20 hours after each pretreatment



Six well plates were incubated for four hours with Herbimycin-A or genistein dissolved in DMSO. For comparison certain plates were thermally preconditioned by incubating them at 43°C for 30 minutes. Control plates were incubated with the vehicle DMSO or simply left in medium. The primary monoclonal antibody recognises the inducible form (hsp72) of the 70 kDa heat shock protein. Lanes from left to right, 1. Herbimycin-A, 2. Herbimycin-A, 3. Control - no intervention, 4. Control - no intervention, 5. Control - DMSO, 6. Blank lane, 7. Genistein, 8. Genistein, 9. Thermal preconditioning, 10. Thermal preconditioning. The protein band representing hsp72 is indicated (*arrow*).

Figure 3.5b **Relative levels of hsp72 as assessed by densitometry (Biorad) normalising to the actin band on a duplicate Coomassie Brilliant Blue R250 (BDH) stained gel**



Bars represent one standard error of the mean. Data are presented as mean values (+SE) from duplicate determinations in five independent experiments.

** $p < 0.0001$.

Key

CON - Control,

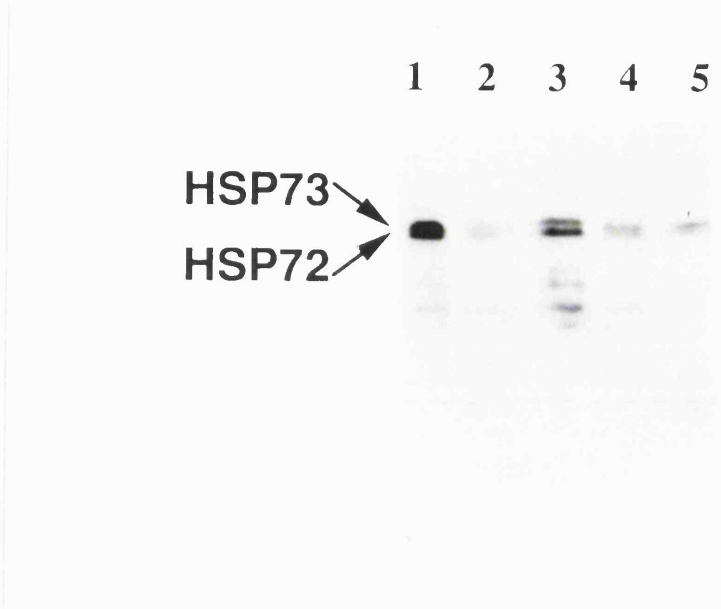
HBA - Herbimycin-A,

GEN - Genistein,

TPC - Thermal preconditioning.

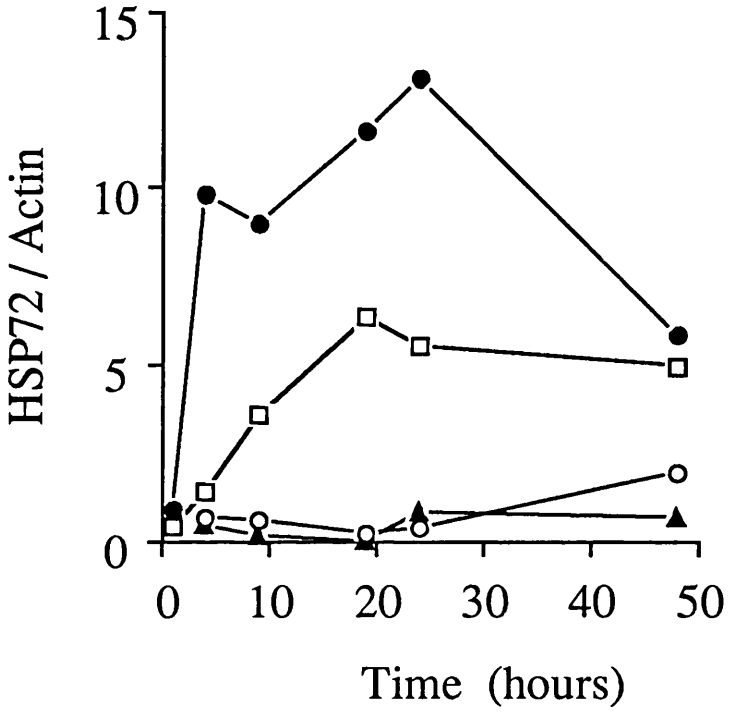
Figure **3.5c**

Inducible and constitutive isoforms of hsp70 (72 and 73 kDa) 20 hours after each pre treatment



Western blot of samples harvested from primary cardiomyocyte cultures 20 hours following treatment with tyrosine kinase inhibitor. Six well plates were incubated for four hours with Herbimycin-A (1μg/ml) or genistein (50μM) dissolved in DMSO. For comparison certain plates were thermally preconditioned by incubating them at 43°C for 30 minutes. Control plates were incubated with the vehicle DMSO or simply left in medium. The primary monoclonal antibody recognises both the inducible (hsp72) and constitutive (hsp73) isoforms of the 70 kDa heat shock protein. Lanes from left to right, 1. Herbimycin-A, 2. Genistein, 3.Thermal preconditioning, 4. Control - no intervention, 5. Control - DMSO. The protein bands representing hsp72 and hsp73 are indicated (*arrow*).

Figure 3.6 The time course of hsp72 induction.



Primary cardiocytes were incubated with Herbimycin-A, or genistein and samples were harvested for Western blotting at intervals between 30 minutes and 48 hours. The primary monoclonal antibody recognises the inducible form of the 70 kDa heat shock protein (hsp72). Maximal levels of hsp72 are seen at 19 to 25 hours for both herbimycin-A treated cells and thermally preconditioned cells.

Key:

- ● Thermal preconditioning
- □ Herbimycin-A
- ○ Genistein
- ▲ Control

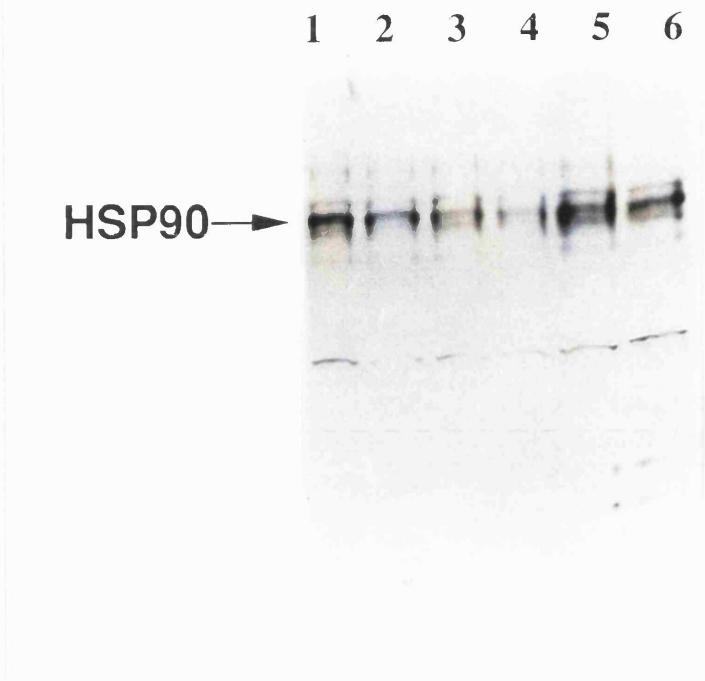
3.5c). Due to the magnitude of induction of 70kDa stress proteins by Herbimycin-A the bands are almost confluent, resolution being limited by the fact that there is no antibody available which recognises hsp73 alone. In addition there was no evidence of increases in hsp90 (Figure 3.7a), hsp60 (Figure 3.7b), or hsp25 (Figure 3.7c) following Herbimycin-A or genistein treatment. Furthermore these treatments did not induce the glucose-regulated protein grp78 (Figure 3.7c). As expected, thermal preconditioning resulted in increased levels of all stress proteins measured. Thus the effect of Herbimycin-A appears to be specific for hsp70 (both constitutive and inducible forms) with no effects on other stress inducible proteins, whereas for thermal preconditioning all the stress proteins studied in these experiments were increased.

3.2 Survival Following "Lethal" Heat Stress

Interestingly, and as might be expected concurrent with an increase in hsp70, treatment of cardiomyocytes with Herbimycin-A led to a significant increase in survival following "lethal" heat stress in comparison to control cells as assessed using trypan blue exclusion. Each experiment was performed five times using five different preparations of cells, with at least three wells per treatment group. As seen in figure 3.8, cell death in Herbimycin-A treated cells was 45.0% (±3.6), whereas cell death in control groups was 69.4% (±3.4), ($p<0.005$, $n=10$). Thus Herbimycin-A treated cells have a statistically significant 30% improvement in survival following "lethal" heat stress. A similar degree of protection was obtained by thermal preconditioning with 52.9 % (±1.8) dead, the difference in improved survival between the Herbimycin-A and the thermal preconditioning groups was not statistically significant. However, genistein

Figure 3.7a

90 kDa stress protein content 20 hours after each pre treatment



Western blot of samples harvested from primary cardiomyocyte cultures 20 hours following a four hour incubation with Herbimycin-A (1 μg/ml) or genistein (50 μM). The primary monoclonal antibody recognises the 90 kDa heat stress protein. Lanes from left to right, 1. Thermal preconditioning, 2. Herbimycin-A, 3. Genistein, 4. Genistein, 5. Control - DMSO, 6. Control - no intervention. The protein band representing hsp90 is indicated (*arrow*). There is no increase in signal in the cultures treated with Herbimycin-A, in contrast to the hsp72 data.

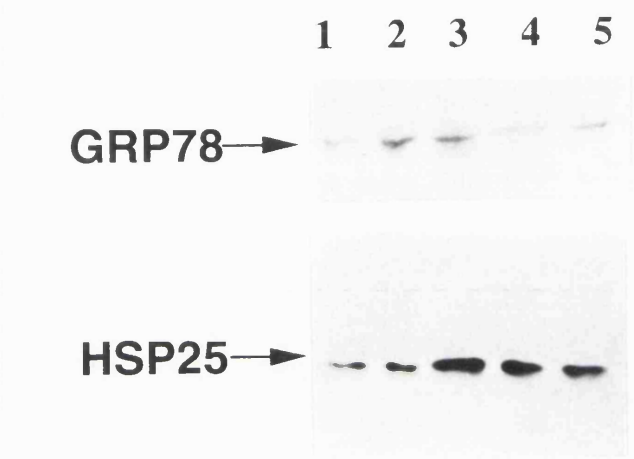
Figure **3.7b**

60 and 72 kDa stress protein content 20 hours after each pre treatment

Western blot of samples harvested from primary cardiomyocyte cultures 20 hours following a four hour incubation with Herbimycin-A (1 μg/ml) or genistein (50 μM). Two primary monoclonal antibodies have been used, one recognising hsp60, the other being specific for the inducible isoform of the 70kDa heat stress protein (hsp72). Lanes from left to right, 1. Herbimycin-A, 2. Herbimycin-A, 3. Genistein, 4. Genistein, 5. Control - DMSO, 6. Control - no intervention. The protein bands representing hsp60 and 72 are indicated (*arrow*).

Figure **3.7c**

Glucose regulated protein (grp) 78 and hsp 25 content 20 hours after each pre treatment



Western blot of samples harvested from primary cardiomyocyte cultures 20 hours following a four hour incubation with Herbimycin-A (1 μg/ml) or genistein (50 μM). Two primary monoclonal antibodies have been used, one recognising grp78, the other being specific for the 25kDa heat stress protein (hsp25). Lanes from left to right, 1. Herbimycin-A, 2. Genistein, 3.Thermal preconditioning, 4. Control - no intervention, 5. Control - DMSO. The protein bands representing grp78 and hsp25 are indicated (*arrow*).

(50μM) treatment led to no significant improvement in survival, 70.9% (±2.0). Comparable results were obtained by analysis of released LDH activity: control cells 20.2 U/L (±2.6), Herbimycin-A group 8.25 U/L (±0.25) ($p<0.05$), thermal preconditioning group 6.8 U/L (±2.50 and genistein group 15.25 U/L (±3.55) (figure 3.9).

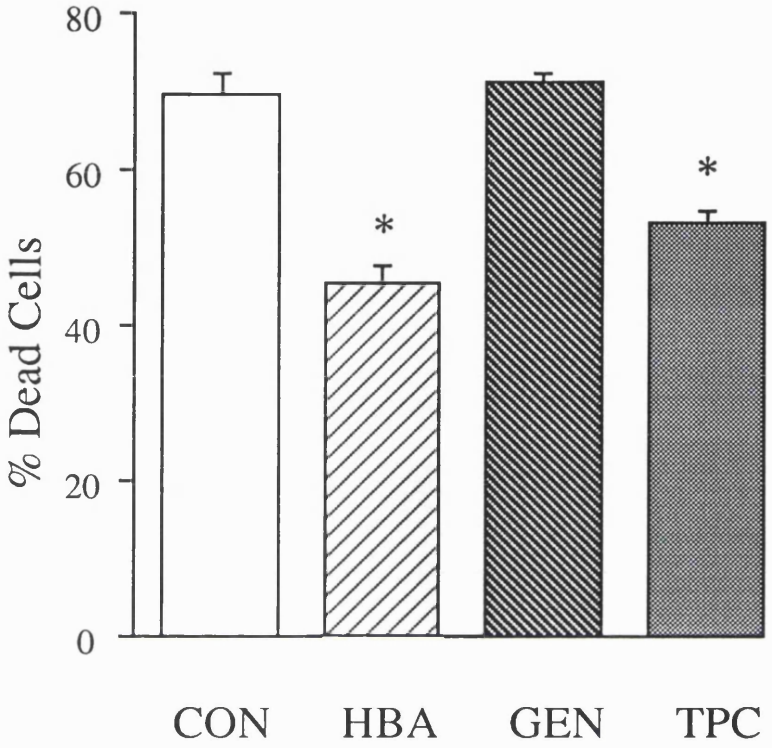
3.3 Survival Following "Lethal" Simulated Ischaemia

Similarly treatment of cardiomyocytes with Herbimycin-A or with thermal preconditioning led to a significant increase in survival against a subsequent "lethal" ischaemic insult in comparison to control cells as assessed by trypan blue exclusion. Duplicate determinations were made in five independent experiments using five different preparation of cells. As shown in figure 3.10, in the Herbimycin-A treated group the percentage of dead cells was 50.6% (±5.6), ($p<0.001$), 50.6% (±1.9), ($p<0.001$) in thermally preconditioned group, and 68.1% (±1.9) in control cells. Again genistein treatment did not alter survival significantly from control values with 62.4% (±2.6) dead cells in this group. Thus Herbimycin-A treated cells have a statistically significant 25% improvement in survival when exposed to a subsequent ischaemic insult.

Results obtained with LDH assay as a marker of cell damage yielded comparable results (figure 3.11). Control cells released significantly more LDH, 15.9U/L (±0.8), than those treated with Herbimycin-A 7.4 U/L (±1.1), ($p<0,001$), or thermal preconditioning 8.0 U/L (±2.0), ($p<0.01$). Genistein (50μM) treated cells had similar levels of LDH activity to controls, 14.8 U/L (±1.1).

Therefore overall the effects of Herbimycin-A treatment are similar to preconditioning in terms of protection against stress, and are consistent with effects previously shown by the upregulation of hsp70 expression in cell culture

Figure 3.8 **Percentage of cell death following "lethal" heat stress as assessed by trypan blue exclusion**



24 hours following pretreatment cells were exposed to 47'C for 90 minutes. Following this "lethal" heat stress, cells were exposed to an equal volume of trypan blue and the percentage of dead (blue) cells is shown. Data are presented as mean values (+ S.E) from duplicate determinations in five independent experiments.

*$p<0.005$.

Key

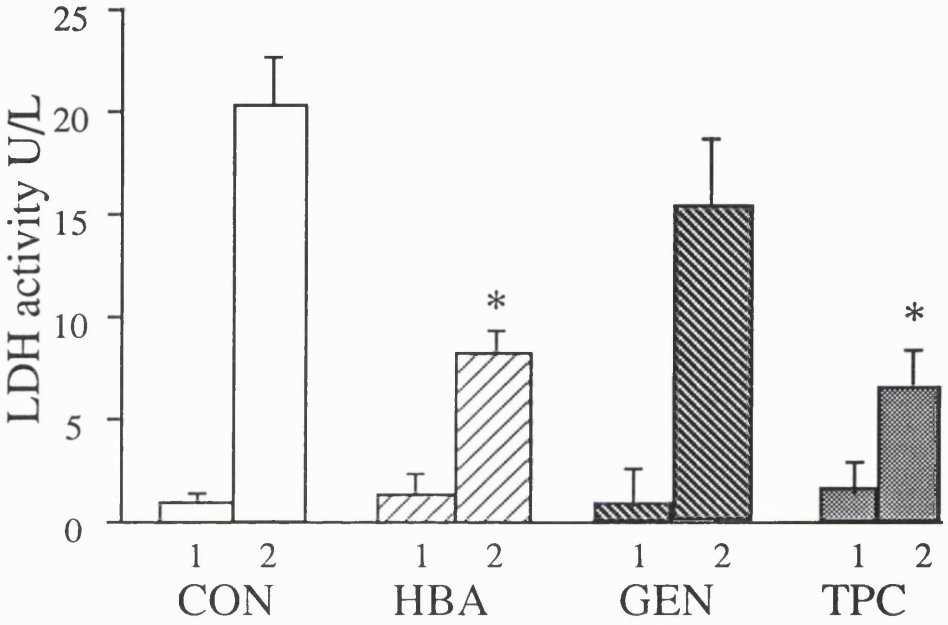
CON - Control,

HBA - Herbimycin-A,

GEN - Genistein,

TPC - Thermal preconditioning.

Figure 3.9 Cell damage following "lethal" heat stress as assessed by released lactate dehydrogenase activity



LDH released into the media as a result of cell damage was determined with an LDH test kit. Values befor "lethal" heat stress are labelled **1** and values after "lethal" heat stress are labelled **2**. Data are presented as mean values (+ S.E.). Significant protection is observed in the Herbimycin-A group and in the the thermal preconditioning group compared to controls. *$p<0.05$.

Key

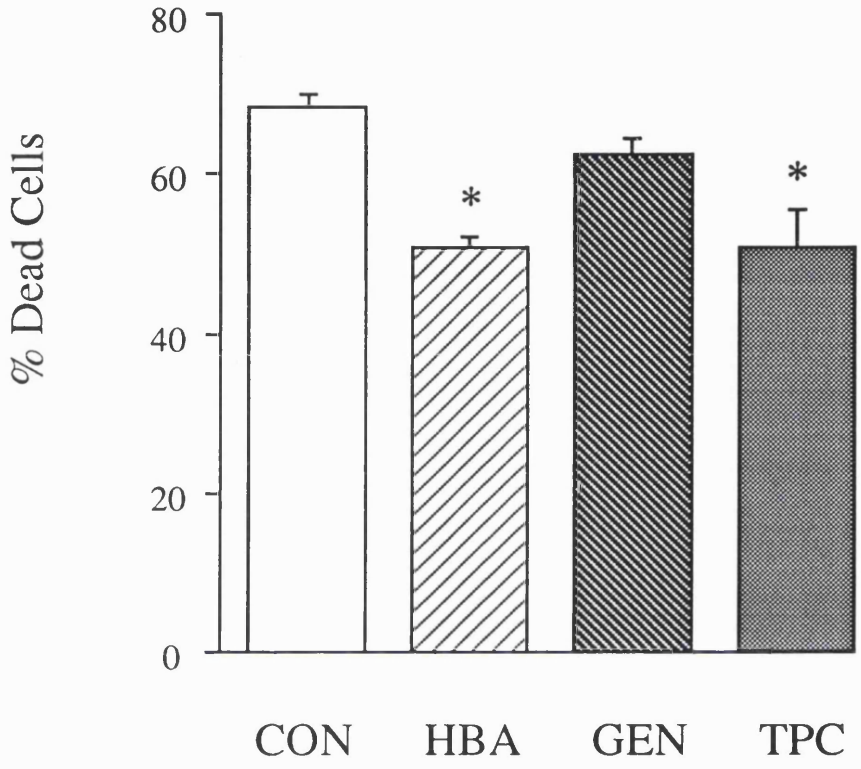
CON - Control,

HBA - Herbimycin-A,

GEN - Genistein,

TPC - Thermal preconditioning.

Figure 3.10 **Cell damage as assessed by trypan blue following "lethal" simulated ischaemia.**



24 hours folllowing pre treatment all groups of cells were transferred to an ischaemic buffer for 90 minutes at 37°C. Cell viability was then determined by trypan blue exclusion. The graph illustrates percentage of cells death (blue cells) presented as mean values (+ SE) from duplicate determinations in five independent experiments. *$p<0.001$.

Key

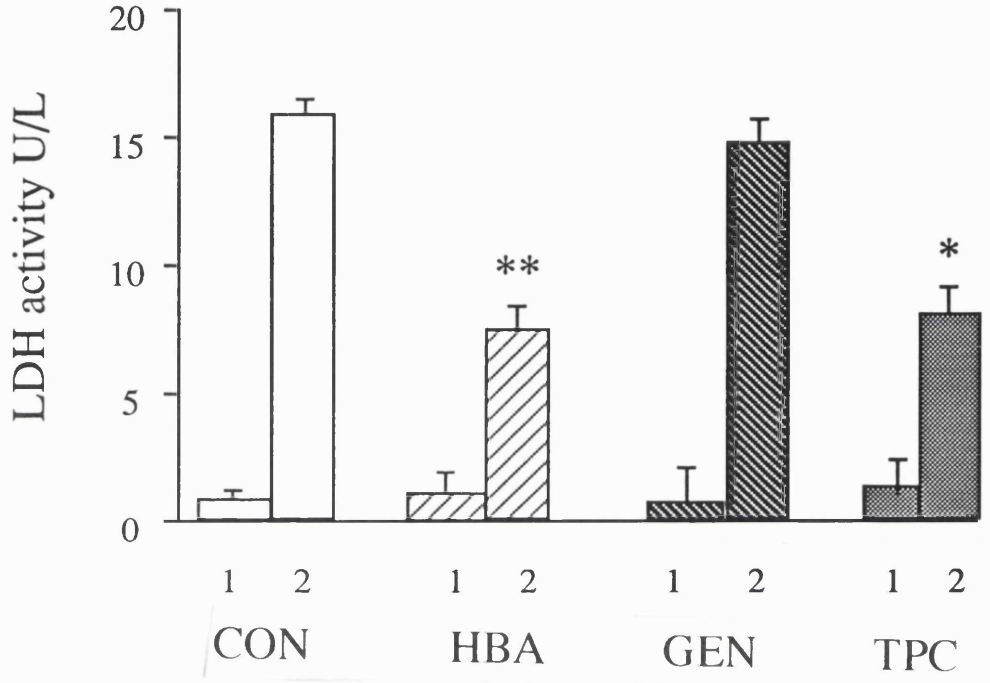
CON - Control,

HBA - Herbimycin-A,

GEN - Genistein,

TPC - Thermal preconditioning.

Figure 3.11 **Cell damage following "lethal" simulated ischaemia as assessed by released LDH activity.**



LDH released into the media as a result of cell damage was determined with an LDH test kit. Values before "lethal" simulated ischaemia are labelled **1**, values after are labelled **2**. Data are presented as mean values (+ SE). Significant protection is observed in the Herbimycin-A group ** $p<0.001$, and also in the thermal preconditioning group ** $p<0.01$.

Key

CON - Control,

HBA - Herbimycin-A,

GEN - Genistein,

TPC - Thermal preconditioning.

(by stable transient transfection (Heads et al 1995)) and transgenic animal models (Marber et al 1995).

3.4 Tyrosine Kinase Activity

In view of the different effects on hsp72 induction observed with the tyrosine kinase inhibitors Herbimycin-A and genistein we wished to confirm that both these inhibitors were having the expected effect on tyrosine kinase activity. Western blotting with monoclonal antiphosphotyrosine antibody showed reduced activity in both Herbimycin-A treated cells and genistein (concentrations 25,50,100 and 150μM) treated cells in comparison to controls, (figure 3.12). Genistein appeared to have a more immediate effect with inhibition of activity occurring after just 10 minutes for all concentrations of drug, whereas Herbimycin-A inhibited tyrosine kinase after 30 minutes of incubation to a similar degree. Thus biologically active levels of both agents were present in both cases.

3.5 Results of transfection experiments:

CAT activity was quantitated using a phospho-imager (Bio-Rad Laboratories) to give percentage values of conversion for each experiment.

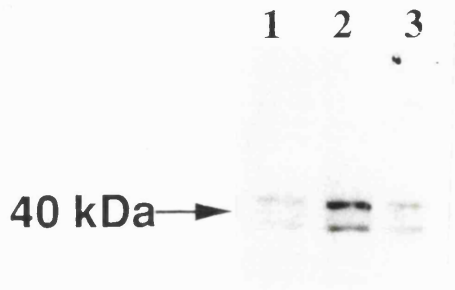
Table 3.1: CAT activity (% conversion) mean of 5 experiments

	LSN	**LSPN**	**RSV**	**No plasmid**
Treatment				
No treatment	0.9	1.1	4	0
Herbimycin A	1.2	0.9	3.7	0
Genistein	1	1.2	3.9	0
TPC	2.1	1.6	4.3	0

See figure 3.13 for representative graph.

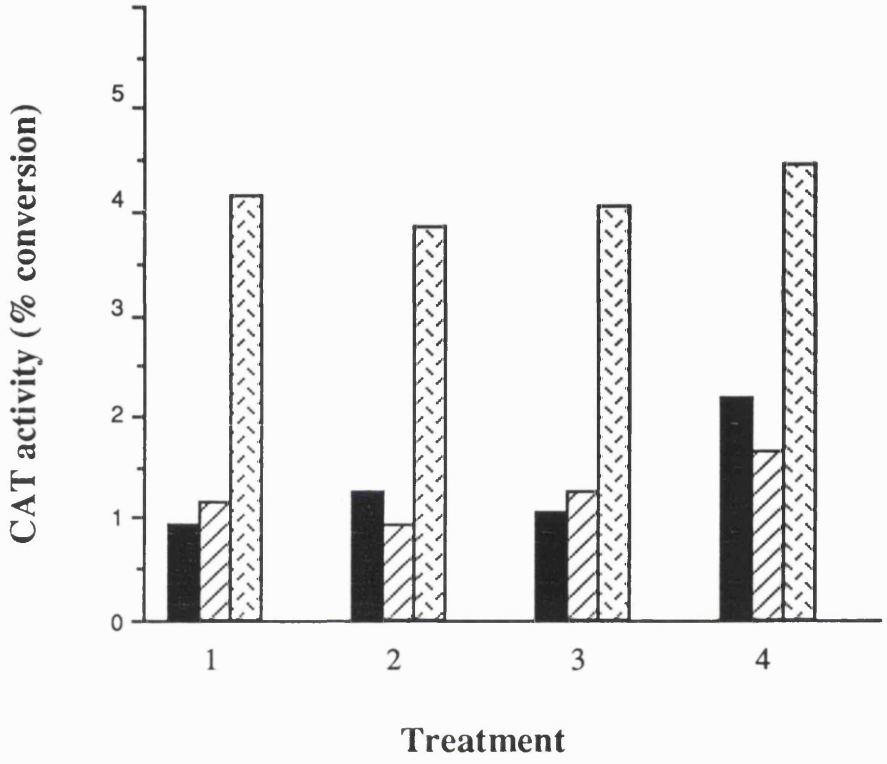
Figure 3.12

Western blot probed with antiphosphotyrosine antibody



Western blot of samples prepared from primary cardiocyte cultures 30 minutes following incubation with Herbimycin-A or genistein. Controls were incubated with the vehicle DMSO. The primary monoclonal antibody is an antiphosphotyrosine. Lanes from left to right, 1. Herbimycin-A, 2. Control, 3. Genistein. Both Herbimycin-A and genistein inhibit the phosphorylation of a prominent protein doublet at 40 kDa.

Figure 3.13 : Transfection experiments - Graph of CAT Activity



Key:

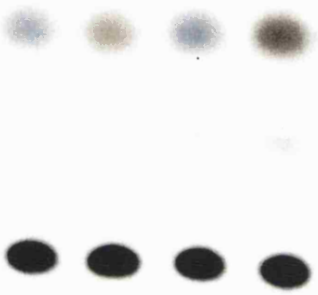
LSN	1 No treatment
LSPN	2 Herbimycin-A
RSV	3 Genistein
No plasmid	4 Thermal preconditioning

The transfection efficiency of cardiocytes using the calcium phosphate procedure was rather low, and thus levels of CAT activity were not high even with control plasmid driving CAT from a strong promoter (RSV - CAT). However experiments were repeated seven times in duplicate and in each case relative activities between the different treatments and plasmids were similar, allowing firm conclusions to be drawn.

Example CAT assays are shown in figure 3.14a-c. In the example shown the small apparent induction of LSPN by Herbimycin-A was not reproducible and was an example of fluctuations between experiments, as was the apparent repression of RSV-CAT by genistein in the example shown.

Thus from the above results it can be seen that CAT activity did not appear to be induced by Herbimycin-A treatment, suggesting that the effect of Herbimycin-A on hsp70 expression does not involve the heat shock element-containing region of the promoter. This is in agreement with our finding that the other hsps are not induced by Herbimycin-A treatment, as would have been expected if induction was dependent of the heat shock element, and that constitutive hsp70 is also induced - unlike in heat stress. Thus these results also suggest a different mechanism to the stress response induced by salicylate which stimulates HSF binding to the HSE (Jurvich 1992). However, as expected thermal preconditioning did induce CAT activity from the HSE - containing plasmid (LSN -CAT) which was considerably reduced when the HSE was absent (LSPN - CAT). Clearly, in order to determine the mechanism by which Herbimycin-A induces stress proteins, further work will need to be done including the use of longer promoter sequences with mutations at various sites.

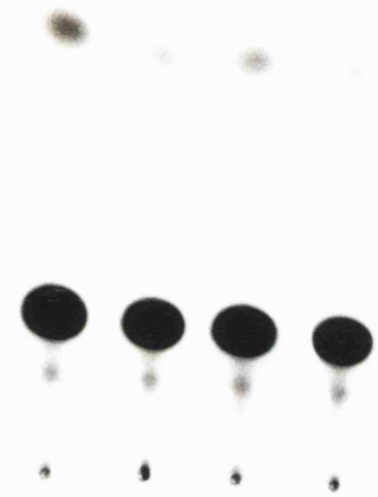
Figure 3.14a
CAT assay radiograph: Plasmid LSN-CAT



C H G T

Key:
C: Control
H: Herbimycin-A
G: Genistein
T: Thermal preconditioning

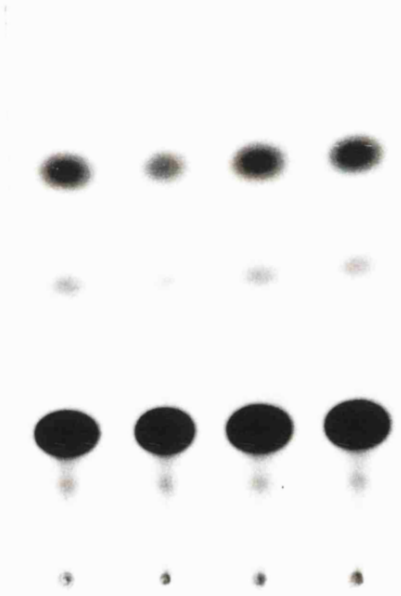
Figure 3.14b
CAT assay radiograph: Plasmid LSPN-CAT



T G H C

Key:
C: Control
H: Herbimycin-A
G: Genistein
T: Thermal preconditioning

Figure 3.14c
CAT assay radiograph: Plasmid RSV-CAT



T G H C

Key:
C: Control
H: Herbimycin-A
G: Genistein
T: Thermal preconditioning

Thus while this result is slightly disappointing, as direct interaction with the HSE would have provided an easily defined mechanism of action, these results are consistent with the findings related to hsp expression, i. e. that Herbimycin-A specifically induced hsp70 and not the whole range of stress proteins as is the case in the stress response. However, recent work (see conclusion) has shown that Herbimycin-A can interact with the transcription factor NFkB (Mahon 1995), suggesting an alternative possible mechanism of action for Herbimycin-A, although due to time limitations this could not be further explored in this thesis.

4 Discussion

4.1 Study findings

Earlier studies have shown that expression of stress proteins from a variety of different environmental stimuli correlates with protection against subsequent adverse stress. We have been able to show that the benzoquinoid ansamycin antibiotic Herbimycin-A is able to induce 70kDa hsps, in primary neonatal rat cardiomyocytes, as well as protecting these cells against lethal heat stress and simulated ischaemia. Genistein, also an inhibitor of tyrosine kinase, neither induced 70kDa hsps nor conferred any protection to these cells. We initially screened a wide range of concentrations of genistein to confirm that its lack of effect on hsp induction and protection was apparent at all concentrations.

In our experiments in cardiac cells Herbimycin-A induced only the 70kDa hsps and not a wide range of other hsps. This is in contrast to the work of Hedge et al (1995) who found that Herbimycin-A induces a range of hsps in fibroblasts,

indicating that Herbimycin-A may have a cell type-specific effect on some hsps. At least in cardiac cells, our results show a correlation between 70kDa hsp induction and tolerance against lethal heat stress and ischaemia, with such protection occurring in the absence of induction of the other hsps. However, we cannot imply from this a directly protective effect of hsp 70, since Herbimycin-A may induce other non hsp proteins; for example other candidates for protection include endogenous antioxidants such as manganese superoxide dismutase (Mn-SOD) (Yamashita et al 1994) which we did not measure in our experiment. However hsp 72 transfection studies in cell lines (Mestril et al 1994, Heads et al 1994 and Williams et al 1993) taken together with a number of transgenic studies (Marber et al 1995, and Plumier et al 1995) in which expression of an hsp 72 transgene in the mouse heart protects against ischaemia/reperfusion injury, as well as the known actions of hsp72 in protein folding and transport, provide strong evidence for a directly protective effect. It is also clear from our results that the induction of 70kDa hsps by a pharmacological route can provide effective protection without any induction of the other hsps.

The time course for induction of hsp72 was similar in both thermally preconditioned groups and those treated with Herbimycin-A, and also the magnitude of the hsp response was comparable in these groups. It is particularly interesting to note that in our experiments in cardiac cells Herbimycin-A did not induce hsp90, 60 or 25. In contrast, the heat shock response results in the synthesis of a number of stress proteins, and in our experiments thermal preconditioning did induce the other hsps as expected. This suggests that the mechanism of hsp72 induction by Herbimycin-A may not be via activation of the heat shock transcription factor, unlike the agents or mechanisms which induce the stress response (Minowada and Welch 1995),

but rather that Herbimycin-A may act via a distinct and possibly less "stressful" pathway for hsp72 induction. This is also supported by the fact that Herbimycin-A appears to strongly induce both isoforms of hsp70 (hsp73 and hsp72) and this contrasts to the pattern of induction evoked by the stress response.

4.2 Mechanism of action of Herbimycin-A

We have been able to show that, despite being used at doses adequate for tyrosine kinase inhibition, genistein was unable to induce any hsps and similarly unable to protect cardiocytes from lethal stress. Hence the tyrosine kinase inhibitory activity of Herbimycin-A is unlikely to be responsible for its action with regard to 70kDa hsp induction and enhanced tolerance against lethal stress. Although genistein and Herbimycin-A are both tyrosine kinase inhibitors their modes of action are quite dissimilar. Herbimycin-A has a benzaquinone moiety and is thought to covalently modify thiol groups on its target kinase (Mahon and O'Niell 1995), and it therefore may have other actions related to this thiol-reactivity. Interestingly recent reports have suggested Herbimycin-A may directly modify the transcription factor NFkB (Mahon and O'Niell 1995) Their results suggest that Herbimycin-A may modify the p50 subunit on cysteine 62 in the NFkB complex, which blocks DNA binding and NFkB driven gene expression. It has also been shown that NFkB activation is inhibited by sodium salicylate which prevents the degradation of IkB (Kopp and Ghosh 1994). IkB is an associated protein which inhibits NFkB activity, on phosphorylation of IkB the complex dissociates and NFkB moves to the nucleus where it activates gene expression (Latchman 1995; and Thanos and Maniaitis 1995). Furthermore sodium salicylate induces the heat shock-responsive chromosomal puffs in Drosophila salivary glands and induces HSF

DNA binding activity in cultured Drosophila cells as well as activating DNA binding by the HSF in cultured human cells (Jurvich et al 1992). This suggests a possible link between NFkB and stress protein transcription, with Herbimycin-A possibly inducing 70kDa hsps via its inhibitory action on NFkB.

4.3 Conclusions

Further elucidation of the pathway by which Herbimycin-A acts may not only enable us to understand the mechanisms of hsp 72 induction, but furthermore lead us to strategies for targeted hsp72 induction and possibly protection against lethal stress particularly ischaemia in a clinically relevant context.

Acute myocardial infarction remains the most common single cause of death in men in the western world. Interventions such as thrombolytic therapy and aspirin have revolutionized the treatment of myocardial infarction. However, the mortality benefit of thrombolytic therapy is diminished if treatment is administered late (GUSTO 1993, and ISIS 2 1988). Hence any intervention that could delay the onset of myocardial necrosis would increase the time available for thrombolytic therapy. The ability to induce a prolonged state of resistance to ischaemia in the myocardium would also be beneficial in situations such as unstable angina, high risk coronary angioplasty, surgery involving cardiopulmonary bypass, and in explanted hearts prior to transplantation - all conditions in which the heart is rendered transiently ischaemic. Our results indicate that it is possible to specifically induce 70kDa hsps in cardiomyocytes and thus protect them from injury. It may eventually be possible to induce hsp70 in vivo and protect the human myocardium from ischaemia via pharmacological manipulation, thus exploiting the endogenous protective mechanisms of the heart.

Work arising from his study has been published in the Journal of Clinical Investigation (Morris 1996).

5 HSP 70 in human myocardium

Primate cells contain an additional member of the family - hsx 70, that is closely related to hsp70 in sequence and antigenically, but is expressed at a significant basal level (Pelham 1986). The gene encoding this protein, unlike other members of the family, is activated by the adenovirus E1a protein, and is also cell cycle regulated; it has no direct counterpart in rodent cells. Hsx70 and hsp70 are indistinguishable in many experiments. McGrath et al (1995) examined heat shock protein expression in patients undergoing cardiac operations. They obtained right atrial biopsy specimens from patients before bypass, after reperfusion and after bypass. They found that although hsp70 concentration was unchanged in hearts after reperfusion and after bypass, the initial pre bypass level of hsp 70 was high. This result is not surprising, for we know that there is a high basal level of hsx70 expression in man and the two are indistinguishable by blotting methods.

Some preliminary experiments were carried out using the samples of human atrium collected from patients undergoing coronary artery bypass grafting. The atrial trabeculae were obtained in the same manner as those used in experiments measuring contractile force (see next chapter). Several different protocols were used in order to study basal levels of hsp70 expression as well as the ability of stress (for example heat stress and simulated ischaemia) and agents such as Herbimycin-A to induce these proteins.

5.1 Measurement of hsp70 in human atrial trabeculae

Atrial trabeculae (diameter 0.9mm, length ≥3mm) were dissected under magnification in a dish superfused with Tyrode's solution, tied at one end with a 7/0 silk suture (Pearsalls sutures, England) and removed together with a small portion of atrial wall at the free end. As described fully in the next chapter they were superfused with a continuous flow of modified Tyrode's solution oxygenated with a 95%O_2/5%CO_2 gas mixture (all gases from British Oxygen Company, London, England), and paced at 1 Hz. After 45 minutes to allow for stabilisation of the trabeculae they were subjected to the following protocols:

Heat Stress: The superfusate was warmed to 43°C for 20 minutes (the superfusate temperature being regulated by a water circulator (Techne C-85a Circulator, Cambridge, England)) , followed by reperfusion with superfusate at 37°C for 6 hours.

Simulated ischaemia: The superfusate was free of substrate and was bubbled with 95%N_2/5%CO_2 to lower the pO_2 in the organ bath to 6-8 kPa. (pH7.24-7.34). The trabeculae were paced at 3 Hz as described in the next chapter. After 3 minutes (the time sufficient for classical preconditioning), the superfusate was returned to oxygenated Tyrode's and trabeculae paced at 1 Hz for 6 hours.

Herbimycin-A: Prior to suspension in the organ bath the trabeculae were exposed to Herbimycin-A at a concentration of 1 μg/ml for 15 minutes. This was followed by pacing of trabeculae at 1Hz

Genistein: Prior to suspension in the organ bath the trabeculae were exposed to Genistein at a concentration of 50 μM for 15 minutes.

All the above protocols were followed by a pacing of trabeculae at 1Hz in oxygenated modified Tyrode's solution at 37°C for 6 ours to allow time for stress protein synthesis. Control trabeculae had no pre treatment .

Measurement of hsp72

The trabeculae were immediately frozen in liquid nitrogen following these experiments prior to analyses of hsp levels. In addition certain trabeculae were frozen in liquid nitrogen immediately following dissection from the atrial appendage. Samples were weighed and then homogenised and subjected to a Bradford assay to quantitate protein levels prior to SDS-Page and western blotting for hsp72 levels which was carried out as described above for rat cardiomyocytes. Blots were probed with monoclonal antibodies specific for the inducible isoform of hsp70 (hsp72) (C92F3A-5, Stressgen, Canada) or with monoclonal antibodies specific for both constitutive and inducible isoforms (hsp73 and 72 respectively) (SPA-820, Stressgen, Canada) at 1:1000 dilution. There is no antibody specific for hsp73 alone or for hsx70.

Initial results with this method showed high levels of hsp72 immediately following dissection from the right atrial appendage and this level of expression did not increase following any of the above pre treatments. These findings are not unexpected - hsx70 is expressed at a high basal level in humans as discussed above and we do not have specific antibodies to distinguish it from any hsp72 that may have been induced. The results are shown in figure 3.15. There is a high level of hsp72 expression in all groups with no significant increase with ischaemia, heat stress or herbimycin-A. However, there is an increase in mobility (reduced molecular weight) in the hsp72 bands from the Herbimycin-A treated groups, suggestive of post-translational modification of this stress protein, such as a reduction in phosphorylation.

Figure 3.15
HSP72: blots derived from human atrial trabeculae

1 2 3 4 5 6 7 8 9 10 11 12 13

Key:

1-2 Control

3-5 Heat stress

6-8 Herbimycin-A

9-11 Genistein

12-13 Ischaemia

The experiments were also carried out without stretching and pacing the trabeculae in the organ bath as the act of stretch in itself may have been sufficient to induce stress proteins. However the results with this method were not different from those above.

It would be interesting to repeat these experiments in the future when more selective antibodies become available.

Chapter 4:

Human Atrial Model of Cardioprotection: the protective effects of ACE inhibitors

1 Introduction: The principles of Superfusion

Muscle preparations with a small diameter, superfused in a an organ bath containing oxygenated physiological buffer, can remain viable without arterial perfusion. Such specimens must be small enough to allow free diffusion of metabolic substrates (oxygen, glucose, and pyruvate) and metabolites (carbon dioxide and perhaps lactate). In practise the diffusion of oxygen is the limiting factor for contractility (Paradise et al 1981). The delivery of a satisfactory amount of oxygen to all of the cells in an isolated muscle preparation is determined by the rate of oxygen utilisation and the distance from the surface of the muscle to the central core, as well as the oxygen tension of the surrounding solution (Paradise et al 1981). If oxygen consumption is high or diffusion distances large, then the core of an isolated preparation may be inadequately oxygenated and contractility will decrease.

The appropriate maximum diameter of isolated superfused muscle preparations has been determined by several different investigators. Using biophysical considerations AV Hill (1928) described a method to determine the critical distance for the diffusion of oxygen. Assuming an oxygen consumption of approximately 1.4ml/min/100g of active muscle bathed in Ringer's solution and ignoring any contributions from myoglobin, a critical radius of 0.466mm has been suggested (Prasad and Callaghan 1969). Snow and Bressler (Snow et al 1977) were able to show that the core of working rabbit papillary muscle paced at 0.8b Hz would not be hypoxic if the diameter was less than or equal to 1.22mm, but these experiments were carried out at 25°C and hence my not be directly extrapolated to this present study. By progressively decreasing the partial pressure of oxygen in the superfusate until a decrease is observed in the force developed by muscles of varying radius, studies by (Paradise et al 1981)

suggested that the critical diameter in superfused cat papillary muscle was 0.96 mm when stimulated at 0.5 Hz at 37°C decreasing to 0.71 mm at 1Hz. Page and Solomon (Page et al 1960) had calculated that a superfused cylindrical cat papillary muscle with a diameter of 1.12 mm would be adequately oxygenated.

The technique of superfusing isolated specimens of human cardiac muscle is well documented, and using ventricular muscle obtained from explanted hearts, there are published studies where the sample diameter is 1-2 mm (Bristow et al 1992 and White et al 1994).

The investigations outlined above, therefore, suggest that the human atrial trabeculae used in this study of diameters ranging from 0.5 to 0.9 mm were adequately oxygenated. However, one cannot exclude the possibility of a degree of core hypoxia in the larger trabeculae, but this should effect all groups equally. The results should still be valid since the study involves the comparison of responses of groups which were similar at baseline.

2 Description of the Apparatus

The apparatus was manufactured from glass, polythene/silicone tubing and non-toxic perspex (see figures 4.1 and 4.2).

2.1 The Organ Bath

The organ bath was manufactured by the physiology workshop at UCL to the design illustrated in figures 4.3a and 4.3b The capacity of the bath was approximately 4ml and perfusate flow was 8ml/min. The deep entry well (2.2cm) was designed to damp the pulsatile perfusate flow delivered by the peristaltic pump (Watson-Marlow 502S, Cornwall, England). Superfusate temperature was monitored continuously using a thermocouple (type K,

Figure 4.1: Schematic representation of the apparatus used to assess contractile force in human right atrial trabeculae

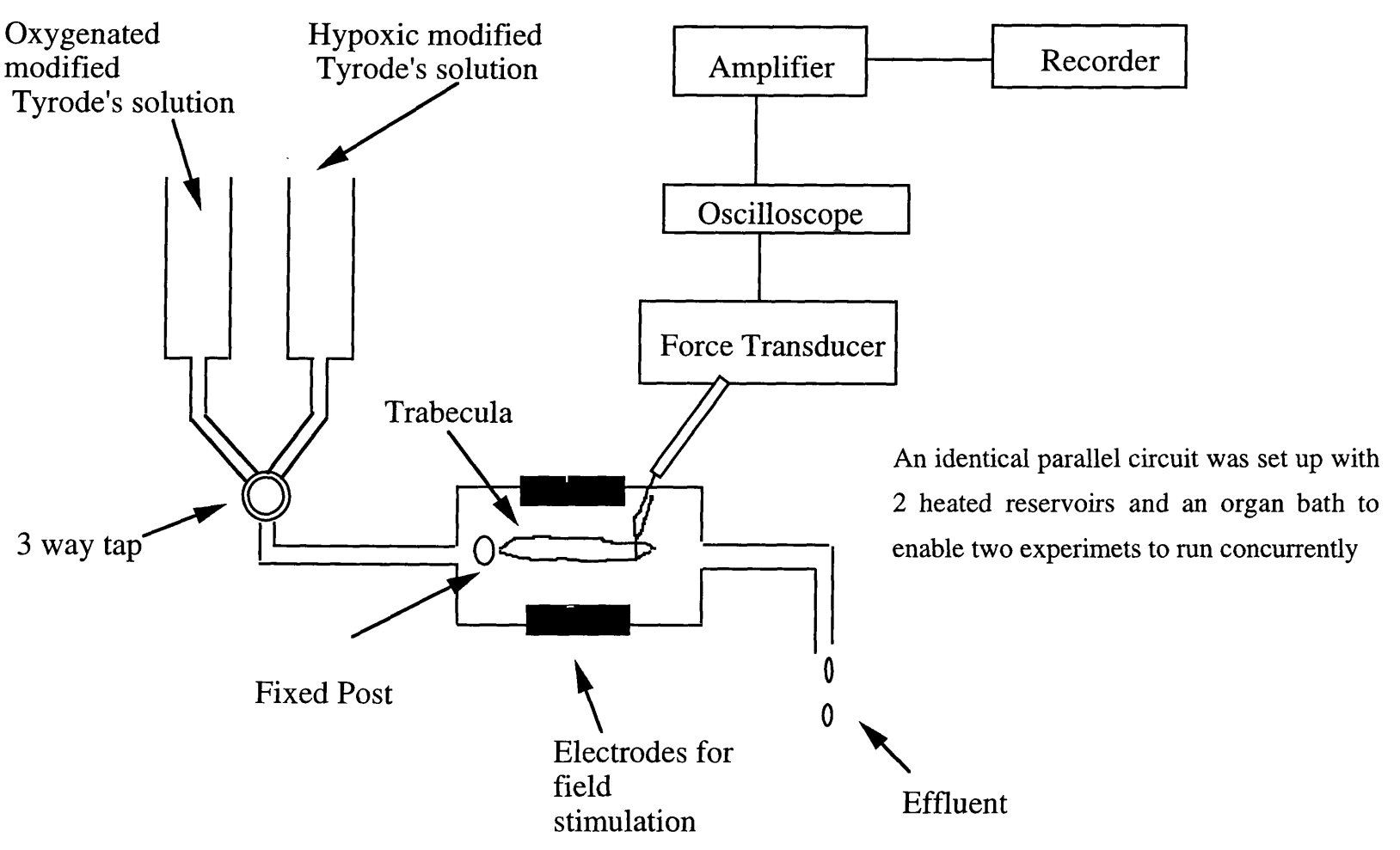
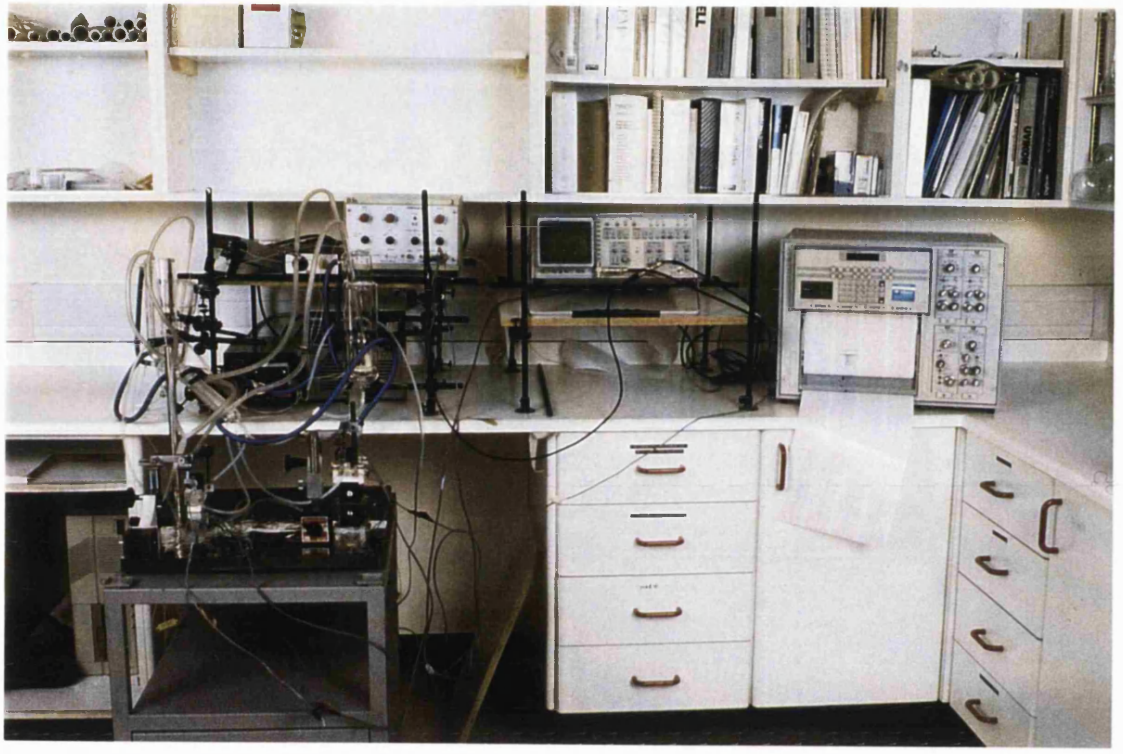


Figure 4.2a

Apparatus for human atrial trabeculae experiments



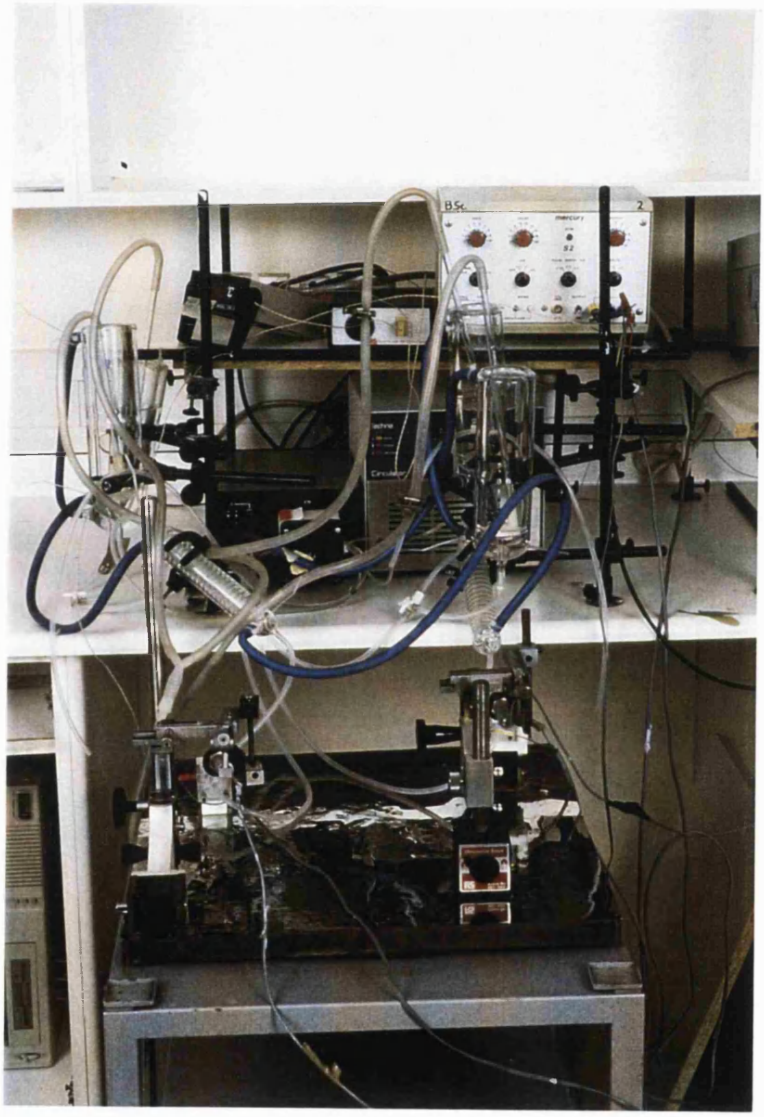
The jacketed glass reservoirs (4 in total - 2 for each organ bath) were heated by a water circulator (Techne C-85a Circulator, Cambridge, England), and the superfusate pumped via glass heat exchangers to the organ baths. A three way tap selector was used to draw different superfusates from either of the glass reservoirs, all connecting tubing was silicone.

The trabeculae were paced by field stimulation via parallel flattened platinum electrodes with an isolated stimulator (Digitimer DS2, Hertfordshire, UK) triggered by a programmable computerised clock. Force envelopes were amplified and recorded on paper (Universal Amplifier and RS3400 ink pen recorder, Gould, Ohio, USA).

Figure **4.2b**

Apparatus for human atrial trabeculae experiments:

Close up view of organ baths and glass reservoirs

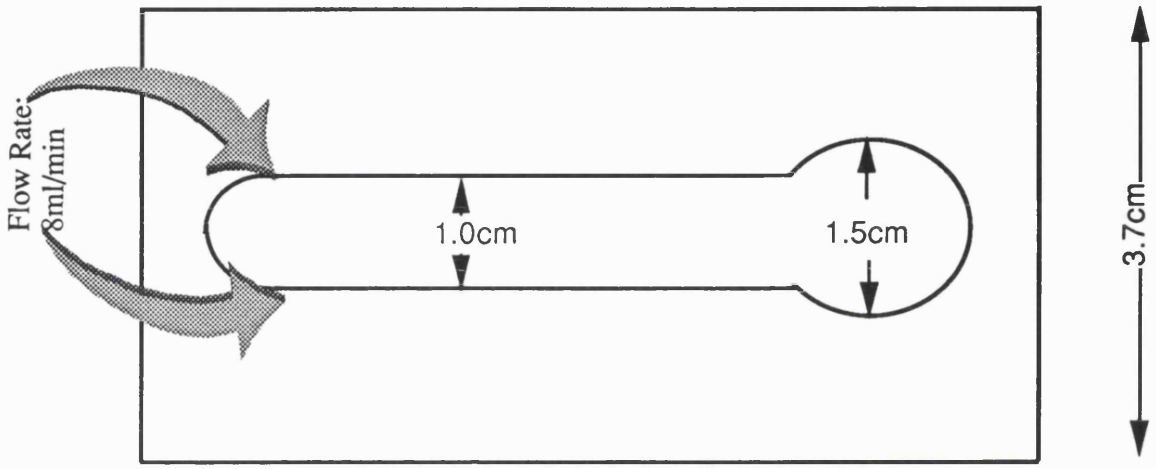


The organ baths were placed on an anti-vibration table. Flow was delivered to the organ baths via a peristaltic pump (Watson-Marlow 502S, Cornwall, England).

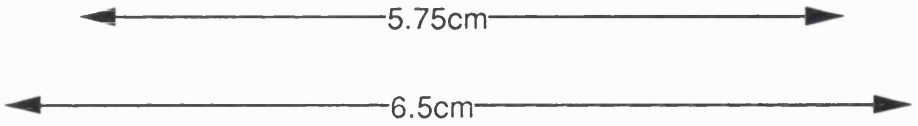
The jacketed glass reservoirs and the heated water circulator can also be seen.

Figure 4.3: Schematic diagram of the organ bath viewed from above (3a) and the side (3b) with relevant dimensions

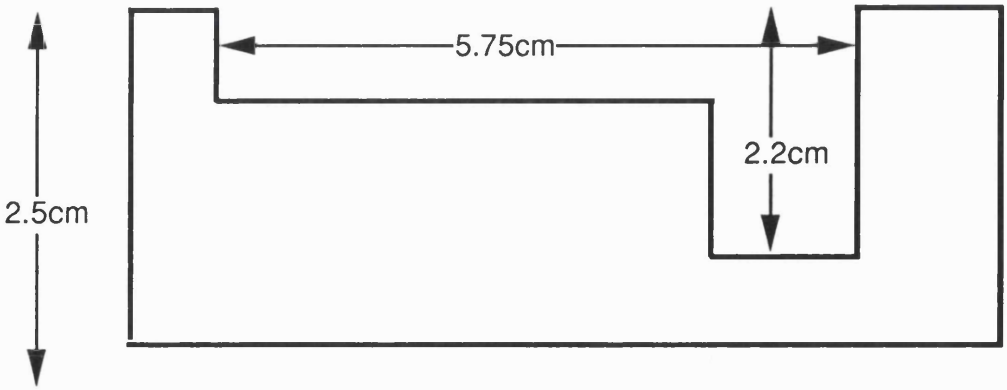
3a



TOP



3b



SIDE

Radiospares, Northants, England) placed close to the trabecula; the temperature being maintained at 37°C by circulating warm water through two parallel bores within the body of the bath. To monitor the pH, pO_2 and pCO_2 in the organ bath, the effluent was analysed at regular intervals with an automatic blood gas analyser (AVL 993, AVL Medical Instruments, Switzerland) and the pH being maintained between 7.35-7.45 (reduced to 7.2 - 7.3 during hypoxia).

The organ bath was covered with a glass cover slip in order to prevent gas exchange with the atmosphere.

2.2 Superfusate Flow and Control

The superfusates were maintained at body temperature in jacketed glass reservoirs (4 in total - 2 for each organ bath) heated by a water circulator (Techne C-85a Circulator, Cambridge, England), and were pumped via glass heat exchangers to the organ baths. A three way tap selector was used to draw different superfusates from either of the glass reservoirs, all connecting tubing was silicone.

2.3 Muscle Stimulation and Recording of Data

Once horizontally suspended, trabecula were paced by field stimulation at 1 Hz via parallel flattened platinum electrodes with an isolated stimulator (Digitimer DS2, Hertfordshire, UK) triggered by a programmable computerised clock. The pulse width was fixed at 5 msec and the pulse amplitude set at twice threshold (6-8 mV). Force envelopes were amplified and recorded on paper (Universal Amplifier and RS3400 ink pen recorder, Gould, Ohio, USA).

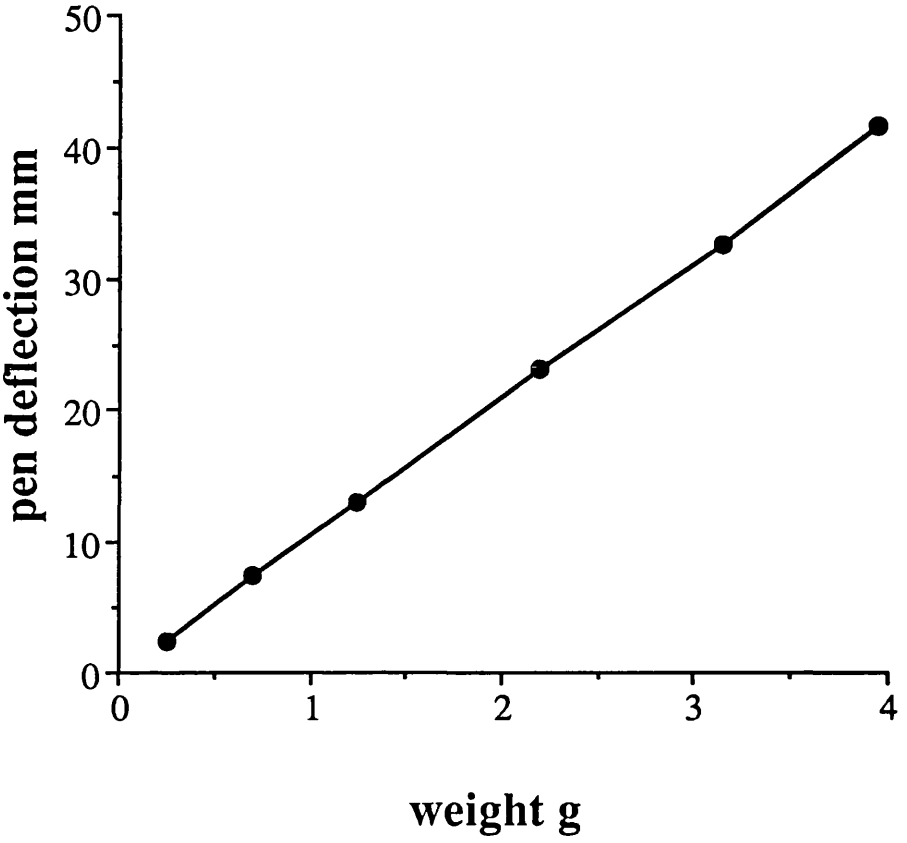
The frequency response of the force transducer and its mechanical linkage was known to be flat to 50 Hz (Walker et al 1983). The range of the transducer, according to the manufacturers specification, was +/- 30g with a displacement

of +/- 0.06 mm at the upper end of this force range. The excitation voltage for the force transducer was provided by a DC bridge amplifier (Digitimer, Neurolog recorder amplifier NL 107). The output of the force transducer was "conditioned" to be within the range of +/- .5 V using the same amplifier and recorded on a "pressure-ink" chart recorder (Gould RS3400 chart recorder, Ohio, USA) at a speed of 5mm/min. Full scale deflection for the transducer amplifier corresponded to 5 V. Zero suppression of the amplifier was performed at the beginning of each experiment, and again was used at the end of each experiment to return the pen deflection to the point corresponding to the unloaded baseline, so that changes in zero suppression could be used to calculate resting force.

The isolated stimulator was also connected to the chart recorder via an amplifier (Gould 13-4615-58, Ohio, USA). This resulted in an uncalibrated artefact on the recorder which indicated that the pacing system was functioning.

The force transducer was calibrated by measuring the pen deflection on the chart recorder in response to different weights suspended from the transducer. The varying weights comprised of differing lengths of coiled lead solder which themselves had been weighed on a calibrated electronic balance accurate to 5 figures (Mettler AJ50, Leicester, England). Figure 4.4 illustrates the linear relationship between force and pen deflection on the chart recorder.

Figure 4.4: Calibration of force transducer



This graph illustrates the relationship between the load on the transducer in grammes and the pen deflection on the chart recorder. This was determined with the DC amplifier set at 1 V full scale deflection and the transducer set at 250 full scale deflection.

3 Superfusion of Trabeculae

3.1 Preparation and Suspension of Trabeculae

Specimens of right atrial appendage were obtained from the right atrial cannulation site in patients undergoing cardiopulmonary artery bypass grafting (46 patients: 37 male and 9 female, age range 39-79, mean age 64). All patients had chronic stable angina, and those with right ventricular failure, atrial arrhythmia's or taking anti-arrhythmic medication were excluded. In addition, patients taking oral hypoglycaemic agents and those receiving ACE inhibitors were also excluded. Ethical approval for this procedure was obtained from the hospital ethics committee. The specimens were transported to the laboratory in oxygenated modified Tyrode's solution.

Atrial trabeculae (diameter 0.9mm, length ≥3mm) were dissected under magnification in a dish superfused with Tyrode's solution, tied at one end with a 7/0 silk suture (Pearsalls sutures, England) and removed together with a small portion of atrial wall at the free end. The silk suture was then used to attach the muscle to a fixed post in an organ bath whilst the free end was attached to a force transducer (Gould Statham UCT 2, Ohio, USA) using a snare around the wedge of atrial wall. The force transducer was attached to a micromanipulator (Prior, Cambridge, England) which allowed the force transducer to be manoeuvred with precision. Both the organ bath and the force transducer were sited on a low vibration table. The muscles were suspended horizontally in the organ bath through which there was a continuous flow of superfusate oxygenated with a 95%O_2/5%CO_2 gas mixture (all gases from British Oxygen Company, London, England).

Length and width of the trabecular muscles were recorded at the end of the experiment using an eyepiece graticule in an overhead microscope (Prior,

England), and all specimens were then weighed. Cross sectional area was calculated by dividing muscle mass by length times density, assuming a cylindrical shape and a density of 1.0mg/mm^3. To ensure that initial comparisons of developed tensions and resting force were not affected by variable muscle size, the muscles' cross sectional area was used to calculate tensions. In addition, all muscles with a cross sectional area in excess of 1.2 mm^2 were excluded from the study.

3.2 Trabecula Stability

Initial pilot experiments were carried out to determine the stability of the preparations. For the first 30 minutes after suspension developed and resting force were unstable, but thereafter force remained stable for up to 8 hours with a fall of in developed force of around 5% maximum.

3.3 Superfusion of Trabeculae

The superfusate was a modified Tyrode's solution of the following composition: NaCl 118.5 mM, KCl 4.8mM , $NaHCO_3$ 24.8mM, KH_2PO_4 1.2mM, $MgSO_4.7H_2O$ 1.44mM, $CaCl_2.2H_2O$ 1.8 mM, glucose 10.0mM, pyruvic acid 10.0mM. In the substrate-free Tyrode's solution, choline chloride 7mM was substituted for glucose and pyruvic acid to maintain constant osmolarity. All reagents were AnalaR grade from BDH Chemicals, Poole, England except pyruvic acid from Sigma Chemicals, St Louis, Missouri, USA. The organ bath was covered to prevent gas exchange with the atmosphere. Gas tensions in the organ bath were analysed intermittently using an automated blood gas analyser (AVL 993, AVL Medical Instruments, Switzerland).

The pH was maintained between 7.35-7.45, the pO_2 between 50-60 kPa and pCO_2 4.0-6.0 kPa. During simulated ischaemia the superfusate was free of substrate and was bubbled with 95%N_2/5%CO_2 to lower the pO_2 in the organ bath to 6-8 kPa. (pH7.24-7.34).

4 The role of ACE inhibitors and bradykinin B_2 receptors in a human atrial trabeculae model

4.1 Introduction

Components of the renin angiotensin system have been found in cardiac tissue and ace itself has been localised in both atria and ventricles using techniques such as quantitative in vitro auto radiography with 125I-351-A radioligand (Yamada et al 1989). As discussed in the chapter 1 there is increasing evidence that ACE inhibitor activity is mediated in part via increased local bradykinin levels. The pathways involved in the formation and destruction of bradykinin is illustrated in chapter 1. Bradykinin is a nonapeptide described as causing slow contractions in the gut (*brady*, slow; *kinin*, movement). It is formed from kininogen by the action of the enzyme kallikrein, which in turn is derived from pre-kallikrein. Bradykinin is, in turn, inactivated by two kininases, kininase 1 and 2, the later being identical with ACE (angiotensin converting enzyme inhibitor). There is evidence for an independent kallikrein kinin system within the heart (Nolly et al 1994), and that cardiac tissue can in fact synthesise and release both kallikrein and kininogen. The presence of this local kallikrein kinin system ensures the ability of the heart to produce bradykinin.

A recently published study by Miki et al (1996) demonstrated the ability of the ACE inhibitor captopril to potentiate the infarct size limiting effect of ischaemic preconditioning in a rabbit model of myocardial ischaemia. They showed that captopril (1mg/kg) when given in addition to preconditioning with 2 minutes of ischaemia (which alone was insufficient to protect) reduced infarct size to 45% of the control value after 30 minutes of regional ischaemia. This protection was abolished by Hoe 140, suggesting a role for bradykinin B2 receptors in

mediating this protection. Does a similar situation exist within the human myocardium, and could this explain some of the cardioprotective effects of ACE inhibitors in clinical practice?

4.2 Aims of the study

The aim of the present study was to determine whether ACE inhibitors, (with and without -SH groups), could contribute to the protective effects of preconditioning in a human atrial trabeculae model. Furthermore, using a specific B_2 receptor antagonist, Hoe140, our aim was to determine whether any protective effect observed is related to enhanced B_2 bradykinin receptor activation following reduced kinin degradation.

4.3 Chemicals

Captopril and lisinopril were obtained from Sigma (St Louis, Missouri, USA). Captopril was dissolved in normal saline and added to Tyrode's solution to give a final concentration of 10mM, and lisinopril was similarly dissolved to give a final concentration of 20μM - concentrations comparable to plasma levels in patients taking these particular ACE inhibitors (Opie 1992). Hoe140 was a gift from Prof. R. N. Zahlten and Prof B. A. Scholkens, Hoechst, Frankfurt, Germany, and was diluted to a final concentration of 20nmol/l, a concentration known to be sufficient to effectively antagonise B_2 receptors.

Captopril:

Captopril, designated chemically as 1-[(2S)-3-mercapto-2-methyl-propionyl]-L-proline, ($C_9H_{15}NO_3S$) is a highly specific competitive inhibitor of angiotensin

1-converting enzyme, the enzyme responsible for the conversion of angiotensin I to angiotensin II

Lisinopril:

Lisinopril, [S]-N@-[1-carboxy-3-phenylpropyl]-lys-pro, ($C_{21}H_{31}N_3O_5$) is also a specific inhibitor of angiotensin I converting enzyme. In contrast to captopril, lisinopril does not contain an -SH group. Both captopril and lisinopril are in the active form.

5 Experimental Protocols

Trabecula were initially stimulated at 1 Hz unstretched for 30 minutes, to allow time for recovery. They were subsequently stretched in a stepwise manner over 15 minutes to a length developing 90% of maximal force, and allowed to equilibrate for a further 30 minutes.

5.1 Initial experiments to define the protocol

Initial experiments were performed to determine the optimal protocols for preconditioning and for the subsequent long ischaemia. These preliminary experiments determined that 3 minutes of simulated ischaemia consisting of superfusion with substrate free hypoxic Tyrode's solution with rapid pacing at 3 Hz, followed by reperfusion with oxygenated Tyrode's solution and a return to 1 Hz resulted in a degree of protection similar to that in other experimental models (see results below). Experiments were carried out to determine the optimal length of time for the long ischaemic insult, so that the resultant contractile impairment was sufficient to demonstrate significant differences in

recovery of function between preconditioned and non preconditioned groups. The optimal time was found to be 90 minutes of substrate free superfusion in hypoxic Tyrode's solution with rapid pacing at 3 Hz.

Further experiments were carried out to determine a sub threshold preconditioning protocol, i. e. one which was just insufficient to result in any improvement in recovery of function compared to control, non preconditioned, groups. This was done by gradually decreasing the length of time of the preconditioning stimulus from 3 minutes to 1 minute. The results of these experiments are shown in table 4.1.

5.2 Final protocols

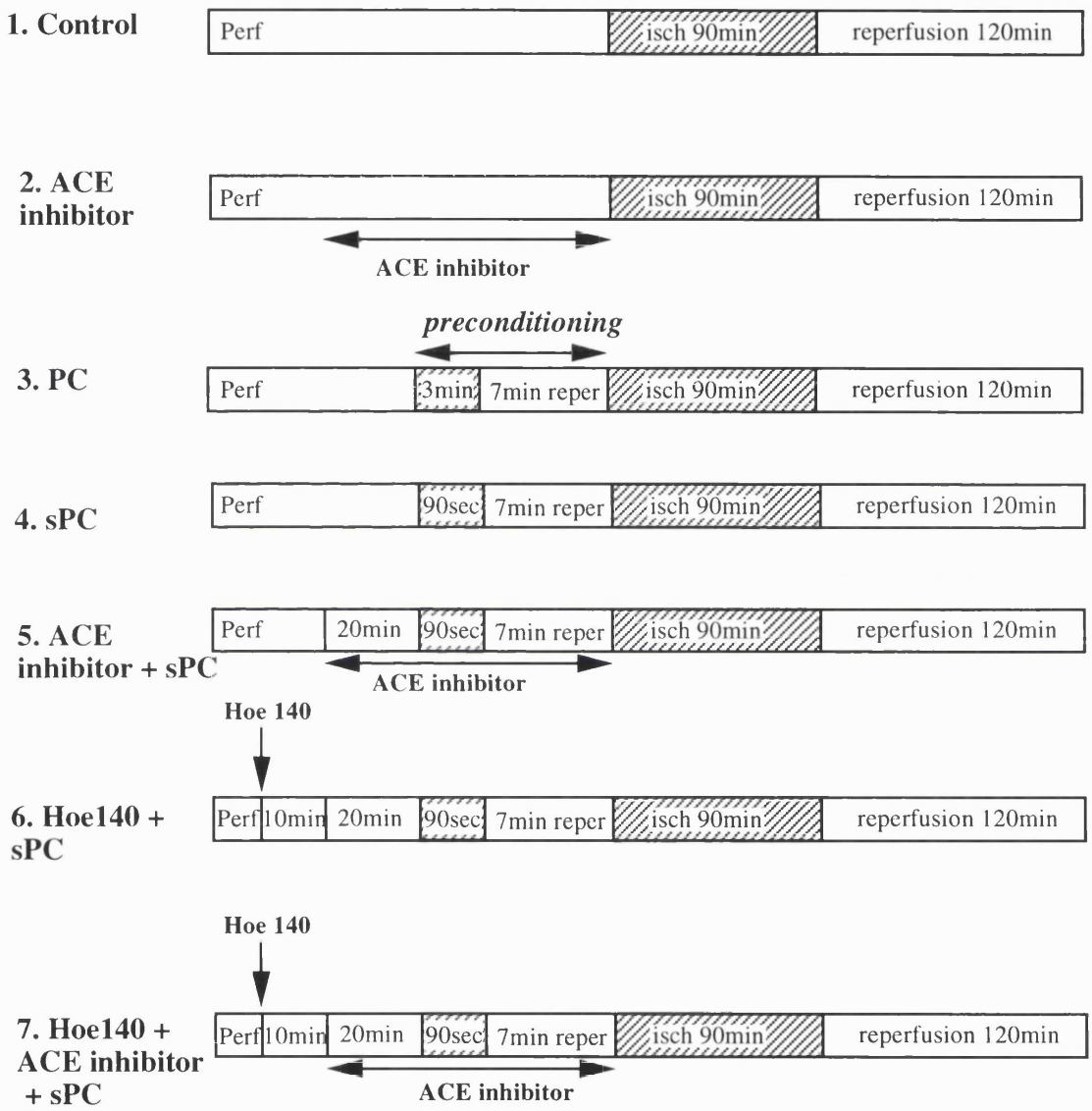
All groups eventually underwent a period of simulated ischaemia (I) which consisted of 90 minutes of hypoxic substrate-free superfusion and rapid pacing at 3 Hz, followed by reperfusion for 120 minutes with oxygenated Tyrode's solution paced at 1 Hz. The preconditioning protocol (PC) consisted of 3 minutes of hypoxic substrate-free superfusion paced at 3 Hz followed by 7 minutes of reperfusion with oxygenated Tyrode's solution at 1 Hz with substrate. A subthreshold preconditioning protocol (sPC) was established which failed to protect alone. This consisted of 90 seconds of pacing at 3 Hz with hypoxic substrate-free perfusate, and was also followed by 7 minutes of reperfusion with oxygenated Tyrode's solution. Figure 4.5 shows the experimental protocols for the 10 groups, all of which were finally subjected to 90 minutes of simulated ischaemia. Figure 1 shows the experimental protocols for the 10 groups. **1. Control**: stabilisation period followed by 90 minutes ischaemia. **2. ACE inhibitor**: superfusion with captopril or lisinopril for 20 minutes prior to 90 minutes of ischaemia. **3. PC**: preconditioning: 3 minutes preconditioning protocol, followed by 90 minutes of simulated ischaemia.

Table 4.1:
Preliminary experiments to define the protocol

Duration of preconditioning ischaemia (PC)	Baseline developed force (DF)	Recovery of DF 120 minutes (% baseline)	Recovery of DF 210 minutes (% baseline)
180 seconds	1.38±0.19	44±2	61±1
150 seconds	1.29±0.21	57±3	55±7
120 seconds	1.42±0.24	46±5	45±9
90 seconds	1.35±0.20	26±3	23±4
60 seconds	1.23±0.19	23±2	24±5
Control (no PC)	1.20±0.16	20±2	22±1

n = 3 for each group

Figure 4.5: Experimental Protocols



KEY:

▨ ischaemia

isch: superfusion with hypoxic, substrate free Tyrode's solution and pacing at 3 Hz

PC: preconditioning

reper: reperfusion with oxygenated Tyrode's solution and pacing at 1 Hz

sPc: subthreshold preconditioning

n=6 for all groups. All protocols were preceded by at least a 45 minutes stablisation period.

4. sPC: subthreshold preconditioning (90 seconds) followed by 90 minutes of simulated ischaemia. **5. ACE inhibitor + sPc**: 20 minutes of superfusion with captopril or lisinopril , prior to the subthreshold preconditioning protocol, and 90 minutes of simulated ischaemia. **6. Hoe140 + sPC**: superfusion with Hoe140 for 20 minutes prior to subthreshold preconditioning, then 90 minutes of ischaemia. **7. Hoe140 + ACE inhibitor + sPC**: superfusion with Hoe140 for 10 minutes prior to the addition of captopril or lisinopril for 20 minutes followed by subthreshold preconditioning. For all these groups n=6, including 6 experiments each for all the captopril and all lisinopril groups.

5.3 Statistical Analysis

Data are expressed as group means ± standard error of the mean (SEM). Statistical differences between groups were evaluated with respect to treatment and time by two-way ANOVA followed by Fisher's protected least significant difference post hoc test (Snedecor and Cochran 1980). A probability value of less than or equal to 0.05 was considered significant. Statistical analyses were performed using the programme "Statview SE + Graphics (Abacus Concepts Inc)" for Apple Macintosh.

6 Results

6.1 Baseline patient data

Trabeculae were obtained from patients with stable ischaemic heart disease undergoing routine coronary artery bypass surgery. The majority of patients were taking low dose aspirin (75 - 150 mg) which was discontinued three days prior to surgery. Other medications were beta blockers, nitrates and calcium antagonists, whilst those taking ACE inhibitors were specifically excluded. In fact all patients were taking at least one anti-anginal medication. Samples were not taken from patients who had atrial arrhythmias or right ventricular failure. In addition patients taking anti-arrhythmic agents or oral hypoglycaemic agents such as glibenclamide were excluded.

Specimens of right atrial appendage were obtained from a total of 51 patients with a maximum of two trabeculae being used per patient. Each trabecula was used for one experiment only.

6.2 Analysis of Data

6.2.1 Baseline characteristics of atrial trabeculae

Three muscles were excluded due to a calculated cross sectional area in excess of 1.2 mm^2. There were no other exclusions and all samples completed the protocol to which they were randomly assigned.

The physical characteristics of the trabecula and baseline functional data were similar in all 10 groups (Table 4.2), including resting and developed force.

Table 4.2: Baseline Characteristics of Atrial Trabeculae

GROUP	Length	Mass	Diameter	Resting Force	Developed Force
	mm	g	mm	g	g
Control	4.4±0.3	0.006±0.001	0.71±0.04	0.65±0.11	1.20±0.16
Cap	4.5±0.3	0.006±0.001	0.73±0.04	0.77±0.10	1.51±0.18
Lis	4.9±0.5	0.005±0.001	0.77±0.06	0.70±0.09	1.43±0.13
PC	4.2±0.3	0.005±0.001	0.71±0.05	0.71±0.10	1.38±0.19
sPC	4.5±0.4	0.005±0.001	0.69±0.05	0.78±0.10	1.35±0.20
Cap + sPC	5.0±0.4	0.006±0.001	0.71±0.05	0.77±0.11	1.26±0.21
Lis + sPC	4.7±0.3	0.006±0.001	0.79±0.02	0.66±0.11	1.19±0.21
Hoe+sPC	4.6±0.4	0.005±0.001	0.69±0.05	0.68±0.12	1.28±0.17
Hoe+Cap+sPC	4.5±0.3	0.006±0.001	0.68±0.06	0.75±0.12	1.47±0.22
Hoe+Lis+sPC	4.7±0.4	0.006±0.001	0.71±0.05	0.67±0.13	1.48±0.19

Values are mean ± SEM

Cap: Captopril, **Lis:** Lisinopril, **PC:** Preconditioning, **sPC:** Subthreshold preconditioning, **Hoe:** Hoe140.

Therefore experimental data on developed force is presented graphically as a percentage of baseline developed force. Developed force was not related to the diameter of the trabeculae (figure 4.6) and so contractility has not been expressed in terms of developed tension. A record of the force measurements obtained during preconditioning, substrate free hypoxia and subsequent re oxygenation is shown in figure 4.6b.

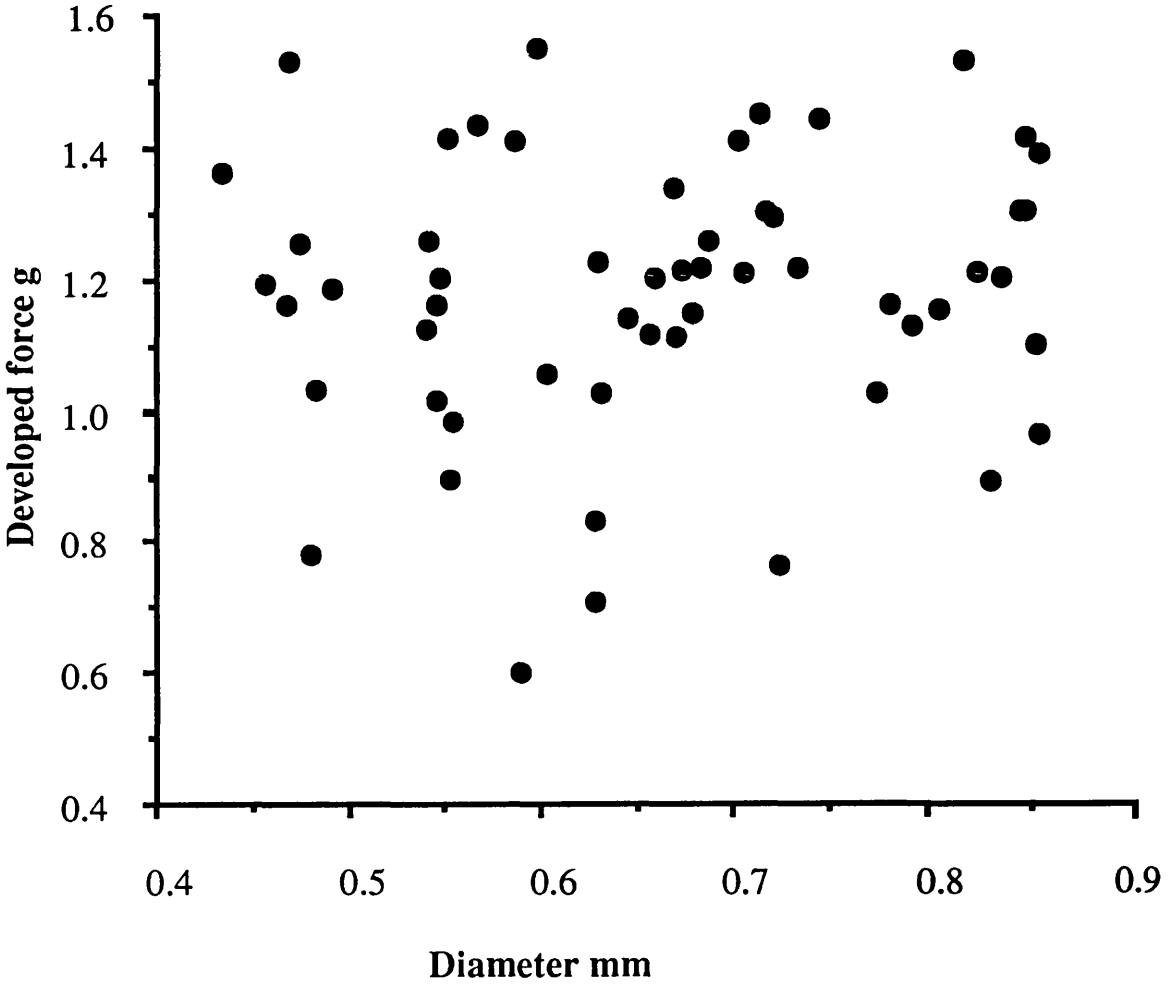
6.2.2.Specific results: changes in contractility

Figures 4.7 - 4.11 illustrate the experimental results which are represented as developed force (as a percentage of baseline) against time. Time 0 indicates the beginning of simulated ischaemia; therefore reperfusion with oxygenated Tyrode's solution extends from 90 to 120 minutes. As indicated in the diagram of the protocols (figure 4.5), simulated ischaemia is preceded in all groups by a stabilisation period and various pretreatments (indicated on the graphs by an arrow). As noted above, there was no significant difference in developed force between the 10 groups at the end of the stabilisation period, and this baseline function is denoted as 100% developed force.

6.2.3 Stability of the preparation

Figure 4.7 illustrates the stability of the isolated superfused trabeculae model over a period of 4 hours. These trabeculae were paced at 1 Hz in oxygenated modified Tyrode's solution with no other intervention. During this time there is a gradual reduction in function of 22±5% (n=4), a value consistent with other investigators (Walker et al 1993)

Figure 4.6
Correlation between atrial trabeculae diameter and developed force

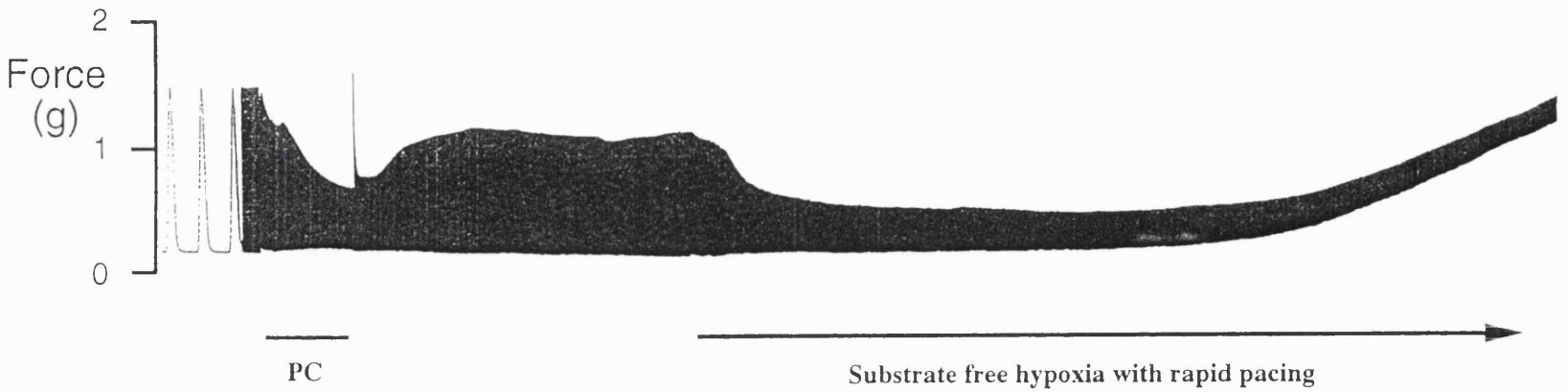


This graph illustrates the lack of relationship between atrial muscle diameter and developed force (r=0.3). Therefore all contractility data are presented as force rather than tension.

One would normally expect force to increase with increasing diameter, but the results could be due to a greater volume of necrosed core tissue in the centre of the larger specimens, i.e the larger the trabecula perhaps the greater the central inactive core.

Figure 4.6b Experimental record of force measurements

i. During preconditioning and with the onset of substrate free hypoxia and rapid pacing
The first one or two contractions after preconditioning showed marked potentiation



ii. During reoxygenation with substrate
Resting force returns toward baseline, more rapidly initially. The initial increase in contractility was transient, but this was followed by a slow sustained increase in developed force.

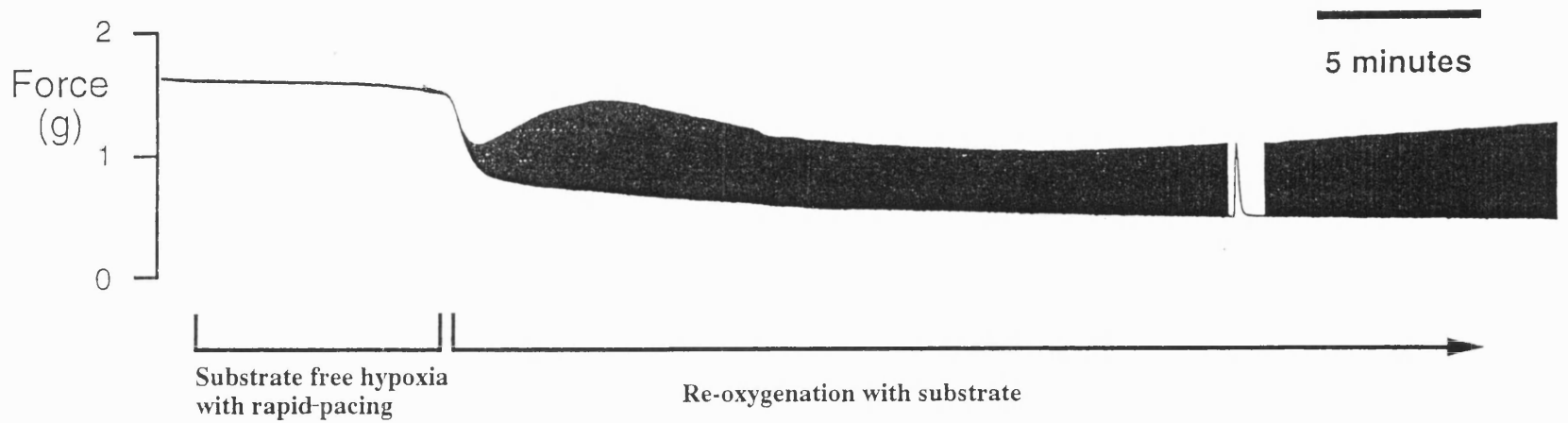


Figure 4.7:

Developed force expressed as a percentage of baseline force over time in minutes. Stability of preparation without intervention.

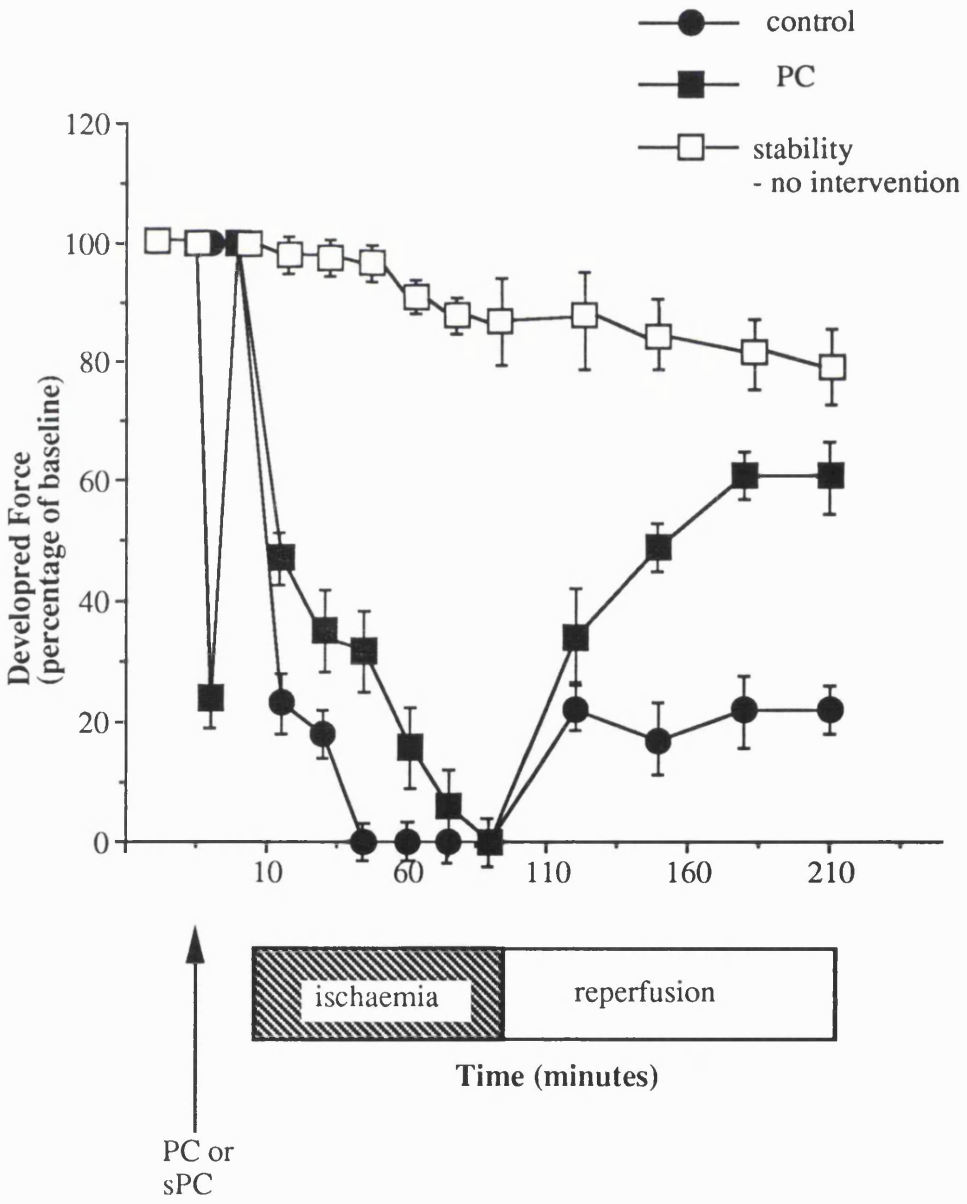


Figure 4.8

Developed force expressed as a percentage of baseline force over time in minutes. Comparison of ACE inhibitor alone with ACE inhibitor combined with subthreshold preconditioning

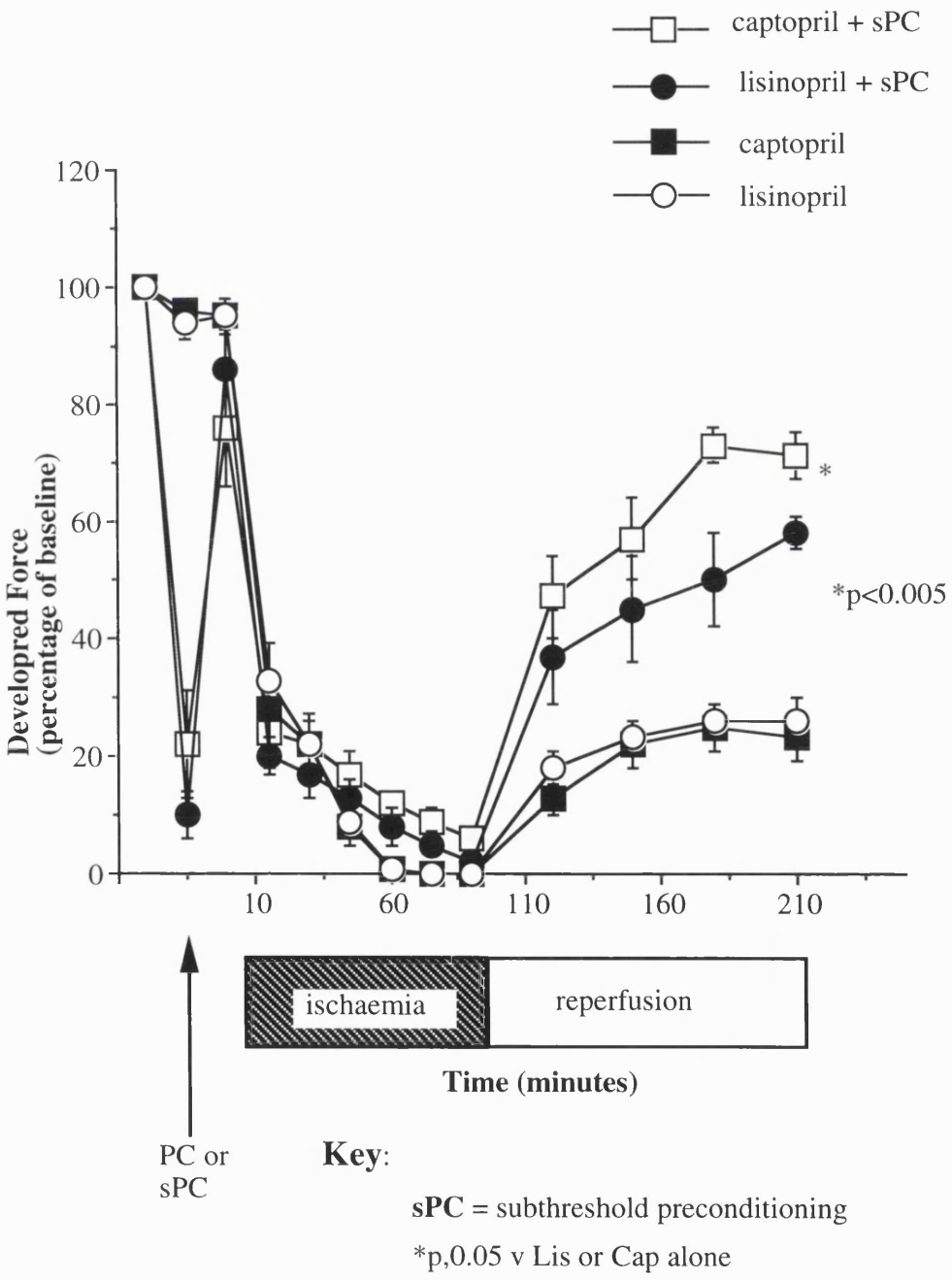
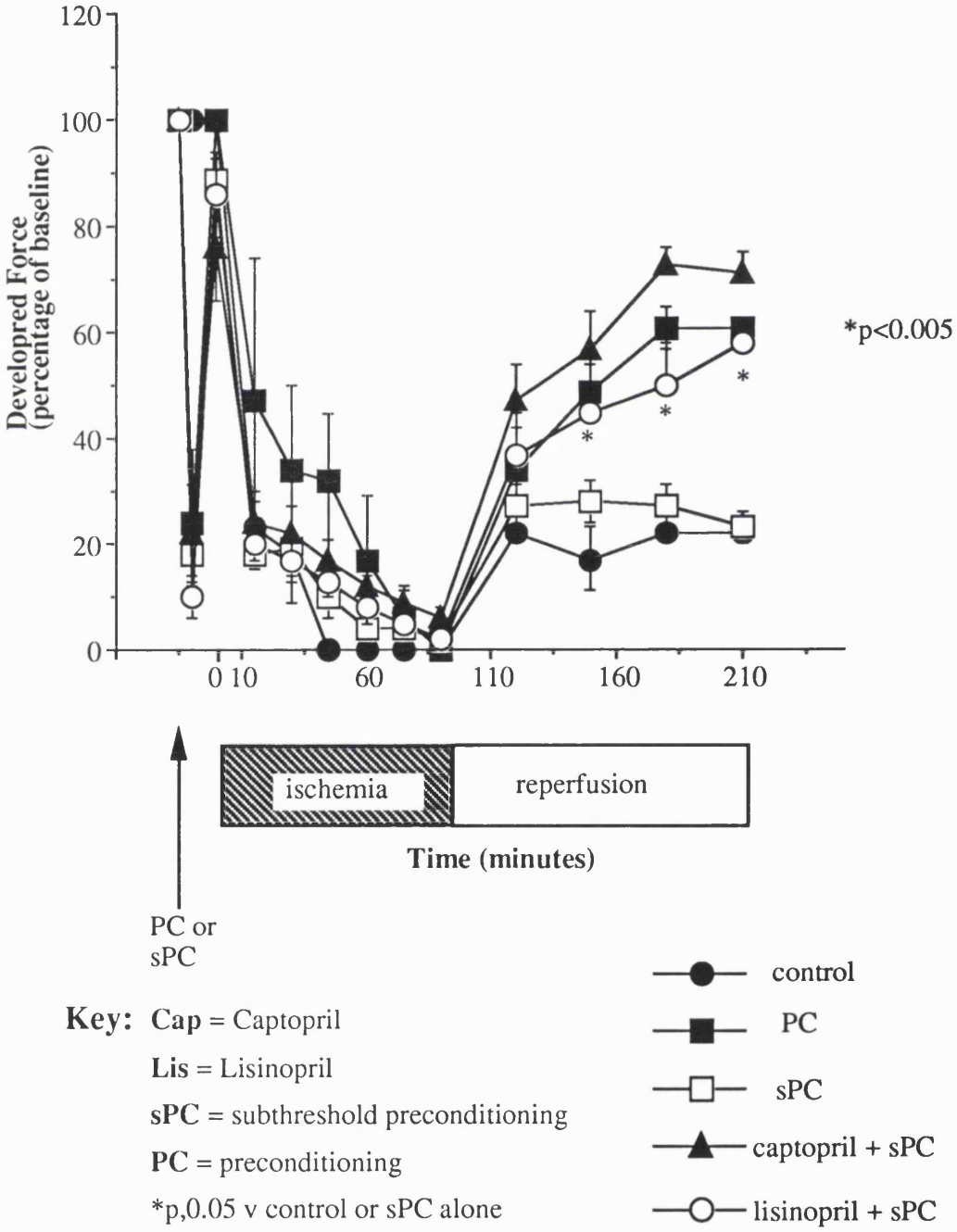


Figure 4.9

Developed force expressed as a percentage of baseline force over time in minutes. Combination of ACE inhibitor with subthreshold preconditioning in comparison to the full preconditioning protocol.



Key: **Cap** = Captopril

Lis = Lisinopril

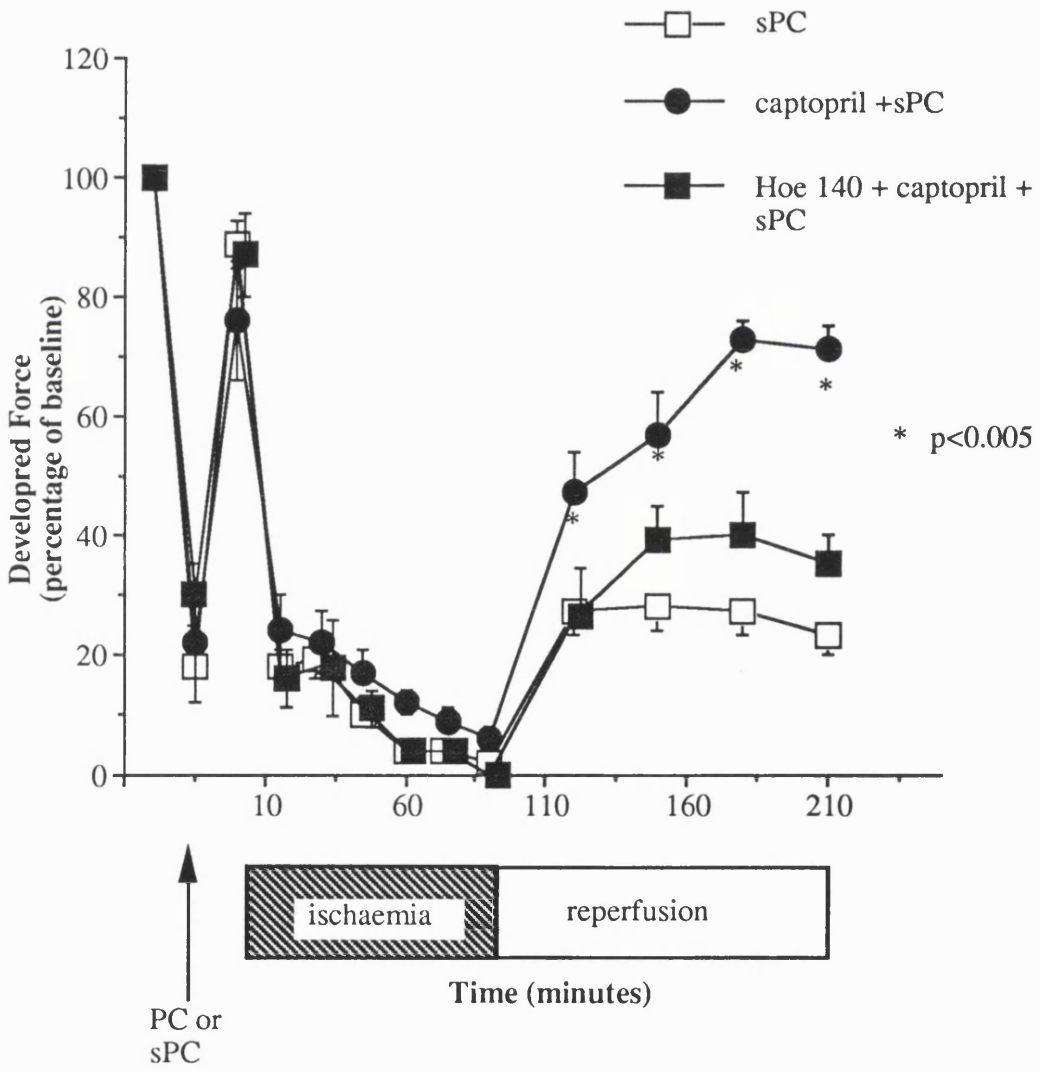
sPC = subthreshold preconditioning

PC = preconditioning

*p,0.05 v control or sPC alone

Figure 4.10

Developed force expressed as a percentage of baseline force over time in minutes. Effect of pre treatment with Hoe 140 on the protective effect of Captopril in combination with subthreshold preconditioning



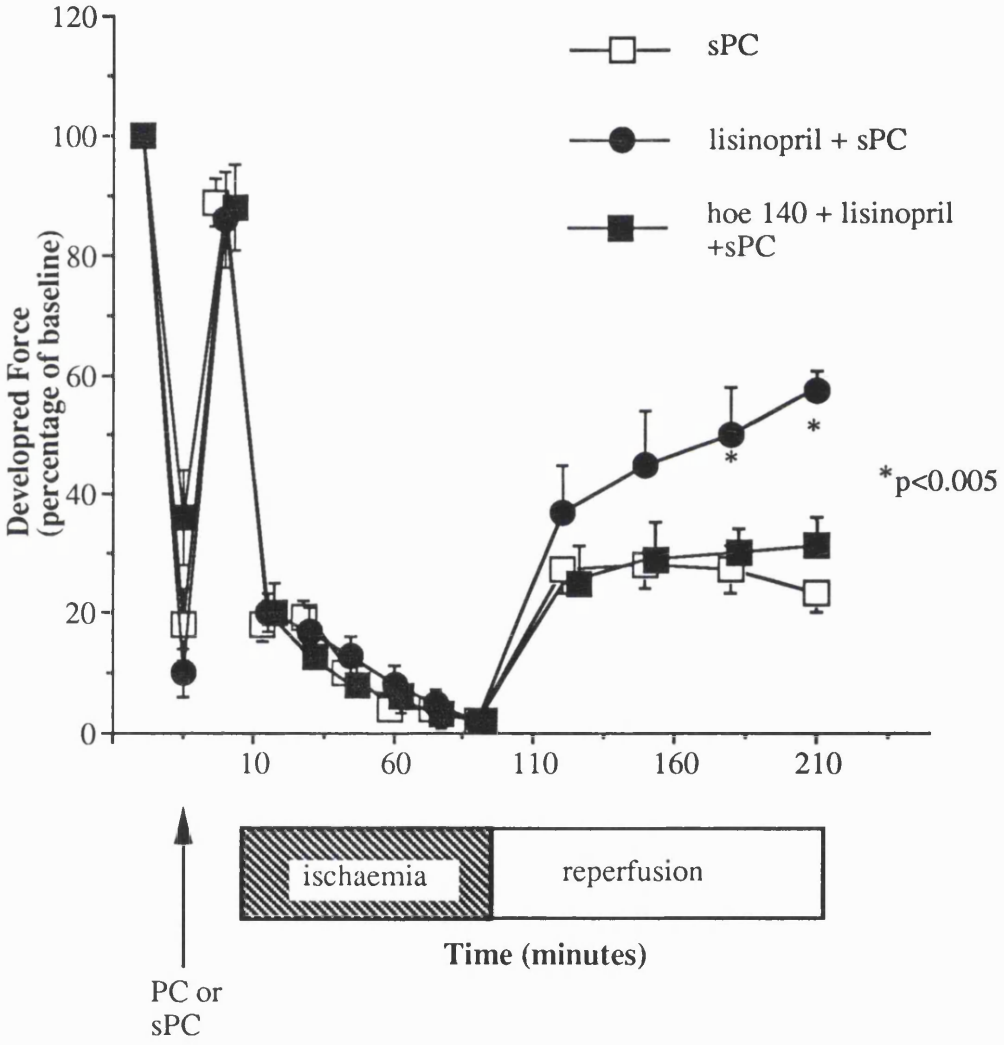
Key: **sPC** = subthreshold preconditioning

Captopril + sPC = treatment with Captopril followed by subthreshold preconditioning

Hoe 140 + Captopril + sPC = treatment with Hoe 140 prior to captopril and subtreshold preconditioning

Figure 4.11

Developed force expressed as a percentage of baseline force over time in minutes. Effect of pre treatment with Hoe 140 on the protective effect of lisinopril in combination with subthreshold preconditioning



Key: **sPC** = subthreshold preconditioning

Lisinopril + sPC = treatment with lisinopril followed by subthreshod preconditioning

Hoe 140 + Lisinopril + sPC = treatment with Hoe 140 prior to lisinopril and subtreshold preconditioning

6.3 Preconditioning protocols

The 3 minute or 90 second preconditioning protocols resulted in a significant reduction in developed force which recovered to baseline on reperfusion prior to the long ischaemic insult. The preconditioning protocol (3 minutes of simulated ischaemia, i.e. superfusion with substrate free Tyrode's solution and rapid pacing of trabeculae at 3Hz resulted in a significant improvement in recovery of function to 61±1% compared to 22±1% in control (non preconditioned) groups.

There was no protection in the group receiving subthreshold ischaemic preconditioning alone (23±4%), this group having a similar level of recovery to the control (no pre treatment) group.

6.3.1 ACE inhibition alone

ACE inhibitor pre-treatment alone had no protective effects in contrast to the marked protection observed when combined with subthreshold preconditioning, as seen in figure 4.8. The values for developed force for ACE inhibition alone are 25±4% for lisinopril and 22±3% for captopril.

6.3.2 ACE inhibition combined with subthreshold preconditioning

Figures 4.8 and 4.9 illustrate the synergistic effect of pre-treatment with an ACE inhibitor in combination with a subthreshold preconditioning stimulus. The pre treatment with either ACE inhibitor did not significantly alter the reduction in function observed during the preconditioning protocol. The trabeculae in these groups (i.e. ACE inhibitor combined with sub threshold

preconditioning) recovered to baseline prior to the long 90 minute period of simulated ischaemia.

The level of protection observed in the ACE inhibitor + sub threshold preconditioning groups is of a similar magnitude to that obtained with the standard 3 minute ischaemic preconditioning protocol, although captopril was significantly more protective than lisinopril at the last two timepoints, i.e. 210 and 180 minutes of reperfusion, ($p<0.05$). Recovery, measured at the end of reperfusion (i. e. 210 minutes), was 71±4% in the cap+sPC group and 58±8% in the lis+sPC group, compared to 22±1% in controls compared to 61±1% in the PC group. $p<0.005$ for cap+sPC versus controls, for lis+sPC versus controls and for PC versus controls.

6.3.3 Investigations involving Hoe 140

Figures 4.10 and 4.11 illustrate the effects of pre treatment with the B_2 receptor antagonist Hoe140 on captopril and lisinopril treated groups respectively. In figure 4.10 it can be seen that Hoe140 clearly abrogates the protective effects of captopril in combination with subthreshold preconditioning with a functional recovery of 35±5% . This was statistically significant: $p<0.005$ for Hoe 140 + captopril + sPC versus captopril + sPC. However, there is some residual protective effect observed although this is not significant. Figure 4.11 also shows that Hoe140 abrogates the protective effects of lisinopril in combination with subthreshold ischaemic preconditioning (31±5 %), again $p<0.005$ for Hoe 140 + lisinopril+ sPC versus lisinopril+ sPC. .

In addition Hoe140 pre treatment alone did not adversely affect recovery in comparison to control groups (20±5%).

6.4 The onset of contracture and peak contracture

Table 4.3 illustrates the values for onset of contracture and time of peak contracture for the different experimental groups. The time to onset of contracture was slightly shorter in the preconditioned groups but the results did not reach statistical significance. There was no significant difference in time to peak contracture between groups. Previous work in isolated rat hearts during global ischaemia found that onset of contracture occurred earlier in preconditioned hearts whereas peak contracture occurred later in preconditioned hearts (Barakat et al 1991). These changes could be due to lower levels of ATP in preconditioned hearts prior to the long ischaemia and the slower rate of ATP utilisation during ischaemia (Murry et al 1990). The results of this present study do not lead to any specific conclusions in this regard.

Table 4.3:
Onset of contracture and peak contracture

GROUP	Onset of contracture min (SEM)	Peak contracture min (SEM)	% Recovery at 210 minutes
Control	25±6.0	53±6.4	22±1
Cap	20±3.5	62±4.0	22±3
Lis	28±4.9	55±5.4	25±4
PC	17±4.9	59±4.4	61±1
sPC	23±4.2	48±6.1	23±4
Cap + sPC	25±5.4	56±7.0	71±4
Lis + sPC	20±2.7	53±5.1	58±8
Hoe+sPC	21±3.8	51±3.9	20±5
Hoe+Cap+sPC	19±4.7	47±5.7	35±5
Hoe+Lis+sPC	23±5.2	46±4.8	31±5

Values are mean ± SEM

Cap: Captopril, **Lis:** Lisinopril, **PC:** Preconditioning,

sPC: Subthreshold preconditioning, **Hoe:** Hoe140.

n =6 for all groups

7 Discussion

7.1 Study findings

This study shows that ACE inhibition in combination with a subthreshold preconditioning stimulus results in a significant degree of protection, in terms of recovery of function, when the trabeculae are subjected to a subsequent 90 minute period of simulated ischaemia. The degree of protection provided by combined subthreshold preconditioning and ACE inhibition is of a comparable degree to that following a full ischaemic preconditioning protocol. Furthermore the cardioprotective effect was abolished by pre treatment with the B_2 receptor antagonist Hoe140. These results support the hypothesis that generation of bradykinin during preconditioning contributes, via B_2 receptor activation, to the protection observed in terms of improved post-ischaemic functional recovery. However pre-treatment with ACE inhibitor alone did not result in protection, perhaps because a degree of ischaemia is required to release bradykinin initially or other mediators, such as adenosine, are required in addition to trigger preconditioning

7.2 Role of bradykinin

Although we did not measure kinin levels in our samples, Hoe140 is the most specific and potent B_2 receptor antagonist currently known (Linz 1995). It has virtually the same affinity to B_2 receptors as bradykinin itself, and does not react with receptors for other peptides. Furthermore kinin release from ischaemic myocardium has been well documented in the isolated rat heart in vitro (Baumgarten 1993, Przyklenk 1993) and the canine myocardium in vivo (Weimer 1994, and Kimura 1973). Baumgarten et al (1993) showed that kinin

release after 15 minutes of coronary occlusion was enhanced by pre-treatment with an ACE inhibitor, ramiprilat, in isolated rat hearts. Furthermore, Nolly et al (1994) have demonstrated that the heart contains an independent kallikrein -kinin system. They demonstrated the presence of kallikrein (a potent kinin generating enzyme) mRNA in rat atria and ventricles, and kallikrein activity in primary cultures of neonatal rat atrial and ventricular cardiocytes and in their incubation medium. Heart slices were shown to release kallikrein without depletion of total tissue kallikrein - suggesting pool replenishment. In fact kininogen was found in epicardial slices and in the Krebs bathing the slices (having been washed and reincubated in Krebs) - and this release was inhibited by pre-treatment with puromycin - suggesting de novo synthesis. Presumably ischaemia results in pH and other changes that cause activation of kallikrein within the myocardium and the subsequent local availability of bradykinin (Goto 1995). Hence, this work supports our hypothesis that local production of kinin contributed to the protection observed.

7.3 Relevance of the -SH moiety

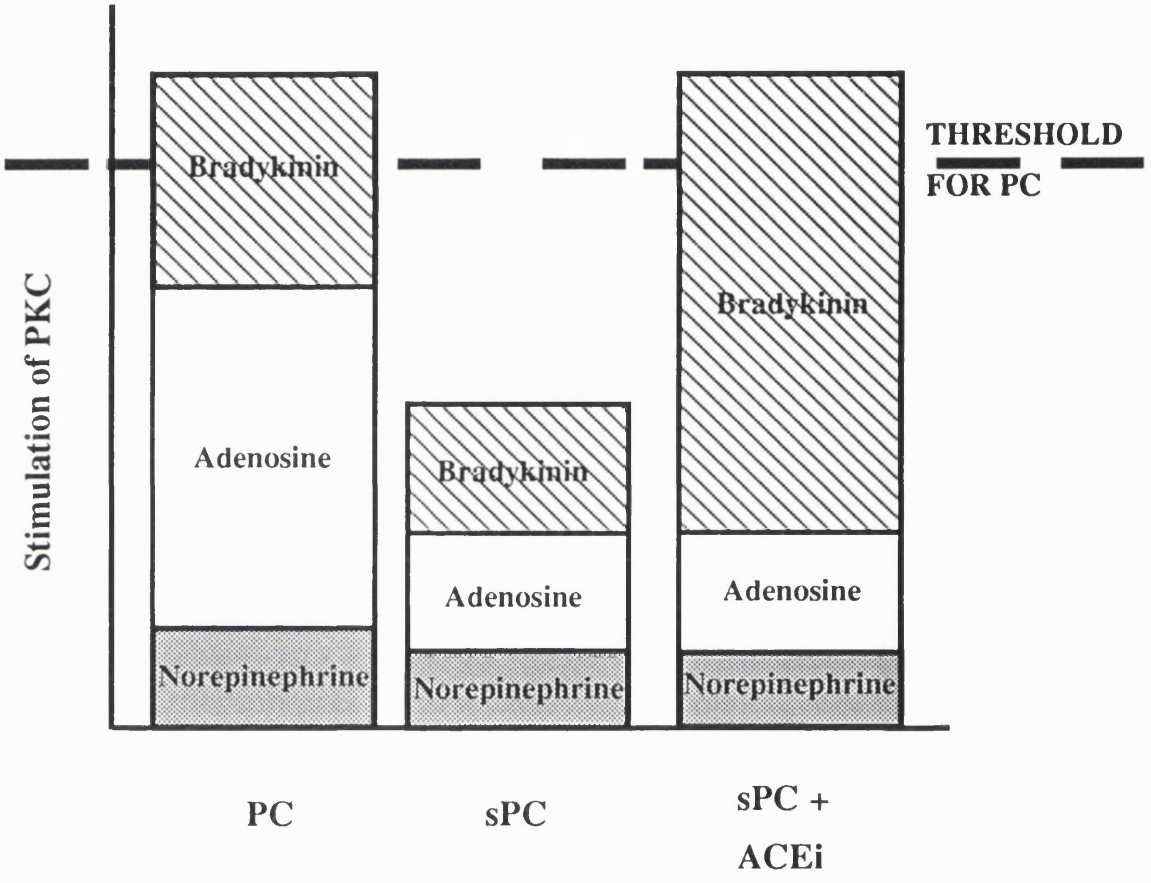
Protection was observed for both an -SH containing ACE inhibitor, captopril, and a non -SH containing one, lisinopril. However for the last 2 timepoints the recovery in the captopril group was significantly greater than in the lisinopril group, although both ACE inhibitors in combination with sPC provided significant protection from 150 minutes of reperfusion onwards. The greater beneficial effect of captopril over lisinopril could theoretically be due to the free radical scavenging ability conferred by the presence of the -SH moiety. Another mechanism by which ACE inhibitors may provide protection is via activation of the K_{ATP} channel. Sargent (1993) found that the -SH containing ACE inhibitor zofenopril induced cardioprotection via modulation of the K_{ATP} channel and others have also reported modulation of this channel by these

agents. Indeed the cardioprotection was abolished by the K_{ATP} blockers glyburide and 5-HD.

7.4 Mechanisms of protection in this model

Interestingly our results show that ACE inhibition alone is insufficient to confer any protection following 90 minutes of ischaemia. This result is consistent with previously published studies (Richard 1993, Daniel 1984, Liang 1982, Miki 1993). However, the situation is not clear cut and there are some reports of infarct size limitation by the same agents (Ertl 1982, and Hartman 1993). Our results are compatible with a current concept of preconditioning as a phenomenon which may have several different endogenous mediators such as adenosine and bradykinin which stimulate protein kinase C to a certain threshold sufficient to trigger the intracellular pathways necessary for providing protection against a further ischaemic insult (Goto 1995). Recently Goto et al (1995) found that Hoe140 blocked the protection from one 5 minute period of preconditioning but could not block protection when a more profound preconditioning stimulus was used in a rabbit model. Hence it is possible that the adenosine and bradykinin released during the subthreshold ischaemia is insufficient to trigger protection unless the level of bradykinin is augmented by ACE inhibition, as illustrated in Figure 4.12. Interestingly it is possible that the interaction between adenosine and bradykinin may not simply be additive, but a study in smooth muscle cells found that stimulation of adenosine A_1 and bradykinin receptors results in a *synergistic* increase in inositol 1,4,5,-triphosphate and free calcium levels (Gerwins 1992).

Figure 4.12: Summary depicting putative mediators involved in triggering PKC in ischaemic preconditioning



Adapted, with permission from Goto et al Circ Res 1995;77:611-21

Key:

PC: preconditioning

sPC: subthreshold preconditioning

ACEi: ACE inhibitor

The present study does not address the issue of the cardioprotective mechanism downstream to activation of the B_2 receptor. This receptor is coupled to phospholipases A_2 and C via G-proteins (Bhoola 1992, and Linz 1995) and stimulation of this receptor leads to the production of two important vasoactive substances, prostacyclin and nitric oxide. However, neither cyclooxygenase inhibitors (Liu 1992) nor the nitric oxide synthase inhibitor, nitro-L-arginine methyl ester (Goto 1994, and Woolfson 1995) attenuated infarct size limitation by preconditioning in a rabbit model of infarction. In contrast Goto et al (1994) demonstrated that polymyxin B, a blocker of protein kinase C, abolished infarct size limitation by treatment with a kinin. Interestingly, it has recently been observed that B_2 receptor stimulation caused a significant inositol triphosphate production in isolated cardiac myocytes (Minshall 1995), suggesting that simultaneously generated diacylglycerol could activate protein kinase C. In summary these observations suggest that PKC activation plays a role in bradykinin-mediated protection.

7.5 Role of angiotensin II

The present study does not take into account the simultaneous suppression of angiotensin II production which has in some recent studies been reported to contribute to ischaemic preconditioning (Bruton 1995); and angiotensin II receptor activation can mimic preconditioning (Liu 1995). Hence, the effect of ACE inhibition in the reduction of angiotensin II levels would tend to lead to an underestimation of the protective effect observed with respect to bradykinin, for some of the angiotensin II mediated component could theoretically be reduced. In terms of implications for future therapy development of a specific kininase inhibitor without the ACE inhibitory component could be valuable in this setting. Furthermore, agents such as angiotensin II receptor antagonists would not have this bradykinin potentiating effect.

8 Critique of methods

8.1 Atrium v Ventricle

The present study used isolated human atrial trabeculae in an attempt to examine the mechanism of preconditioning in human myocardium. Atrial specimens make stable preparations and are generally disease free and sampling was part of the routine procedure for coronary artery bypass grafting. However, there are differences between atrial and ventricular tissue and it must be appreciated that results may not be applicable from one to the other.

8.2 Hypoxia v ischaemia

This present study used a period of hypoxic superfusion in combination with rapid pacing to simulate ischaemia, rather than the "true" ischaemia of ischaemic preconditioning. However there is a great deal of evidence in animal models of both regional and global hypoxia and in cell culture that hypoxia is as effective as ischaemia in inducing preconditioning (Lasley et al 1993, Shizukuda et al 1992, Engleman et al 1995, and Webster et al 1995). For example, Lasley et al demonstrated that hypoxia and ischaemia were equally effective in preconditioning isolated rat hearts. Using either 5 minutes of hypoxia or 5 minutes of ischaemia, the improvement in post ischaemic functional recovery was similar in both groups - suggesting that the limitation of oxygen delivery is more important than metabolite accumulation in producing preconditioning. Engelman et al (1995) also used an isolated rat heart model and demonstrated the effectiveness of 10 minutes of hypoxia in improving functional recovery and a reduction in cell necrosis as assessed by LDH release. Shizukuda et l (1992) demonstrated that hypoxia and ischaemia are equally effective in inducing

protection in an in vivo dog model - in terms of reduction in infarct size and recovery of contractile function. Webster et al (1995) used hypoxic preconditioning to induce protection in an in vitro model of cultured rat neonatal cardiomyocytes.

8.3 Necrosis v stunning

We know that preconditioning improves contractile function as well as reducing infarct size. For example Jenkins et al (1995) who measured both infarct volume and functional recovery after ischaemia, showed that the improved recovery of global left ventricular function produced by preconditioning is proportional to a reduction in infarction, and Cohen et al (1991) and Przyklenk et al (1993) were able to correlate improved recovery of systolic shortening with reduced infarct size in vivo models of regional ischaemia. There is no direct evidence in atrial tissue that recovery in function is proportional to infarct volume, but these studies demonstrate that the enhanced recovery of contractile function due to preconditioning which follows a prolonged period of ischaemia, is due to a reduction in infarct size and is not due to a reduction in stunning. Our model uses a 90 min period of simulated ischaemia as the ischaemic insult, a period more likely to result in cell death rather than stunning, which is induced by shorter periods of 5-15 min of ischaemia.

Furthermore if isoprenaline is added to the superfusate at the end of the experiment there is minimal functional reserve.

Hence, there is evidence that hypoxia can be as effective as ischaemia in preconditioning, that recovery of contractile function is a good correlate for the degree of myocardial necrosis, and that this experimental model is representative of the preconditioning phenomenon.

9 Conclusions

In summary, therefore, we have demonstrated in a human model that ACE inhibitors can potentiate the effects of a subthreshold preconditioning stimulus and that B_2 bradykinin receptors may play a role in this protection. This is the first time that a potential role for bradykinin as a mediator in human preconditioning has been alluded to and furthermore gives a possible clue to the mechanisms involved in the reduction in fatal ischaemic events in patients treated with ACE inhibitors observed in the multicenter trials.

This study has been published in the Journal of the American College of Cardiology (Morris 1997).

Further studies

These experiments specifically excluded patients who were taking ACE inhibitors and it would clearly be of interest to study trabeculae from these patients. Are patients on ACE inhibitors already protected? or perhaps the trabeculae from these patients would be more readily preconditioned.

Chapter 5
General Discussion and Conclusions

1 Summary of results

2 Ongoing investigations

3 Implications of the work described

1 Summary of Results

Upregulation of stress proteins as a route to cytoprotection

The studies presented in this thesis have demonstrated that in primary rat neonatal cardiomyocytes upregulation of stress proteins, particularly hsp 70 occurs during thermal stress, and this is associated with enhanced resistance to a subsequent severe insult, such as ischaemia or heat stress. Furthermore, the tyrosine kinase inhibitor, Herbimycin A, can specifically induce hsp70 in this model resulting in a significant degree of protection. However another tyrosine kinase inhibitor, genistein, did not up regulate stress proteins and did not protect the cardiocytes, suggesting another feature of herbimycin-A, other than its tyrosine kinase inhibition could be responsible for the effects observed.

How does this data add anything new to the field?

Numerous pervious studies have shown that increases in the levels of stress proteins are sufficient to render increased protection to the cell. However, the findings presented in this thesis are novel in relation to several different important aspects regarding the stress response.

The results shown suggest that Herbimycin-A does not act through the usual route of stress protein induction, and is significantly different from other agents which have been shown to induce stress proteins. Herbimycin-A treatment resulted in specific upregulation of the 70 kDa stress proteins, unlike the pattern of upregulation of stress proteins that usually results from the stress response. Furthermore, transfection experiments comparing the effects of Herbimycin-A on intact and disabled heat shock elements suggest that the effect of Herbimycin-A on hsp70 expression does not involve the heat shock element-containing region of the promoter. This is in agreement with our finding that

the other hsps are not induced by Herbimycin-A treatment, as would have been expected if induction was dependent of the heat shock element, and that constitutive hsp70 is also induced - unlike in heat stress. The evidence presented is in favour of Herbimycin-A acting through some type of novel mechanism to induce stress protein expression

ACE inhibitors in cardioprotection

The human atrial trabeculae studies have demonstrated that ACE inhibitors may play a role in cardioprotection by augmenting the effects of preconditioning. The experiments with this human atrial trabeculae model have shown that it is a stable preparation and is capable of being preconditioned in a similar manner to classical preconditioning which is well documented in animal models of myocardial ischaemia and infarction. Furthermore the agents which were shown to augment preconditioning (lisinopril and captopril) are in widespread use in clinical practise. It is likely from our results that this protection may relate to reduced bradykinin breakdown which is a possible mediator of preconditioning in vivo.

In summary, therefore, the results demonstrate in a human model that ACE inhibitors can potentiate the effects of a subthreshold preconditioning stimulus and that B_2 bradykinin receptors may play a role in this protection. This is the first time that a potential role for bradykinin as a mediator in human preconditioning has been alluded to and furthermore gives a possible clue to the mechanisms involved in the reduction in fatal ischaemic events in patients treated with ACE inhibitors observed in the multicenter trials.

2 Ongoing Investigations

Further extrapolation of these experiments to human ventricular tissue would reinforce the relevance of these results. Development of a human ventricular model of infarction, using strips of ventricle derived from patients undergoing mitral valve replacement or from Fallots repair surgery (which will have the inherent advantage of being healthy tissue) is a clear possibility. Development of human ventricular cardiomyocyte culture to investigate the delayed phase of protection in man would also be of great value. This awaits further advances in cell culture technology. This also applies to the detection of different members of the human hsp 70 family by more selective antibodies which are not yet available.

Other experiments to determine the possibility of superimposing both types of protection to give an enhanced state of resistance to stress would be of interest. For example in cell culture it could be possible to induce stress proteins and then superimpose a classical preconditioning - like stimulus.

Finally, further work on the mechanism of action of Herbimycin-A may eventually lead to the development of specific agents to up regulate cytoprotective proteins in clinical practise.

3 Implications of the work described

There is enormous potential for therapeutic manipulation of endogenous cardioprotective mechanisms. The work in this thesis outlines two possible approaches which may ultimately be of use in a clinical setting. Myocardial infarction remains a major challenge despite advances in achieving adequate reperfusion. The ability to delay the onset and slow the progression of myocardial necrosis would increase time available for effective reperfusion.

Similar considerations apply in patients undergoing high risk coronary artery bypass grafting or high risk coronary angioplasty.

The problems of ischaemia and tissue necrosis are not only of importance in the myocardium. Another major cause of morbidity and mortality is cerebrovascular events and there is evidence that some of these endogenous cytoprotective mechanisms also exist within the central nervous system.

Angiotensin converting enzyme inhibitors are already in widespread clinical use and as shown in this thesis may well have cardioprotective effects. In the future manipulation of stress proteins may also be added to the therapeutic armamentarium available to cardiologists and possibly other specialists.

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Appendix 1
Solutions for primary cardiocyte culture

1 Ads buffer

2 Plating medium

3 Maintenance medium

1 Ads Buffer 1 litre

6.8g NaCl

4.76g Hepes

0.12g NaH_2PO_4

1.0g glucose or dextrose

0.4g KCl

0.1g$MgSO_4$

pH is adjusted to 7.35=/-0.5 with 1N NaOH, and then made up to 1litre with distilled water.

The solution was filtered with a 0.22 micron filter.

2 Plating Medium

340ml DMEM with 10ml penicillin/streptomycin

85ml medium199

50ml horse serum

25ml fetal calf serum

3 Maintenance Medium

400mlDMEM

100ml medium 199

10ml penicillin/streptomycin

Appendix 2
Solutions used for SDS-PAGE and Western Blotting

1 Sample Buffer (Two-Times)

This solution was used to solublize cardiomyocytes, and has a high specific gravity allowing samples to sink into the wells in the stacking gel.

Comprises 20% Glycerol, 6% SDS in 0.12 M TRIS at pH 6.8

Glycerol		20ml
SDS*	6g	
TRIS‡		1.4g

the above was made up to 100ml with D water

Prior to use 800mcl of 2-times buffer is added to 200mcl of beta-mercaptoethanol and 5 µl of Bromophenol Blue dye 8% in distilled water (w/v).

*SDS = sodium dodecylsulphate (Laural Sulphate, Sigma Chemicals)

‡TRIS = tris(hydroxymethyl)methylamine (Sigma Chemicals, Mousourri, USA)

2 Acrylamide Gel Solution

The gels were prepared from varying proportions of acrylamide 30% (w/v in distilled water), TRIS buffered gel stock solution at pH 6.8 or 8.8 (see below) and distilled water. Temed and APS were added to promote polymerisation and crosslinking.

	Stacking Gel	**Resolving Gel**	
Concentration %	5	10	12.5
30% Acrylamide* (mls)	2ml	8.4	12
Tris pH 8.8 (mls) (resolving)	9ml	9	9
Tris pH 6.8 (stacking)	3ml	-	-

Distilled water (mls)	7ml	18	15	
10% APS‡	100µl	225	180	(µl)
TEMED§	10µl	15	15	(µl)

*30% Acrylamide with 1% bisacrylamide (Protogel solution, National diagnostics, New Jersey, USA)

‡APS = Ammonium persulphate (BDH chemicals, Dorset, England), made up in distilled water 10% w/v

§TEMED = NNNN-Tetraethylethalinediamine (sigma Chemicals, Moussourri, USA)

3 TRIS Stock Solutions for SDS-PAGE Gels

3.1 Resolving (Base) Gel

Comprised 1.5 M TRIS, 0.4% SDS pH adjusted to 8.8 with HCL

100ml	SDS 0.4g	500ml	SDS 2.0g
	TRIS 18.16g		TRIS 90.9g
made up to:	100ml with D water		500ml with D water

3.2 Stacking (Top) Gel

Comprised 0.5M TRIS, 0.4% SDS pH adjusted to 6.8 with HCL

100ml	SDS 0.4g	500ml	SDS 2.0g
	TRIS 6.05g		TRIS 30.25g
made up to:	100ml with D water		500ml with D water

4 SDS-PAGE Running Buffer

This was made up as a 10 times concentrated stock solution and diluted in distilled water prior to use. The electrophoresis apparatus required approximately 2 litres of solution to fill upper and lower reservoirs. The natural pH of this solution was critical, and if it deviated from pH 8.8 proteins would fail to resolve adequately. It was prepared by mixing reagents in the following proportions.

	1000ml	500ml
glycine	144.2g	72.1g
SDS	10.0g	5.0g
TRIS	30.3g	15.15g

5 Coomasie Blue Staining Solution

This solution was used to stain SDS-PAGE gels to visualise protein. The stain was prepared by mixing the following constituents.

Methanol	250ml

D water	25ml
Acetic acid	35ml
Coomasie blue	1.07g

6 Coomasie Blue De-Staining Solution

This solution was used to wash non-specific Coomasie blue stain from SDS-PAGE gels. The solution needed to be changed frequently as dye leeched out of the acrylamide. It comprised 10% acetic acid, 50% methanol and 40% distilled water.

Acetic acid	300ml
Methanol	1500ml
D water	1200ml

Any gel shrinkage was re-expanded by agitating in distilled water with 7% acetic acid.

7 Ten-Times Blotting Buffer for Western Blotting

A ten-times concentrated stock solution was made as follows

	1000ml	500ml
TRIS	30.3g	15.2g
Glycine	144.2g	72.1g
made up to: water	1000ml with D water	500ml with D

Prior to Western blotting 300mls of ten-times blotting buffer was added to 600ml of methanol and 2100ml of D water to make one-times buffer. The standard blotting tank required 3 litres of reconstituted blotting buffer.

Publications arising from the work in this thesis

Publications

Morris S. D, Yellon D. M. ACE inhibitors potentiate preconditioning via bradykinin B2 receptors in the human heart. *J Am Coll Cardiol* 1997;**29**:1599-606.

Morris S. D, Cumming D. V. E, Latchman D. S, Yellon D, M. Specific induction of 70kDa hsps by Herbimycin-A protects cardiomyocytes: A pharmacological route to cytoprotection. *J Clin Invest* 1996;**97**:706-712.

Morris SD, Yellon DM, Marber M. Stress proteins: a future role in cardioprotection? *Heart* Editorial 1996;**76**:97-98

Morris S. D, Cumming D. V. E, Latchman D. S, Yellon D, M. Induction of hsp72 by the tyrosine kinase inhibitor Herbimycin protects cardiomyocytes from a subsequent stressful stimulus. *Br Heart J. Abstr.* 1995; **73**:suppl 3: 66.

Morris S. D, Cumming D. V. E, Latchman D. S, Yellon D, M. Specific induction of hsp72 by Herbimycin-A protects cardiomyocytes from a subsequent stress. *J Mol Cell Cardiol* Abstr. 1995; **27**: A136.

Morris S. D, Cumming D. V. E, Latchman D. S, Yellon D, M. Specific induction of hsp72 by Herbimycin-A protects cardiomyocytes: A pharmacological route to cytoprotection. *Circulation.* Abstr.1995;**92**:I-652; 3131.

Morris S. D, Yellon, D. M. Captopril potentiates the protective effect of preconditioning in the human heart. *J Mol Cell Cardiol* 1996;**28**:A71.

Oral Presentations

Specific induction of hsp72 by Herbimycin-A protects cardiomyocytes: A pharmacological route to cytoprotection.
American Heart Association. 68th Scientific Sessions. 1995.

Induction of hsp72 by the tyrosine kinase inhibitor Herbimycin protects cardiomyocytes from a subsequent stressful stimulus.
British Cardiac Society 1995

The protective effect of preconditioning in human cardiac muscle is potentiated by ACE inhibition.
XVIIIth Congress of the European Society of Cardiology. 1996

Preconditioning in human cardiac muscle is potentiated by ACE inhibition via a B2 bradykinin receptor pathway.
American Heart Association. 69th Scientific Sessions. 1996.

Specific Induction of the 70-kD Heat Stress Proteins by the Tyrosine Kinase Inhibitor Herbimycin-A Protects Rat Neonatal Cardiomyocytes

A New Pharmacological Route to Stress Protein Expression?

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Abstract

Heat shock protein (hsp) induction by stressful stimuli such as heat and ischemia is known to protect cardiac cells from severe stress. The ability to induce hsp's in the heart directly by "nonstressful" means would potentially have important clinical implications. In noncardiac cells, the tyrosine kinase inhibitor herbimycin-A has been shown to induce the 72-kD hsp. We therefore examined whether herbimycin-A and another tyrosine kinase inhibitor, genistein, could induce 70-kD hsp's in primary cultures of rat neonatal cardiomyocytes, and whether these treatments protect against severe stress. Primary cardiomyocytes were incubated with herbimycin-A or genistein. hsp induction was measured 16–20 h later by Western blotting. Cell survival after subsequent lethal heat stress or simulated ischemia was assessed using trypan blue exclusion and released lactate dehydrogenase activity. Our results indicate that, in cardiac cells, herbimycin-A induces 70-kD hsp's but not hsp90, -60, -25, or glucose-regulated protein 78, whereas genistein has no effect on hsp's. Moreover, hsp induction correlated with the ability of herbimycin-A to protect cells against severe stress, whereas genistein had no protective effects. This suggests that herbimycin-A may induce 70-kD hsp's via a tyrosine kinase–independent mechanism. These results indicate the possibility of a pharmacological approach to HSP70 induction and cardiac protection, which may ultimately be of clinical relevance. (*J. Clin. Invest.* 1996. 97:706–712.) Key words: myocardial protection • heat shock proteins • myocardial infarction • primary cardiomyocyte

Introduction

Over the past few years investigations have shown that part of the myocardium subjected to ischemic damage is potentially salvageable. Restoration of normal cardiac function would require removal of denatured cardiac protein and reestablishment of normal cardiac protein synthesis. The heat stress proteins, which are synthesized in all organisms in response to stresses such as heat and ischemia, may well play a pivotal role in providing protection from a subsequent stress (1, 2). The heat stress response is known to be initiated by activation of the heat shock factor (HSF)[1] 1, which is normally present in unstressed cells and rapidly trimerizes in response to metabolic stress. This enables HSF-1 to bind to the heat shock element located within the promoter of the genes encoding the heat shock proteins, resulting in a high level of transcription of these genes (1).

Previous investigators have found that the induction of heat shock proteins (hsp's) coincides with the appearance of cytoprotection. For example, exposure of cells to sublethal hyperthermia results in induction of hsp's and the acquisition of thermotolerance (3). Furthermore, the induction of hsp's by one insult, such as toxin exposure, may confer protection from a subsequent different insult, such as heat shock challenge (4). This potentially exploitable property of cells may allow a therapeutic approach to minimizing cellular injury during myocardial ischemia. In cardiac tissues, a wide variety of insults results in the synthesis of stress proteins, including myocardial ischemia (5, 6), trauma (7), and hyperthermia (8). Further studies have shown a direct correlation between the amount of the inducible 70-kD heat stress protein and the degree of myocardial protection. For example, we have examined the effects of whole body heat stress using an isolated rabbit papillary muscle model (9), concluding that the degree of contractile recovery after 30 min of hypoxia was related to the content of hsp72. In addition, Hutter et al. (10) were able to show that myocardial infarct size in the rat 24 h after different severities of whole body heat stress was inversely correlated with myocardial hsp72 content. Moreover, evidence that myocardial stress proteins are directly protective is provided by the observation that transfected myocyte lines overexpressing HSP70, but not hsp90, have enhanced resistance to hypoxic stress (11–13).

There is compelling evidence, therefore, that stress proteins may directly influence the resistance of the heart to ischemia, and hence any pharmacological intervention that would raise stress protein levels within the cell by a direct and "nonstressful" means would have important therapeutic implications. Interestingly, herbimycin-A, a benzoquinoid ansamycin antibiotic that inactivates p-60$^{v\text{-}src}$ tyrosine kinase, has been

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1. *Abbreviations used in this paper:* BHK, baby hamster kidney; grp, glucose-regulated protein; HSF, heat shock factor; hsp, heat shock protein; hsp25, the 25-kD heat stress protein; hsp60, the 60-kD heat stress protein; hsp72, inducible member of the 70-kD heat stress protein family; hsp73, constitutive member of the 70-kD heat stress protein family; hsp90, the 90-kD heat stress protein family; LDH, lactate dehydrogenase.

shown to induce hsp72 in a range of cells, including A431 human epidermoid carcinoma cells, HeLa S3 cells, chick embryo fibroblasts, and N1H3T3 cells (14). As yet the exact mechanism of action of herbimycin-A in this regard is uncertain, but preliminary evidence suggests that hsp induction is not secondary to cellular damage (15).

We have therefore attempted to ascertain the ability of herbimycin-A to induce hsp's in primary cultured neonatal rat cardiomyocytes, using genistein, another tyrosine kinase inhibitor, for comparison. Furthermore, we investigated whether these drugs were able to afford any degree of protection against "lethal" heat stress or lethal simulated ischemia. In addition, we compared these agents to "thermal preconditioning," which is an established method of hsp induction (16, 17).

To clarify some of the terms used in this paper, the following definitions apply. (*a*) Nonstressful: does not induce a wide range of stress proteins: (*b*) mild stress: does not cause major lethality, i.e., known to be sufficient to induce stress proteins but not significant cell death; and (*c*) severe stress: kills a high proportion of cells.

Methods

Animals

This investigation was performed in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986* published by HMSO 1986, London.

Cell culture

Isolated cardiac myocyte model. Ventricular myocytes from the hearts of neonatal Sprague Dawley rats < 2 d old were cultured using previously described methods (18–20). The cells were dispersed in a nominally calcium-free, Hepes-buffered salt solution containing 0.6 mg/ml pancreatin (GIBCO BRL, Gaithersburg, MD) and 0.5 mg/ml type II collagenase (~ 266 U/mg) (Worthington Biochemical Corp., Freehold, NJ) via a series of incubations at 37°C. The dispersed cells were preplated for at least 30 min to allow the fibroblasts to adhere to the plate, and the myocytes remained unattached. The myocyte-enriched unattached cells were replated on six-well gelatine coated plates at a density of 1.5–2 million cells per well. The cardiac myocytes were cultured at 37°C, 5–7% CO_2, in 4:1 DME/Medium 199 (GIBCO BRL) supplemented with 10% horse serum, 5% FCS, and 1% penicillin/streptomycin. After 24 h, the medium was replaced with serum-free medium to reduce fibroblast contamination. Cardiocyte cultures under these conditions start to beat synchronously within 72 h, and the percentage of beating cells exceeds 85% for the duration of the experiment.

Baby hamster kidney (BHK) cell culture. BHK (C13) cells were cultured at 37°C, 5–7% CO_2 in DME supplemented with 10% FCS (21).

Experimental protocols

Once the cultured cardiomyocytes were beating synchronously, the following protocols were carried out. Six-well plates were incubated for 4 h with herbimycin-A (1 μg/ml) or with genistein (50 μM), both agents having been dissolved in DMSO. Control plates were incubated with the vehicle DMSO or were simply left in medium. The cells were then washed with medium and returned to the incubator at 37°C. As a comparison, certain plates of cells were thermally preconditioned by incubation at 43°C for 30 min. The following day, cells were harvested for hsp levels or subjected to severe heat shock or "simulated" ischemia.

PAGE and Western blotting

20 h after the above treatments, the cells were harvested for analysis of HSP levels: ~ 2×10^6 cells were harvested in 100 μl of 2× concentrated SDS-PAGE sample buffer, and proteins were separated by SDS-PAGE (10% acrylamide) (22). Proteins were transferred to nitrocellulose membranes (Hybond C; Amersham International, Little Chalfont, UK), and blots were probed with mAbs specific for the inducible isoform of hsp70 (hsp72) (C92F3A-5; Stressgen, Victoria B.C., Canada) or with mAbs specific for both constitutive and inducible isoforms (hsp73 and -72, respectively) (SPA-820; Stressgen) at 1:1,000 dilution. In addition, blots were probed with mAbs to hsp90 (AC88, a gift from Dr. D. Toft, Mayo Clinic, Rochester, NY) at a concentration of 12.15 μg/ml, to hsp60 (clone 4B9/89; Affinity Bioreagents, Neshanic Station, NJ) at 5 μg/ml, or to hsp25 (H0148; Sigma Chemical Co., Poole, UK) at a 1:500 dilution. Certain blots were also probed with an mAb specific for glucose-regulated protein (grp) 78 (SPA-827M; Bioquote, York, North Yorkshire, UK) at a dilution of 1:500. The blots were subsequently washed in PBS/0.5% Tween and then incubated with a peroxidase-conjugated rabbit anti–mouse IgG antibody (DAKOPATTS, Copenhagen, Denmark) at a dilution of 1:2,000, followed by detection using an enhanced chemiluminescence kit (Amersham International). The relative levels of hsp were determined using densitometry (Bio-Rad Laboratories, Richmond, CA), normalizing to the actin band on a duplicate Coomassie brilliant blue R250 (BDH Chemicals Ltd., Poole, UK)–stained gel. This procedure adjusts for slight variations in protein loading between samples. Initially, to determine the optimum time for further experimentation, the cells were harvested at intervals between 30 min and 48 h after treatment with tyrosine kinase inhibitor or thermal preconditioning to determine hsp levels. Maximal induction occurred at 20 h after either treatment; therefore, protection and further quantification of hsp levels were sought at this time point.

Tyrosine kinase inhibition

To demonstrate tyrosine kinase inhibition by herbimycin-A and genistein, at the doses used in these experiments, the following protocol was carried out. Herbimycin-A (1 μg/ml) or genistein (25, 50, 100, 150 μM) was added to wells of cardiocytes and incubated at 37°C, and controls were incubated with the vehicle DMSO or were left untreated. Initially, this experiment was performed with insulin-like growth factor 1 added to each well to stimulate tyrosine kinase activity, but there was sufficient intrinsic tyrosine kinase activity in control cells without growth factor for this experiment to be carried out on unstimulated cells. Samples were harvested with SDS-PAGE sample buffer at the following time intervals after the addition of drug: 10, 30, 60, 120, and 240 min. PAGE and Western blotting were carried out as above using a monoclonal antiphosphotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at a dilution of 1:1,000. The secondary antibody was a peroxidase-conjugated rabbit anti–mouse IgG antibody (DAKOPATTS), diluted to 1:2,000, and enhanced chemiluminescence was used as above. Again, relative levels of phosphotyrosine were determined by densitometry normalizing to the actin band.

Lethal heat stress

20 h after incubation with herbimycin-A, genistein, or DMSO, or after thermal preconditioning, the cultures were subjected to a severe thermal stress by incubating them at 47°C for 90 min.

Lethal simulated ischemia

20 h after incubation with herbimycin-A, genistein, or DMSO, or after thermal preconditioning, the cardiocytes were transferred to an ischemic buffer adapted from Esumi et al. (23): (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2.2\ H_2O$, 4 mM Hepes) supplemented with 10 mM deoxyglucose, 0.75 mM sodium dithionate, 12 mM KCl, and 20 mM lactate, pH 6.5, and incubated for 90 min at 37°C. This buffer is designed to simulate the extracellular milieu of myocardial ischemia, with the approximate concentrations of potassium, hydrogen, and lactate ions occurring in vivo.

Determination of cardiocyte viability

Trypan blue exclusion. After lethal heat stress or simulated ischemia, the cells were washed with PBS, trypsinized for 2 min in 0.25 mg/ml trypsin in versine (GIBCO BRL), and then neutralized with neonatal calf serum. Cells were then centrifuged, the supernatant was aspirated, and the cardiocytes were resuspended in 300 μl of PBS. A 20-μl aliquot of cell suspension was then added to an equal volume of trypan blue, and the percentage of dead (blue) cells was determined using a hemocytometer. To establish that the administration of these drugs did not have a directly toxic effect upon the cardiocytes, trypan blue exclusion was also performed at 4 and 24 h after incubation with herbimycin-A or genistein, in comparison with controls, without lethal stress.

Lactate dehydrogenase (LDH) assay. LDH activity released from cardiomyocytes both before and after subjection to lethal stress, was determined using an LDH test kit (Sigma Chemical Co.). This test relies on the fact that LDH catalyzes the reduction of pyruvate to lactate, resulting in an equimolar amount of NADH oxidized to NAD. The oxidation of NADH results in a decrease in the absorbance at 340 nm. The rate of decrease in absorbance at 340 nm is directly proportional to LDH activity in the sample. Therefore, using a spectrophotometer measuring absorbance at 340 nm, the mean absorbance change per minute was determined in the media from each experimental group. This method of assessing cell damage was chosen because it is reliable even in the presence of ischemic buffer, unlike creatinine kinase activity (24).

Statistical analysis

All values are expressed as mean±SEM. The unpaired Student's *t* test was used to identify significant differences between experimental and control groups. Statistical significance was assumed at the $P < 0.05$ level.

Results

Incubation with herbimycin-A or genistein at all concentrations studied, or treatment by thermal preconditioning, did not result in significant cell death compared with controls as assessed by trypan blue exclusion (< 3%). In addition, light microscopic appearances were unchanged, and the cells continued to beat synchronously (data not shown). Furthermore, released LDH activity was negligible in treated cells and controls when measured immediately after addition of tyrosine kinase inhibitor or treatment with thermal preconditioning, and when measured at intervals until subjecting the cardiomyocytes to lethal stress (see below).

Heat shock protein induction. After 4 h of incubation with herbimycin-A, there was a significant increase in hsp72 from almost negligible levels to > 100× that found in control cells ($P < 0.0001$) when samples were normalized to the actin band from a Coomassie-stained gel (Fig. 1, *A* and *B*). This level of hsp72 increase was of a similar magnitude to that induced by thermal preconditioning, and there was no statistically significant difference between amounts of hsp72 induced by herbimycin-A or thermal preconditioning. Genistein treatment at a wide range of doses did not lead to any significant hsp induction. A study of the time course of hsp72 induction over 48 h showed maximal induction between 19 and 24 h after herbimycin-A treatment or thermal preconditioning, but again there was no evidence of hsp induction by genistein up to 48 h (Fig. 2).

To examine the effect of herbimycin-A on the constitutive 70-kD hsp (hsp73), we used the antibody that recognizes hsp72 and -73, since there is no antibody specific for hsp73 alone. In this experiment, herbimycin-A induced both constitutive and

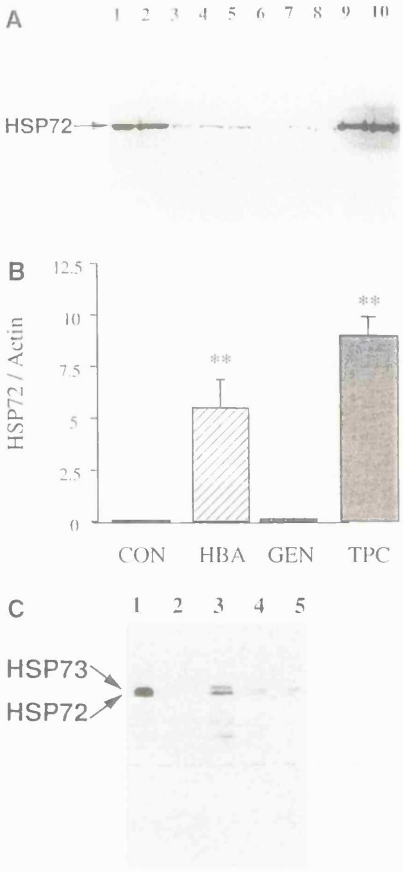

Figure 1. (*A*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after treatment with tyrosine kinase inhibitor. Six-well plates were incubated for 4 h with herbimycin-A or genistein dissolved in DMSO. For comparison, certain plates were thermally preconditioned by incubating them at 43°C for 30 min. Control plates were incubated with the vehicle DMSO or simply left in medium. The primary mAb recognizes the inducible form (hsp72) of the 70-kD heat shock protein. Lanes from left to right: *1*, herbimycin-A; *2*, herbimycin-A; *3*, control, no intervention; *4*, control, no intervention; *5*, control, DMSO; *6*, blank lane; *7*, genistein; *8*, genistein; *9*, thermal preconditioning; *10*, thermal preconditioning. The protein band representing hsp72 is indicated (*arrow*). (*B*) Relative levels of hsp72 as assessed by densitometry (Bio-Rad Laboratories) normalizing to the actin band on a duplicate Coomassie brilliant blue R250–stained gel. Bars represent 1 SEM. Data are presented as mean values (±SE) from duplicate determinations in five independent experiments. *CON*, control; *HBA*, herbimycin-A; *GEN*, genistein; *TPC*, thermal preconditioning. $^{**}P < 0.0001$ (unpaired Student's *t* test). (*C*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after treatment with tyrosine kinase inhibitor. Six-well plates were incubated for 4 h with herbimycin-A (1 μg/ml) or genistein (50 μM) dissolved in DMSO. For comparison, certain plates were thermally preconditioned by incubating them at 43°C for 30 min. Control plates were incubated with the vehicle DMSO or simply left in medium. The primary mAb recognizes both the inducible (hsp72) and constitutive (hsp73) isoforms of the 70-kD heat shock protein. Lanes from left to right: *1*, herbimycin-A; *2*, genistein; *3*, thermal preconditioning; *4*, control, no intervention; *5*, control, DMSO. The protein bands representing hsp72 and -73 are indicated (*arrow*).

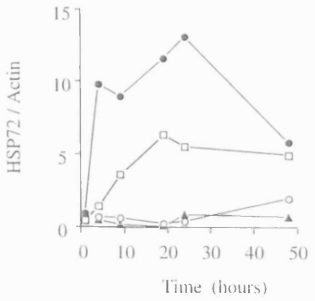

Figure 2. The time course of hsp72 induction. Primary cardiocytes were incubated with herbimycin-A or genistein for 4 h, and samples were harvested for Western blotting at intervals between 30 min and 48 h. Control plates were incubated with DMSO or simply left in medium. The primary mAb recognizes the inducible form of the 70-kD heat shock protein (hsp72). —□—, herbimycin-A; —●—, thermal preconditioning; —○—, genistein; —▲—, control.

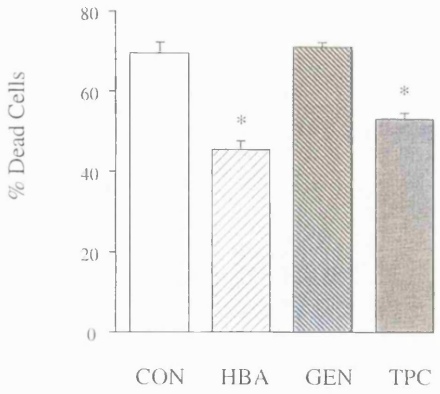

Figure 4. Percentage of cell death after lethal heat stress as assessed by trypan blue exclusion. Six-well plates of primary cardiocyte cultures were incubated with herbimycin-A, genistein, or DMSO (control) for 4 h. For comparison, certain plates were thermally preconditioned by incubating them at 43°C for 30 min. 24 h later, cells were exposed to 47°C for 90 min. After this lethal heat stress, cells were exposed to an equal volume of trypan blue, and the percentage of dead (blue) cells is shown. Data are presented as mean values (±SE) from duplicate determinations in five independent experiments. *CON*, control; *HBA*, herbimycin-A; *GEN*, genistein; *TPC*, thermal preconditioning. $*P < 0.005$ (unpaired Student's *t* test).

inducible isoforms of hsp70 (hsp73 and -72, respectively), whereas, in the thermal preconditioning group, the induction is mainly observed in the hsp72 band, as expected (Fig. 1 *C*). Because of the magnitude of induction of 70-kD stress proteins by herbimycin-A, the bands are almost confluent, resolution limited by the fact that there is no antibody available that recognizes hsp73 alone. In addition, there was no evidence of increases in hsp90 (Fig. 3 *A*), hsp60 (Fig. 3 *B*), or hsp25 (Fig. 3 *C*) after herbimycin-A or genistein treatment. Furthermore, these treatments did not induce the glucose-regulated protein grp78 (Fig. 3 *C*). However, as expected, the hsp's were induced by thermal preconditioning, as was grp78, which has also been shown to be induced by heat stress (25).

To compare these results in noncardiac cells, we followed the same protocol with BHK cells. After 4 h of incubation with herbimycin-A, there was a significant increase in hsp72 levels as well as a slight increase in hsp73 (Fig. 3 *D*). In contrast to our findings in cardiomyocytes, there was also a modest induction of other hsp's when these noncardiac cells were treated with herbimycin-A.

Survival after lethal heat stress. Treatment of cardiomyocytes with herbimycin-A led to a significant increase in survival after lethal heat stress in comparison with control cells as assessed using trypan blue exclusion. Each experiment was performed five times using five different preparations of cells, with at least three wells per treatment group. As seen in Fig. 4, cell death in herbimycin-A–treated cells was 45.0% (±3.6), whereas cell death in control groups was 69.4% (±3.4) ($P < 0.005$, $n = 10$). Thus, herbimycin-A–treated cells have a statistically significant 30% improvement in survival after lethal heat stress. A similar degree of protection was obtained by thermal preconditioning with 52.9% (±1.8) dead; the difference in improved survival between the herbimycin-A–treated and the thermal preconditioning groups was not statistically significant. However, genistein (50 μM) treatment led to no significant improvement in survival (70.9% [±2.0]).

Comparable results were obtained by analysis of released LDH activity. None of the pretreatments resulted in significant LDH release, indicating that they did not produce significant cell damage. However, after subsequent lethal heat stress,

Figure 3. (*A*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after a 4-h incubation with herbimycin-A (1 μg/ml) or genistein (50 μM). The primary mAb recognizes the 90-kD heat stress protein. Lanes from left to right: *1*, thermal preconditioning; *2*, herbimycin-A; *3*, genistein; *4*, genistein; *5*, control, DMSO; *6*, control, no intervention. The protein band representing hsp90 is indicated (*arrow*). (*B*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after a 4-h incubation with herbimycin-A (1 μg/ml) or genistein (50 μM). Two primary mAbs have been used, one recognizing hsp60, the other specific for the inducible isoform of the 70-kD heat stress protein (hsp72). Lanes from left to right: *1*, herbimycin-A; *2*, herbimycin-A; *3*, genistein; *4*, genistein; *5*, control, DMSO; *6*, control, no intervention. The protein bands representing hsp60 and -72 are indicated (*arrow*). (*C*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after a 4-h incubation with herbimycin-A (1 μg/ml) or genistein (50 μM). Two primary mAbs have been used, one recognizing grp78, the other specific for the 25-kD heat stress protein (hsp25). Lanes from left to right: *1*, herbimycin-A; *2*, genistein; *3*, thermal preconditioning; *4*, control, no intervention; *5*, control, DMSO. The protein bands representing grp78 and hsp25 are indicated (*arrow*). (*D*) Western blot of samples harvested from noncardiac BHK cells 20 h after a 4-h incubation with herbimycin-A (1 μg/ml). The mAbs used recognize hsp73/72, -60, and -25. Lanes from left to right: *1*, control; *2*, control; *3*, herbimycin-A; *4*, herbimycin-A.

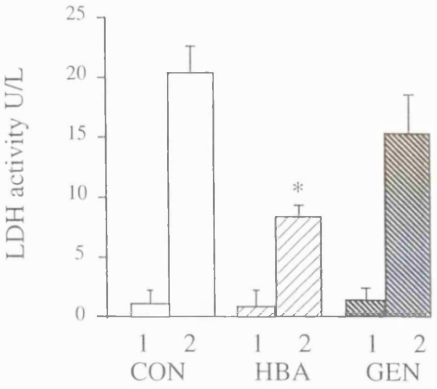

Figure 5. Cell damage after lethal heat stress as assessed by released LDH activity. 24 h after incubation with herbimycin-A, genistein, or control (either DMSO or no intervention), cells were exposed to 47°C for 90 min. Values before lethal stress are labeled 1, and values after lethal heat stress are labeled 2. LDH released into the media as a result of cell damage was determined with an LDH test kit. Data are presented as mean values (±SE). *CON*, control; *HBA*, herbimycin-A; *GEN*, genistein. Significant protection is observed in the herbimycin-A group. $^{*}P < 0.05$.

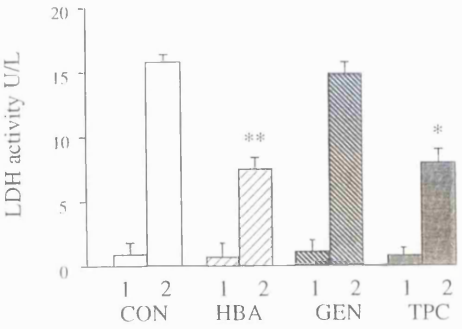

Figure 7. Cell damage after lethal simulated ischemia as assessed by released LDH activity. 24 h after incubation with drug or thermal preconditioning (43°C for 30 min), cells were transferred to an ischemic buffer for 90 min at 37°C. Values before lethal stress are labeled 1, and values after lethal simulated ischemia are labeled 2. LDH released into the media as a result of cell damage was determined with an LDH test kit. Data are presented as mean values (±SE). *CON*, control; *HBA*, herbimycin-A; *GEN*, genistein; *TPC*, thermal preconditioning. Significant protection is observed in the herbimycin-A group ($^{**}P < 0.001$) and also in the thermal preconditioning group ($^{**}P < 0.01$).

significantly more LDH activity was observed in control cells, 20.2 U/liter (±2.6), compared with the herbimycin-A–pretreated group (8.25 U/liter [±0.25] [$P < 0.05$]). Genistein-pretreated cells gave levels of LDH activity that were similar to controls (15.25 U/liter [±3.55]) (Fig. 5) (thermal preconditioning data not available).

Survival after lethal simulated ischemia. Similarly, treatment of cardiomyocytes with herbimycin-A or with thermal preconditioning led to a significant increase in survival against a subsequent lethal ischemic insult in comparison with control cells as assessed by trypan blue exclusion. Duplicate determinations were made in five independent experiments using five different preparations of cells. As shown in Fig. 6, in the herbimycin-A–treated group, the percentage of dead cells was 50.6% (±5.6) ($P < 0.001$), 50.6% (±1.9) ($P < 0.001$) in the thermally preconditioned group, and 68.1% (±1.9) in control cells. Again, genistein treatment did not alter survival significantly from control values with 62.4% (±2.6) dead cells in this group. Thus, herbimycin-A–treated cells have a statistically significant 25% improvement in survival when exposed to a subsequent ischemic insult.

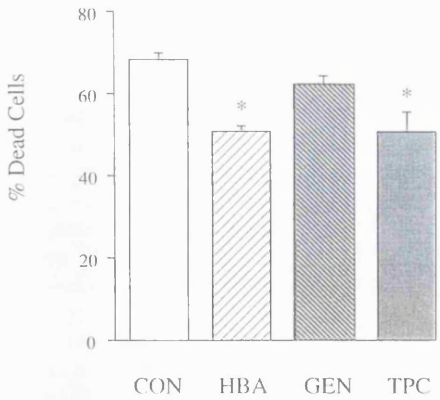

Figure 6. Cell damage as assessed by trypan blue after lethal simulated ischemia. Six-well plates were incubated with herbimycin-A, genistein, or DMSO (control) for 4 h. For comparison, certain plates were thermally preconditioned by incubating them at 43°C for 30 min. 24 h later, all groups of cells were transferred to an ischemic buffer for 90 min at 37°C. Cell viability was then determined by trypan blue exclusion. The graph illustrates percentage of cell death (blue cells) presented as mean values (±SE) from duplicate determinations in five independent experiments. *CON*, control; *HBA*, herbimycin-A; *GEN*, genistein; *TPC*, thermal preconditioning. $^{*}P < 0.001$.

Results obtained with LDH assay as a marker of cell damage yielded comparable results (Fig. 7). None of the pretreatments resulted in significant LDH release, indicating that they did not produce significant cell damage. However, after subsequent lethal simulated ischemia, control cells released significantly more LDH, 15.9 U/liter (±0.8), than those pretreated with herbimycin-A (7.4 U/L [±1.1] [$P < 0.001$]), or thermal preconditioning (8.0 U/liter [±2.0] [$P < 0.01$]). Genistein (50 μM)–pretreated cells had similar levels of LDH activity to controls (14.8 U/liter [±1.1]).

Tyrosine kinase activity. In view of the different effects on hsp72 induction observed with the tyrosine kinase inhibitors herbimycin-A and genistein, we wished to confirm that both these inhibitors were having the expected effect on tyrosine kinase activity. Western blotting with monoclonal antiphosphotyrosine antibody showed reduced activity in both herbimycin-A–treated cells and genistein (concentrations 25, 50, 100, and 150 μM)–treated cells in comparison with controls (Fig. 8). Genistein appeared to have a more immediate effect, with inhibition of activity occurring after just 10 min for all concentra-

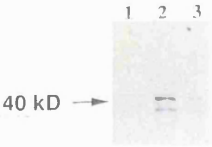

Figure 8. Western blot of samples prepared from primary cardiocyte cultures 30 min after incubation with herbimycin-A or genistein. Controls were incubated with the vehicle DMSO. The primary mAb is an antiphosphotyrosine. Lanes from left to right: *1*, herbimycin-A; *2*, control; *3*, genistein. Both herbimycin-A and genistein inhibit the phosphorylation of a prominent protein doublet at 40 kD.

tions of drug, whereas herbimycin-A inhibited tyrosine kinase after 30 min of incubation.

Discussion

Earlier studies have shown that expression of stress proteins from a variety of different environmental stimuli correlates with protection against subsequent adverse stress. We have been able to show that the benzoquinoid ansamycin antibiotic herbimycin-A is able to induce 70-kD hsp's in primary neonatal rat cardiomyocytes, as well as protecting these cells against lethal heat stress and simulated ischemia. Genistein, also an inhibitor of tyrosine kinase, neither induced 70-kD hsp's nor conferred any protection to these cells. We initially screened a wide range of concentrations of genistein to confirm that its lack of effect on hsp induction and protection was apparent at all concentrations.

In our experiments in cardiac cells, herbimycin-A induced only the 70-kD hsp's and not a wide range of other hsp's. However, in noncardiac BHK cells, we did observe a wider range of hsp induction, in agreement with the work of Hedge et al. (15), who found that herbimycin-A induces a range of hsp's in fibroblasts, indicating that herbimycin-A may have a cell type–specific effect on some hsp's. At least in cardiac cells, our results show a correlation between 70-kD hsp induction and tolerance against lethal heat stress and ischemia, with such protection occurring in the absence of induction of the other hsp's. However, we cannot assume from this a directly protective effect of hsp70, since herbimycin-A may induce other non-hsp proteins. For example, other candidates for protection include endogenous antioxidants such as manganese superoxide dismutase (26), which we did not measure in our experiment. However, hsp72 transfection studies in cell lines (11–13), taken together with a number of transgenic studies (27, 28) in which expression of an hsp72 transgene in the mouse heart protects against ischemia/reperfusion injury, as well as the known actions of hsp72 in protein folding and transport, provide strong evidence for a directly protective effect. It is also clear from our results that the induction of 70-kD hsp's by a pharmacological route can provide effective protection without any induction of the other hsp's.

The time course for induction of hsp72 was similar in both thermally preconditioned groups and those treated with herbimycin-A, and the magnitude of the hsp response was also comparable in these groups. It is particularly interesting to note that, in our experiments in cardiac cells, herbimycin-A did not induce hsp90, -60, or -25. In contrast, the heat shock response results in the synthesis of a number of stress proteins, and in our experiments thermal preconditioning induced the other hsp's as expected. This suggests that the mechanism of hsp72 induction by herbimycin-A may not occur via activation of the heat shock transcription factor, unlike the agents or mechanisms that induce the stress response (1), but, rather, that herbimycin-A may act via a distinct and possibly less "stressful" pathway for hsp72 induction. This is also supported by the fact that herbimycin-A appears to induce both isoforms of hsp70 (hsp73 and -72) strongly, and this contrasts with the pattern of induction evoked by the stress response.

We have been able to show that, although used at doses adequate for tyrosine kinase inhibition, genistein was unable to induce any hsp's and was similarly unable to protect cardiocytes from lethal stress. Hence, the tyrosine kinase–inhibitory activity of herbimycin-A is unlikely to be responsible for its action with regard to 70-kD hsp induction and enhanced tolerance against lethal stress. Although genistein and herbimycin-A are both tyrosine kinase inhibitors, their modes of action are quite dissimilar. Herbimycin-A has a benzoquinone moiety and is thought to modify thiol groups on its target kinase covalently (29), and it therefore may have other actions related to this thiol reactivity. Interestingly, recent reports have suggested herbimycin-A may directly modify the transcription factor NFκB (30). It has also been shown that NFκB activation is inhibited by sodium salicylate, which prevents the degradation of IκB (31). IκB is an associated protein that inhibits NFκB activity; on phosphorylation of IκB, the complex dissociates and NFκB moves to the nucleus, where it activates gene expression (32, 33). Furthermore, sodium salicylate induces the heat shock–responsive chromosomal puffs in *Drosophila* salivary glands and induces HSF DNA binding activity in cultured *Drosophila* cells as well as activating DNA binding by the HSF in cultured human cells (34). This suggests a possible link between NFκB and stress protein transcription, with herbimycin-A possibly inducing 70-kD hsp's via its inhibitory action on NFκB.

Further elucidation of the pathway by which herbimycin-A acts may not only enable us to understand the mechanisms of hsp72 induction but also lead us to strategies for targeted hsp72 induction and possibly protection against lethal stress, particularly ischemia, in a clinically relevant context.

Acute myocardial infarction remains the most common single cause of death in men in the Western world. Interventions such as thrombolytic therapy and aspirin have revolutionized the treatment of myocardial infarction. However, the mortality benefit of thrombolytic therapy is diminished if treatment is administered late (35, 36). Hence, any intervention that could delay the onset of myocardial necrosis would increase the time available for thrombolytic therapy. The ability to induce a prolonged state of resistance to ischemia in the myocardium would also be beneficial in situations such as unstable angina, high risk coronary angioplasty, surgery involving cardiopulmonary bypass, and in explanted hearts before transplantation—all conditions in which the heart is rendered transiently ischemic. Our results indicate that it is possible to specifically induce 70-kD hsp's in cardiomyocytes and thus protect them from injury. It may eventually be possible to induce hsp70 in vivo and protect the human myocardium from ischemia via pharmacological manipulation, thus exploiting the endogenous protective mechanisms of the heart.

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HEART

Editorial

Stress proteins: a future role in cardioprotection?

Myocardial infarction remains a major challenge despite effective interventions which allow rapid reperfusion of jeopardised myocardium. This is in part because benefits diminish as treatment is delayed. The longer the duration of ischaemia, the more complete the necrosis and the less the salvage on reperfusion. Hence any intervention that could reduce the rate of necrosis before thrombolysis would preserve left ventricular function and reduce mortality. The rate of myocardial necrosis is determined by the collateral blood supply to the ischaemic zone and the inherent resistance of the myocardium to ischaemia.[1] After occlusion of an infarct related artery distal pre-formed collaterals are likely to be operating maximally. The need is therefore to understand the processes that determine the inherent myocardial resistance to infarction.

It is now known that the resistance of the myocardium to ischaemia can be enhanced both by classic preconditioning (short periods of ischaemia with intermittent reperfusion) and by the upregulation of cytoprotective proteins. Despite its first description over 10 years ago classic preconditioning (the subject of an editorial in the *British Heart Journal* in 1995[2]) has not led to the development of specific cardioprotective pharmacological agents, partly because of temporal limitations, tachyphylaxis, and the lack of a definitive end effector. Hence, an exploration of the endogenous cytoprotective proteins, particularly the heat shock proteins (hsps), may ultimately prove more fruitful.

Heat stress proteins

The stress response is a universally conserved cellular defence programme consisting of the upregulation of stress proteins. Any agent or treatment which induces the stress response will reduce the injury caused by exposure to a subsequent related or in some cases unrelated stressor. There are five major groups of hsps based on molecular size—70, 90, 50–60, 20–30, and 100–110 kDa.[3] Much of the work on hsps within the myocardium has focused on hsp70, as it is the most abundant and inducible stress protein.

hsp70 as a molecular chaperone

All members of the hsp70 family bind to ATP through a highly conserved amino-terminal nucleotide binding domain, as well as binding to both unfolded proteins and short polypeptides in vitro. When an hsp70 family member is released, a process requiring ATP, the target protein starts folding and/or assembly.

The function of hsp70 gives us some clues as to the mechanisms by which these proteins may result in myocardial protection. During ischaemia the cellular internal milieu changes profoundly with the intracellular accumulation of protons and sodium ions. These changes are compounded by the free radical stress and the marked increase in intracellular calcium associated with reperfusion. Under these circumstances the tertiary structure of proteins changes sufficiently to alter their function. In the presence of an excess of hsp70 these adverse conformational changes may be prevented or reassembly of denatured proteins may be promoted.

hsp70 and myocardial protection

It is now eight years since the association between heat stress proteins and myocardial protection was first described by Currie *et al.*[4] They showed that 24 hours after raising the temperature of rats to 42°C for at least 15 minutes both cardiac hsp70i and catalase activity were increased, while at this time point hearts became resistant to ischaemia/reperfusion injury. In heat stressed hearts (compared with control hearts) post-ischaemic contractile recovery was enhanced and creatine kinase (CK) efflux was reduced. These findings were subsequently confirmed by other groups.[5] Of greater pathophysiological relevance was the observation that ischaemia itself could result in hsp70i (the inducible form of hsp70) induction and cardioprotection,[6] but increases also occurred in a 60kDa stress protein and in another myocardial antioxidant enzyme, superoxide dismutase.[6 7] Thus these studies still fell short of proving a causal relation between hsp70 induction and cardioprotection.

Compelling evidence that upregulation of hsp70 in myocardial cells affords significant protection comes from recent genetic modification studies in which transfected myocyte lines overexpressing hsp70, but not hsp60 or 90, had enhanced resistance to hypoxic stress,[8] and in which hearts from transgenic mice overexpressing the inducible hsp70 gene had enhanced resistance to ischaemic injury.[9] In transgene positive compared with transgene negative hearts, the zone of infarction was reduced by 40%, contractile function at 30 minutes of reflow was doubled, and efflux of CK was halved.[9] In addition overexpression of hsp70 did not alter the macroscopic phenotype of the mouse, contractility of the heart, or antioxidant protein content of the myocardium.[9] This study provides direct evidence that an increase in myocardial hsp70 increases the inherent resistance of the myocardium to infarction.

Regulation of the stress response

Many of the heat shock proteins are in fact expressed constitutively in normal or "unstressed" cells where they play a fundamental part in several important biological processes. A diverse array of metabolic insults induce a

stress response—many of these agents share the common property of being "protein chaotropes," they adversely affect the proper conformation and therefore function of proteins.[10] The heat shock factor (HSF 1) present as an inactive monomer in the normal unstressed cell, rapidly trimerises in response to metabolic stress enabling it to bind to the heat shock element and activate transcription of the genes encoding stress proteins. The activation of the heat shock genes within myocardium is known to occur in response to brief periods of cardiac ischaemia in vivo.[6] In these instances it is thought that HSF activation is a consequence of intracellular ATP depletion.[11]

During ischaemia, substrate deprivation and metabolite accumulation results in a significant breakdown of adenosine triphosphate (ATP), causing the accumulation of interstitial adenosine that gives rise to the sensation of angina.[12] In addition, animal studies indicate that the cellular ATP content remains low for many hours after a brief period of ischaemia—hence periods of ischaemia as short as five minutes are sufficient to trigger hsp gene activation in animal studies. The obvious question is, does protection follow episodes of angina in patients?

Myocardial adaptation

It is interesting to speculate whether myocardial adaptation, perhaps by hsp70i induction, follows an episode of sublethal ischaemia or angina in humans. A history of angina for at least seven days before an acute myocardial infarction seems to predict a less complicated in-hospital course and reduced mortality.[13] This observation is complicated by differences that may exist between symptomatic and non-symptomatic patients, particularly in terms of collateral vessel formation and concomitant medication. However, a recent analysis of a large and well documented thrombolysis trial database controlled for these variables and found that the protective benefits of a 48 hour history of angina before infarction reduced mortality independently of any of the standard predictors of outcome.[14] The magnitude of the advantage associated with preinfarction angina is substantial. For example the absolute risk of death in anterior infarction without antecedent angina can be increased as much as twofold,[15] with the enzyme derived myocardial damage similarly increased by a factor of 1·5–2.[14 15] In these studies the temporal relation between angina and infarction is more consistent with protection by the upregulation of cytoprotective proteins than with classic preconditioning.

Therapeutic implications

Thus there is compelling evidence from animal studies that stress proteins, in particular hsp70, can increase the resistance of the myocardium to infarction. In addition, our understanding of the mechanisms regulating stress protein expression is increasing.

These factors are providing an impetus to manipulate the regulation of the genes encoding hsp70 to confer a clinical advantage. For example, agents could be developed which may be able to bypass the usual stress response and directly upregulate the 70 kDa stress proteins without concomitant cell damage, thus providing a pharmacological route to cytoprotection.[16] The critical question is whether these protective mechanisms are already operative in patients with acute myocardial infarction, and, if so, are they amenable to further manipulation?

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Angiotensin-Converting Enzyme Inhibitors Potentiate Preconditioning Through Bradykinin B_2 Receptor Activation in Human Heart

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Objectives. This study was designed to determine whether angiotensin-converting enzyme (ACE) inhibitors play a role in cardioprotection in a human model of preconditioning.

Background. Recent studies have suggested that bradykinin may contribute to the protective effects of preconditioning in animal models. ACE inhibitors are known to inhibit the degradation of bradykinin and hence may be able to potentiate the effect of preconditioning.

Methods. We examined the effects of the ACE inhibitors captopril and lisinopril in combination with a subthreshold preconditioning stimulus (i.e., insufficient to have any protective effects alone). Human atrial trabeculae were superfused with Krebs buffer and paced at 1 Hz. They were subjected to a full or subthreshold preconditioning stimulus consisting of either 3 or 1.5 min of simulated ischemia and 7 min of reoxygenation. In each instance, this stimulus was followed by 90 min of simulated ischemia and 2 h of reoxygenation. In addition, the subthreshold preconditioned group had 20 min of previous ACE inhibitor treatment.

Results. Recovery of contractile function (percent of baseline) was 22 ± 1% (mean ± SEM) in the control group versus 61 ± 1% in the preconditioned group. The subthreshold preconditioned group and the ACE inhibitor-alone groups did not exhibit any protection; however, in combination, the protection was significant (71 ± 4% in the captopril group, 58 ± 8% in the lisinopril group, $p < 0.005$) compared with the control group. There was no significant difference between these values and recovery after the full preconditioning stimulus. Furthermore, Hoe 140, a specific bradykinin B_2 receptor antagonist, abolished the protection.

Conclusions. To our knowledge, these are the first results in human muscle to suggest that ACE inhibitors may augment ischemic preconditioning, possibly through B_2 receptor activation.

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The introduction of angiotensin-converting enzyme (ACE) inhibitor therapy has had an enormous impact on clinical practice, far beyond initial expectations when first introduced as antihypertensive agents. As well as their use in the management of heart failure, they are now being implicated as myocardial and vascular "protective" agents that not only prevent left ventricular remodeling, but also reduce myocardial ischemic events. These anti-ischemic and antiremodeling effects were somewhat unexpected, and the exact mechanisms involved are uncertain. Large trials, such as the Studies of Left Ventricular Dysfunction (SOLVD) (1) and Survival and Ventricular Enlargement (SAVE) (2) trials, have shown a significant effect of ACE inhibitors on the prevention of myocardial ischemic events. More recent evidence comes from trials such as Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico III (GISSI-3) (3), in which the lisinopril-treated group had a significant reduction in early mortality (6 weeks), and Fourth International Study of Infarct Survival (ISIS-4) (4), which showed a 7% decrease in 5-week mortality in the captopril group.

There is accumulating evidence for a direct protective role for ACE inhibitors in the ischemic/reperfused myocardium (5–9), although the published reports are conflicting. Experiments to date have largely demonstrated this protective effect if the ACE inhibitor is given as pretreatment before infarction. It was proposed that ACE inhibitors protect in this manner by inhibiting the breakdown of bradykinin (9) because Hoe 140 has been shown to reverse the protective effect (6,7). A recent study by Miki et al. (10), showed that captopril potentiates the infarct size–limiting effect of ischemic preconditioning through bradykinin B_2 receptors in a rabbit model.

Ischemic preconditioning is another phenomenon that offers the myocardium an extremely powerful means of self-protection. During ischemia, locally produced mediators, such as adenosine, catecholamines and bradykinin, may act as triggers of preconditioning, in which resistance to ischemic injury is enhanced by previous exposure to a brief episode of ischemia followed by reperfusion. The importance of these mediators requires further elucidation, although the involvement of the adenosine receptor in the human heart was previously confirmed by our group (11). The question remains, Is there a role for some of the other putative mediators of preconditioning in the human heart?

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Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
K_{ATP} = ATP-dependent potassium channel
PKC = protein kinase C

There is now increasing awareness that ACE inhibitor activity is mediated in part through increased local bradykinin levels. Intracardiac production of bradykinin has been shown to be increased in myocardial ischemia (8,12,13), and, furthermore, recent reports suggest that infarct size limitation in the rabbit is blocked by the B_2 receptor antagonist Hoe 140 and mimicked by kinin administration (7).

It has also been reported that direct intracoronary infusion of bradykinin can reduce infarct size in dogs (14) and also mimic preconditioning by reducing the severity of ischemia-induced arrhythmias (5). These effects have been proposed to be mediated through prostacyclin or nitric oxide, or both, and increasing cyclic guanosine monophosphate.

We recently developed a model of preconditioning using isolated superfused isometrically contracting human atrial trabeculae. This model has the advantage of avoiding complications of collateral flow, and the mix of necrotic and viable tissue within each specimen is comparable to the situation in evolving acute myocardial infarction. Previous studies have established that in this model, the protective effects of preconditioning can be induced by activation of adenosine A_1 receptors (11) and that protein kinase C (PKC) and the activation of the ATP-dependent potassium channel (K_{ATP}) are also involved (15), confirming results obtained in animal studies.

The aim of the present study was to determine whether ACE inhibitors, (with and without sulfhydryl groups) could contribute to the protective effects of preconditioning. Furthermore, using a specific B_2 receptor antagonist, Hoe 140, our aim was to determine whether any protective effect observed is related to enhanced bradykinin B_2 receptor activation after reduced kinin degradation.

Methods

Experimental model. Experiments were performed in trabeculae derived from human atrium. Specimens of right atrial appendage were obtained from the right atrial cannulation site in patients undergoing coronary artery bypass grafting (46 patients, 37 men, 9 women; 39 to 79 years old, mean age 64). All patients had chronic stable angina, and those with right ventricular failure or atrial arrhythmias or taking antiarrhythmic medication were excluded. Patients taking oral hypoglycemic agents and those receiving ACE inhibitors were also excluded. Ethical approval for this procedure was obtained from the hospital ethics committee. The specimens were transported to the laboratory in oxygenated modified Tyrode's solution.

Atrial trabeculae (diameter 0.9 mm, length ≥ 3 mm) were dissected under magnification in a dish superfused with Tyrode's solution, tied at one end with a 7/0 silk suture and removed together with a small portion of atrial wall at the free end. The silk suture was then used to attach the muscle to a fixed post in an organ bath while the free end was attached to a force transducer (Gould Statham UCT 2) using a snare around the wedge of atrial wall. The muscles were suspended horizontally in the organ bath through which there was a continuous flow of superfusate oxygenated with a 95% O_2/5% CO_2 gas mixture. The superfusate was a modified Tyrode's solution of the following composition (mmol/liter): NaCl 118.5, KCl 4.8, $NaHCO_3$ 24.8, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.44, $CaCl_2 \cdot 2H_2O$ 1.8, glucose 10.0 and pyruvic acid 10.0. In the substrate-free Tyrode's solution, 7 mmol/liter choline chloride was substituted for glucose and pyruvic acid to maintain constant osmolarity. All reagents were AnalaR grade from BDH Chemicals, Poole, England, except for pyruvic acid from Sigma Chemicals. The organ bath was covered to prevent gas exchange with the atmosphere. Gas tensions in the organ bath were analyzed intermittently using an automated blood gas analyzer (AVL 993, AVL Medical Instruments, Switzerland).

The pH was maintained between 7.35 and 7.45, the partial pressure of O_2 between 50 and 60 kPa and partial pressure of CO_2 between 4.0 and 6.0 kPa. During simulated ischemia, the superfusate was free of substrate and was bubbled with 95% N_2/5% CO_2 to lower the partial pressure O_2 in the organ bath to 6 to 8 kPa. (pH 7.24 to 7.34). The organ bath and preorgan bath heat exchanger were both water jacketed and circulated (Techne circulator C 85-A, Cambridge, UK) to maintain a constant temperature of 37.0 ± 0.1°C, which was monitored through a thermocouple in the bath. The length and width of the trabecular muscles were recorded at the end of the experiment using an eyepiece graticule in an overhead microscope (Prior, UK), and all specimens were then weighed. The cross-sectional area was calculated by dividing muscle mass by length times density, assuming a cylindric shape and a density of 1.0 mg/mm^3. To ensure that initial comparisons of developed tensions and rest force were not affected by variable muscle size, cross-sectional area of the muscles was used to calculate tensions. In addition, all muscles with a cross-sectional area >1.2 mm^2 were excluded from the study (16).

Muscle stimulation and recording of data. Once horizontally suspended, trabeculae were paced by field stimulation at 1 Hz by parallel flattened platinum electrodes with an isolated stimulator (Digitimer DS2, Hertfordshire, UK) triggered by a programmable computerized clock. The pulse width was fixed at 5 ms and the pulse amplitude set at twice threshold (6 to 8 mV). Contractile force was amplified and recorded on paper (Universal Amplifier and RS3400 ink pen recorder, Gould).

Chemicals. Captopril and lisinopril were obtained from Sigma. Captopril was dissolved in normal saline and added to Tyrode's solution to give a final concentration of 10 mmol/liter, and lisinopril was similarly dissolved to give a final concentra-

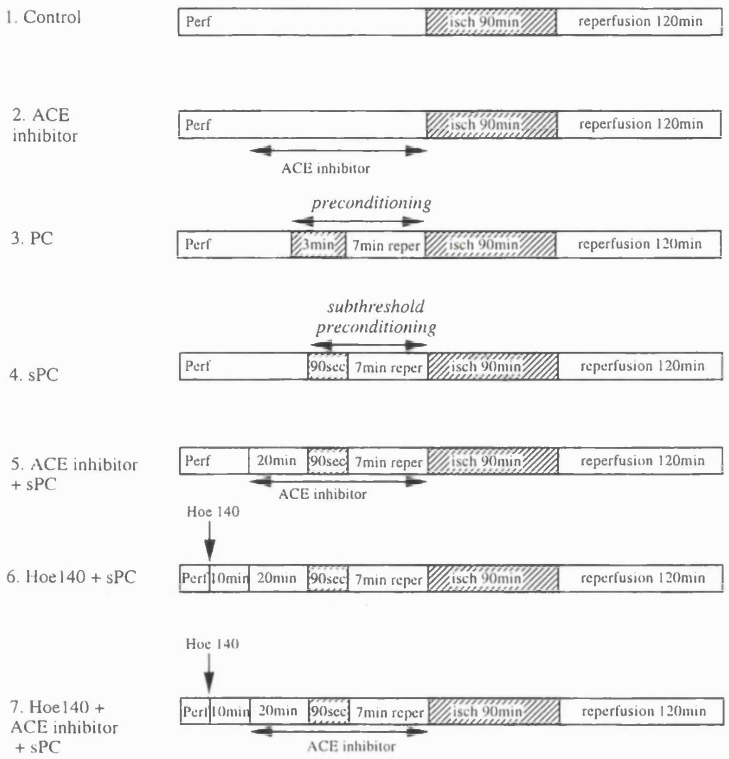


Figure 1. Experimental protocols (n = 6 for all groups). All protocols were preceded by at least a 45-min stabilization period (Perf). **Hatched areas** = ischemia; isch = superfusion with hypoxic substrate-free Tyrode's solution and pacing at 3 Hz; PC = preconditioning; reper = reperfusion with oxygenated Tyrode's solution and pacing at 1 Hz; sPC = subthreshold preconditioning.

tion of 20 μmol/liter—concentrations comparable to plasma levels in patients taking these particular ACE inhibitors (17). Hoe 140 was diluted to a final concentration of 20 nmol/liter, a concentration known to be sufficient to effectively antagonize B_2 receptors.

Experimental protocols. Trabeculae were initially stimulated at 1 Hz unstretched for 30 min to allow time for recovery. They were subsequently stretched in a stepwise manner over 15 min to a length developing 90% of maximal force and allowed to equilibrate for a further 30 min. All groups eventually underwent a period of simulated ischemia that consisted of 90 min of hypoxic substrate-free superfusion and rapid pacing at 3 Hz, followed by reperfusion for 120 min with oxygenated Tyrode's solution and pacing of trabeculae at 1 Hz. The preconditioning protocol consisted of 3 min of hypoxic substrate-free superfusion with trabeculae paced at 3 Hz, followed by 7 min of reperfusion with oxygenated Tyrode's solution with substrate and pacing at 1 Hz. A subthreshold preconditioning protocol was established that failed to protect alone. This protocol consisted of 90 s of pacing at 3 Hz with hypoxic substrate-free perfusate and was also followed by 7 min of reperfusion with oxygenated Tyrode's solution. Figure 1 shows the experimental protocols for the 10 groups, all of which were finally subjected to 90 min of simulated ischemia: 1) *Control protocol* = stabilization period followed by 90 min of ischemia; 2) *ACE inhibitor protocol* = superfusion with captopril or lisinopril for 20 min before 90 min of ischemia; 3) *preconditioning protocol* = preconditioning followed by 90 min of ischemia; 4) *subthreshold preconditioning protocol* = subthreshold preconditioning followed by ischemia; 5) *ACE inhibitor plus subthreshold preconditioning protocol* = 20 min of superfusion with captopril or lisinopril, followed by subthreshold preconditioning; 6) *Hoe 140 plus subthreshold preconditioning protocol* = superfusion with Hoe 140 for 20 min before subthreshold preconditioning; and 7) *Hoe 140 plus ACE inhibitor plus subthreshold preconditioning protocol* = superfusion with Hoe 140 for 10 min before addition of captopril or lisinopril for 20 min, followed by subthreshold preconditioning. For all these groups n = 6, including six experiments for each ACE inhibitor, with a maximum of two trabeculae from each heart.

Statistical analysis. Results are presented as mean value ± SEM. Statistical differences between groups were evaluated by two-way analysis of variance followed by a Fisher protected least significant difference post hoc test with respect to treatment and time; $p \leq 0.05$ was considered significant.

Table 1. Physical Characteristics and Baseline Functional Data of Trabeculae Study Groups

Group	Length (mm)	Mass (g)	Diameter (mm)	Rest Force (g)	Developed Force (g)
Control	4.4 ± 0.3	0.006 ± 0.001	0.71 ± 0.04	0.65 ± 0.11	1.20 ± 0.16
Cap	4.5 ± 0.3	0.006 ± 0.001	0.73 ± 0.04	0.77 ± 0.10	1.51 ± 0.18
Lis	4.9 ± 0.5	0.005 ± 0.001	0.77 ± 0.06	0.70 ± 0.09	1.43 ± 0.13
PC	4.2 ± 0.3	0.005 ± 0.001	0.71 ± 0.05	0.71 ± 0.10	1.38 ± 0.19
sPC	4.5 ± 0.4	0.005 ± 0.001	0.69 ± 0.05	0.78 ± 0.10	1.35 ± 0.20
Cap+sPC	5.0 ± 0.4	0.006 ± 0.001	0.71 ± 0.05	0.77 ± 0.11	1.26 ± 0.21
Lis+sPC	4.7 ± 0.3	0.006 ± 0.001	0.79 ± 0.02	0.66 ± 0.11	1.19 ± 0.21
Hoe+sPC	4.6 ± 0.4	0.005 ± 0.001	0.69 ± 0.05	0.68 ± 0.12	1.28 ± 0.17
Hoe+Cap+sPC	4.5 ± 0.3	0.006 ± 0.001	0.68 ± 0.06	0.75 ± 0.12	1.47 ± 0.22
Hoe+Lis+sPC	4.7 ± 0.4	0.006 ± 0.001	0.71 ± 0.05	0.67 ± 0.13	1.48 ± 0.19

Data presented are mean value ± SEM. Cap = captopril; Hoe = Hoc 140; Lis = lisinopril; PC = preconditioning; sPC = subthreshold preconditioning.

Results

Exclusions. Three muscles were excluded because of a calculated cross-sectional area >1.2 mm^2. There were no other exclusions, and all samples completed the protocol to which they were randomly assigned.

Analysis of data. The physical characteristics of the trabeculae and baseline functional data were similar in all 10 groups (Table 1), including rest and developed force. Therefore, experimental data on developed force are presented graphically as percent of baseline developed force.

The addition of an ACE inhibitor at the beginning of the experiment led to a minor, but nonsignificant, reduction in developed force. The 3-min or 90-s preconditioning protocols resulted in a significant reduction in developed force that recovered to baseline on reperfusion before the long ischemic insult.

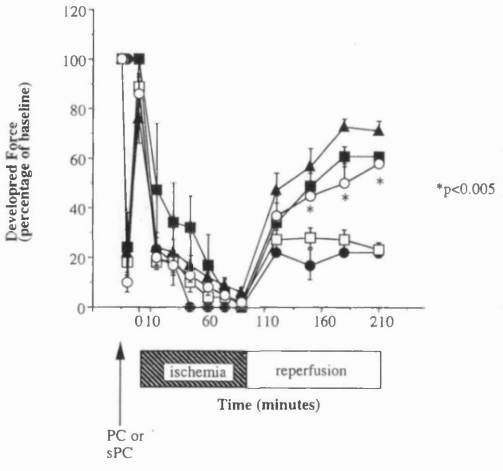
Figures 2 to 5 show the results for developed force as a percent of baseline. Time 0 indicates the beginning of simulated ischemia; therefore, reperfusion with oxygenated Tyrode's solution extends from 90 to 210 min. As indicated in the diagram of the protocols (Fig. 1), simulated ischemia is preceded in all groups by a stabilization period and various pretreatments (indicated on the graphs by an arrow). As noted, there was no significant difference in developed force between the 10 groups at the end of the stabilization period, and this baseline function is denoted as 100% developed force.

Figure 2 illustrates the synergistic effect of pretreatment with an ACE inhibitor in combination with a subthreshold preconditioning stimulus. The level of protection observed is of a similar magnitude to that obtained with the standard 3-min ischemic preconditioning protocol, although captopril was significantly more protective than lisinopril at the last two time points. Recovery was 22 ± 1% in the control group compared with 61 ± 1% in the preconditioned group, 71 ± 4% in the captopril plus subthreshold preconditioned group and 58 ± 8% in the lisinopril plus subthreshold preconditioned group (at the end of reperfusion, i.e., 210 min). There was no protection in the group receiving subthreshold ischemic preconditioning alone (23 ± 4%); this group had a level of recovery similar to that of the control group (no pretreatment).

ACE inhibitor pretreatment alone had no protective effects in contrast to the marked protection observed when combined with subthreshold preconditioning, as seen in Figure 3. In addition, Hoe 140 pretreatment alone did not adversely affect recovery compared with that in the control group, with a maximal recovery in this group of 20 ± 5%.

Figures 4 and 5 illustrate the effects of pretreatment with the B_2 receptor antagonist Hoe 140 on the captopril- and

Figure 2. Developed force expressed as percent of baseline force over time (min), illustrating that the combination of an ACE inhibitor with subthreshold preconditioning (sPC) results in a similar degree of recovery in the group receiving the full preconditioning protocol (PC [**solid squares**]). Recovery of the subthreshold preconditioning-alone group (**open squares**) is not statistically significant from control group (**solid circles**). **Solid triangles** = captopril plus subthreshold preconditioning; **open circles** = lisinopril plus subthreshold preconditioning. *p < 0.005 versus control and subthreshold preconditioning.

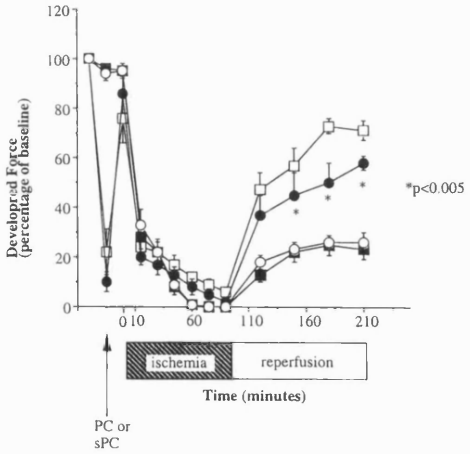


lisinopril-treated groups, respectively. In Figure 4 it can be seen that Hoe 140 clearly abrogates the protective effects of captopril in combination with subthreshold preconditioning, with a functional recovery of 35 ± 5%. However, there is some residual protective effect observed, although this was not significant. Figure 5 also shows that Hoe 140 abrogates the protective effects of lisinopril in combination with subthreshold ischemic preconditioning (31 ± 5%).

Discussion

Study findings. This study shows that ACE inhibition in combination with a subthreshold preconditioning stimulus resulted in a significant degree of protection, in terms of recovery of function, when the trabeculae were subjected to a subsequent 90-min period of simulated ischemia. The degree of protection provided by combined subthreshold preconditioning and ACE inhibition is of a comparable degree to that after a full ischemic preconditioning protocol. Furthermore, the cardioprotective effect was abolished by pretreatment with the B_2 receptor antagonist Hoe 140. These results support the hypothesis that generation of bradykinin during preconditioning contributes, through B_2 receptor activation, to the protection observed in terms of improved postischemic functional recovery. However, pretreatment with ACE inhibitor alone did not result in protection, perhaps because a degree of ischemia is required to release bradykinin initially, or other mediators, such as adenosine, are required in addition to trigger preconditioning.

Figure 3. Developed force expressed as percent of baseline force over time (min), comparing the combination of ACE inhibitor plus subthreshold preconditioning (sPC) with ACE inhibitor alone. The percent of recovery in the ACE inhibitor plus subthreshold preconditioning groups is significantly greater than that in the ACE inhibitor-alone groups (*p < 0.005). **Open circles** = lisinopril; **solid circles** = lisinopril plus subthreshold preconditioning; **solid squares** = captopril; **open squares** = captopril plus subthreshold preconditioning.



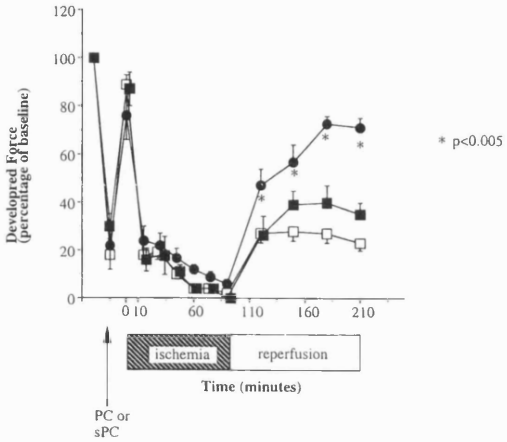


Figure 4. Developed force expressed as percent of baseline force over time (min), illustrating that pretreatment with Hoe 140 abrogates the protective effect of captopril in combination with subthreshold preconditioning (sPC) (**solid circles**) (*p < 0.005). Recovery of the Hoe 140-pretreated group was not statistically significant from that for the control group. **Solid squares** = Hoe 140 plus captopril plus subthreshold preconditioning; **open squares** = subthreshold preconditioning; PC = preconditioning.

There are several clinical studies suggesting that preconditioning occurs in humans (18–20), with evidence from in vitro studies using isolated human ventricular cardiomyocytes that were protected against a 90-min period of simulated ischemia by a preconditioning protocol (21). The other studies are from our own group (11,15) using the same model as that in the present study. These studies have demonstrated that preconditioning of human atrial trabeculae results in a significantly better postischemic recovery of contractile function than nonpreconditioned trabeculae and, in common with animal models of preconditioning, have confirmed a role for adenosine receptors and PKC (11,15).

Human atrial model. The present study used isolated human atrial trabeculae to examine the mechanism of preconditioning in human myocardium. Atrial specimens make stable preparations, and sampling was part of the routine procedure for coronary artery bypass graft surgery. However, there are differences between atrial and ventricular tissue, and the results from one may not be applicable to the other.

The present study used a period of hypoxic superfusion in combination with rapid pacing to simulate ischemia rather than the "true" ischemia of ischemic preconditioning. However, there is a great deal of evidence (22–25) in animal models of both regional and global hypoxia and in cell cultures that hypoxia is as effective as ischemia in inducing preconditioning. We know that preconditioning attenuates contractile dysfunction as well as reducing infarct size. For example, Jenkins et al. (26), who measured both infarct volume and functional recovery after ischemia, showed that the improved recovery of

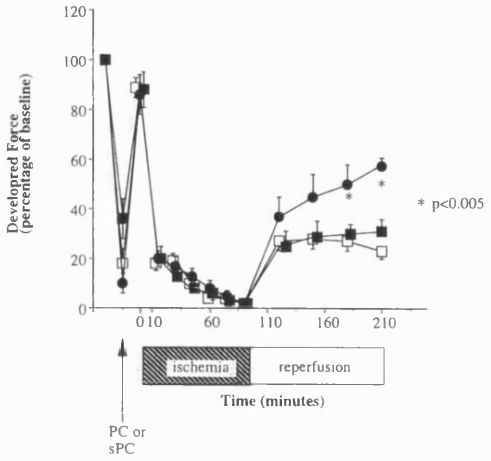


Figure 5. Developed force expressed as percent of baseline force over time (min), illustrating that pretreatment with Hoe 140 abrogates the protective effect of lisinopril in combination with subthreshold preconditioning (sPC) (**solid circles**) (*$p < 0.005$). **Solid squares** = Hoe 140 plus lisinopril plus subthreshold preconditioning; **open squares** = subthreshold preconditioning; PC = preconditioning.

global left ventricular function produced by preconditioning is proportional to a reduction in infarction, and Cohen et al. (27) and Przyklenk et al. (28) were able to correlate improved recovery of systolic shortening with reduced infarct size in in vivo models of regional ischemia. Clearly, these studies demonstrate that the enhanced recovery of contractile function due to preconditioning that follows a prolonged period of ischemia results from a reduction in infarct size and is not due to a reduction in stunning. Our model used a 90-min period of simulated ischemia as the ischemic insult, a period more likely to result in cell death than stunning, which is induced by shorter periods of 5 to 15 min of ischemia. Hence, there is evidence that hypoxia can be as effective as ischemia in preconditioning, that recovery of contractile function is a good correlate for the degree of myocardial necrosis and that this experimental model is representative of the preconditioning phenomenon.

Role of bradykinin. Although we did not measure kinin levels in our samples, Hoe 140 is the most specific and potent B_2 receptor antagonist currently known. It has virtually the same affinity for B_2 receptors as bradykinin itself and does not react with receptors for other peptides. Furthermore, kinin release from ischemic myocardium has been well documented in the isolated rat heart in vitro (13,29) and the canine myocardium in vivo (30,31). Baumgarten et al. (13) showed that kinin release after 15 min of coronary occlusion was enhanced by pretreatment with an ACE inhibitor, ramiprilat, in isolated rat hearts. Furthermore, Nolly et al. (32) demonstrated that the heart contains an independent kallikrein–kinin system. They demonstrated the presence of kallikrein (a potent kinin-generating enzyme) mRNA in rat atria and ventricles and kallikrein activity in primary cultures of neonatal rat atrial and ventricular cardiocytes and in their incubation medium. Heart slices were shown to release kallikrein without depletion of total tissue kallikrein, suggesting pool replenishment. In fact, kininogen was found in epicardial slices and in the Krebs solution bathing the slices (which were washed and reincubated in Krebs solution)—and this release was inhibited by pretreatment with puromycin—suggesting primary synthesis. Presumably, ischemia results in pH and other changes that cause activation of kallikrein within the myocardium and the subsequent local availability of bradykinin (33). Hence, this work supports the hypothesis that local production of kinin contributed to the protection observed.

Relevance of the sulfhydryl moiety. Protection was observed for both a sulfhydryl-containing ACE inhibitor, captopril, and a non–sulfhydryl-containing one, lisinopril. However, for the last two time points, recovery in the captopril group was significantly greater than that in the lisinopril group, although both ACE inhibitors in combination with subthreshold preconditioning provided significant protection from 150 min of reperfusion onward. The greater beneficial effect of captopril over lisinopril could theoretically be due to the free radical scavenging ability conferred by the presence of the —SH moiety. Another mechanism by which ACE inhibitors may provide protection is through activation of the K_{ATP} channel. Sargent et al. (34) found that the sulfhydryl-containing ACE inhibitor zofenopril induced cardioprotection through modulation of the K_{ATP} channel. Indeed, the cardioprotection observed in ischemic rat hearts with zofenopril was abolished by the K_{ATP} blockers glyburide and 5-hydroxydecanoate.

Our results show that ACE inhibition alone is insufficient to confer any protection after 90 min of ischemia. This result is consistent with previously published studies (35–38). However, the situation is not clear-cut, and there are some reports of infarct size limitation by the same agents (6,39). Our results are compatible with a current concept of preconditioning as a phenomenon that may have several different endogenous mediators, such as adenosine and bradykinin, that stimulate PKC to a certain threshold sufficient to trigger the intracellular pathways necessary for providing protection against a further ischemic insult (33). Recently, Goto et al. (33) found that Hoe 140 blocked the protection from one 5-min period of preconditioning but could not block protection when a more profound preconditioning stimulus was used in a rabbit model. Hence, it is possible that the adenosine and bradykinin released during the subthreshold ischemia is insufficient to trigger protection unless the level of bradykinin is augmented by ACE inhibition, as illustrated in Figure 6.

It is possible that the interaction between adenosine and bradykinin may not simply be additive, but a study (40) in smooth muscle cells found that stimulation of adenosine A_1 and bradykinin receptors results in a synergistic increase in inositol 1,4,5,-triphosphate and free calcium levels.

Intracellular signaling pathways. The present study does not address the issue of the cardioprotective mechanism

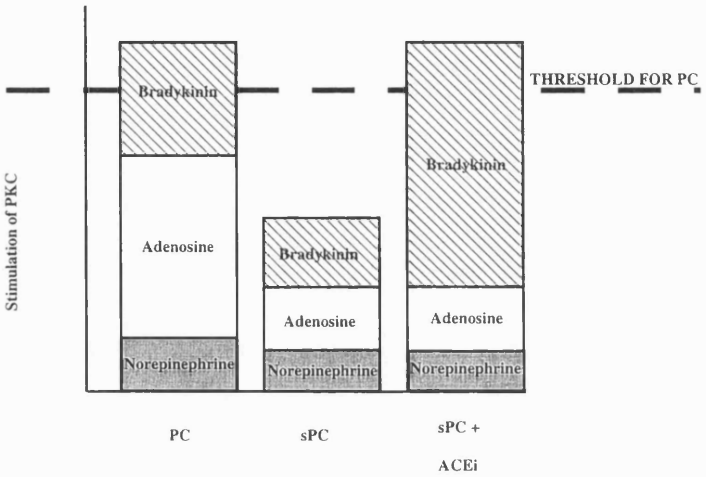


Figure 6. Summary depicting putative mediators involved in triggering PKC in ischemic preconditioning (PC), ultimately leading to myocardial protection. ACEi = ACE inhibitor; sPC = subthreshold preconditioning. Adapted, with permission, from Goto et al. (33).

downstream to activation of the B_2 receptor. This receptor is coupled to phospholipases A_2 and C through G proteins (41,42), and stimulation of this receptor leads to the production of two important vasoactive substances—prostacyclin and nitric oxide. However, neither cyclooxygenase inhibitors (43) nor the nitric oxide synthase inhibitor nitro-L-arginine methyl ester (12,44) attenuated infarct size limitation by preconditioning in a rabbit model of infarction. In contrast Goto et al. (12) demonstrated that polymyxin B, a blocker of PKC, abolished infarct size limitation by treatment with a kinin. Furthermore, it was recently observed (45) that B_2 receptor stimulation caused significant inositol triphosphate production in isolated cardiac myocytes, suggesting that simultaneously generated diacylglycerol could activate PKC. Hence, these observations suggest that PKC activation plays a role in bradykinin-mediated protection.

Role of angiotensin II. The present study does not take into account the simultaneous suppression of angiotensin II production, which has been reported in some recent studies (46) to contribute to ischemic preconditioning; and angiotensin II receptor activation can mimic preconditioning (47). Hence, the effect of ACE inhibition in the reduction of angiotensin II levels would tend to lead to an underestimation of the protective effect observed with respect to bradykinin because some of the angiotensin II–mediated component could theoretically be reduced. In terms of implications for future therapy, development of a specific kininase inhibitor without the ACE inhibitory component could be valuable in this setting. Furthermore, agents such as angiotensin II receptor antagonists would not have this bradykinin-potentiating effect.

Conclusions. We demonstrated in a human model that ACE inhibitors can potentiate the effects of a subthreshold preconditioning stimulus and that B_2 bradykinin receptors may play a role in this protection. To our knowledge, this is the first report to allude to a potential role for bradykinin as a mediator in human preconditioning, giving a possible clue to the mechanisms involved in the reduction of fatal ischemic events in patients treated with ACE inhibitors observed in the multicenter trials.

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