Apelin a cardioprotective adipocytokine

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Abstract

The epidemic of obesity has led to increased interest in its role in the pathogenesis of cardiometabolic disease. Adipose tissue, formerly regarded as purely an energy storage site, is now regarded as an important endocrine organ. It produces various peptide hormones, including the adipocytokines which are implicated in metabolic control and disease. Whilst some adipocytokines may contribute to the development of cardiovascular disease, others e.g. adiponectin, may protect against it.

The recently identified ligand for the G protein coupled receptor APJ, apelin, is a unique vasoactive adipocytokine. Both apelin and APJ mRNA are highly expressed in the cardiovascular system. Apelin has been found to modulate cardiovascular function, fluid homeostasis and inflammation. To date, however, apelin has not been investigated in the context of ischemia-reperfusion and its benefits in this clinical setting are not yet established.

APJ/apelin activates the cell survival cascades Akt/PKB and ERK-1/2 which are associated with the pro-survival Reperfusion Injury Salvage Kinase (RISK) pathway. Apelin also promotes mitogenesis, a feature commonly exhibited by cardioprotective agents. We, therefore, hypothesised that apelin may protect the heart via the RISK pathway in an ischemia/reperfusion (I/R) model. We investigated if apelin has potential as a cardioprotective agent employing murine models of ischemia-reperfusion injury and rat cardiomyocytes, in which mitochondrial permeability transition pore (mPTP) opening was examined. Apelin-13 was found to produce a concentration-dependent decrease in infarct size with a maximal effect being observed at 1000nM. The physiologically less active peptide, apelin-36, also reduced infarct size but to lesser extents than seen with the shorter isoform. LY294002 and UO126, inhibitors of the PI3K-Akt, p44/42, abolished the effects of apelin-13. Further evidence for the involvement of these pathways in the cardioprotective actions of apelin was obtained on Western blot analysis. Apelin-13 delayed mPTP opening which was blocked by LY294002 and

MEK inhibitor 1, an alternative inhibitor of p44/42. This is the first study to demonstrate that apelin has a direct cardioprotective action involving the PI3K-Akt, and p44/42 signalling pathways.

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1 INTRODUCTION

1.1 Ischaemic Heart Disease

Cardiovascular disease is the main cause of death in the United Kingdom, accounting for just over 216,000 deaths in 2004; 4 out of every 10 deaths. There are approximately 87,000 myocardial infarctions a year in the UK. Data from the Oxford Myocardial Infarction Incidence Study (OXMIS; Volmink et al. 1998) shows that of those that die within 28 days of myocardial infarction, three quarters die within the first 24 hours. Methods to restore blood flow to ischaemic areas and protect the myocardium are vital if we are to prevent further damage and improve survival.

Myocardial infarction is part of the acute coronary syndrome, which describes the spectrum of clinical manifestations that result from coronary thrombosis (Fox et al. 2004). The word *infarction* comes from the Latin *infarcire* meaning "to plug up, to stuff" or "to cram" and refers to the clogging, or plugging, of the artery. Myocardial infarction during a “heart attack” can be a pathophysiological process in which an atheromatous plaque becomes eroded or ruptures. This activates a chain of events which culminates in intraluminal thrombosis, causing partial or complete obstruction of the vessel (see Figure 1.1)

The clinical manifestations of the acute coronary syndrome caused by partial or complete occlusion can range from transmural myocardial infarction, in which the necrotic area includes the entire thickness of the ventricular wall, through to smaller subendocardial myocardial infarction and unstable angina, where the platelet thrombi form and then break up. This instability is usually the prelude to acute myocardial infarction, and indicates that therapy needs to be instigated urgently to avoid further progression.

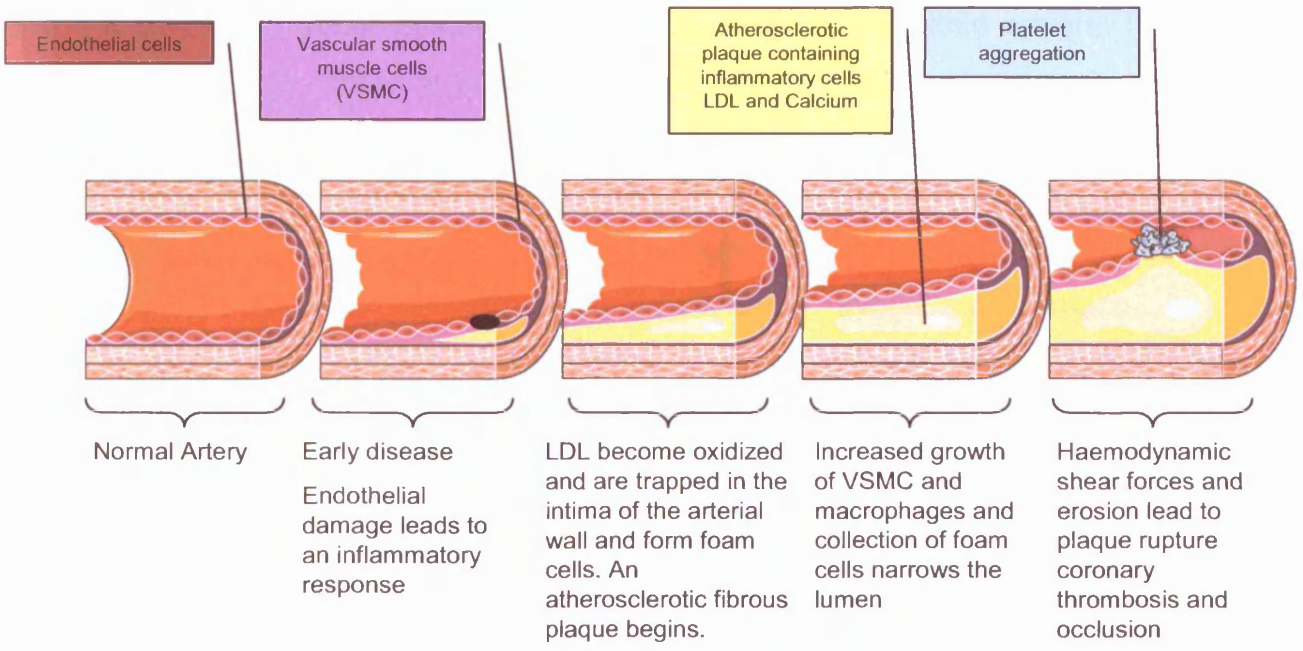

Figure 1-1 The Atherosclerotic process

Atherosclerosis is an inflammatory response to endothelial dysfunction. Primary risk factors lead to endothelial dysfunction and the formation of an atherosclerotic plaque. Endothelial permeability leads to infiltration of inflammatory cytokines and harmful low density lipoprotein (LDL) cholesterol. With time the plaque grows, fat and other inflammatory products accumulate until stress on the weak calcium cap leads to its rupture. This ultimately leads to luminal occlusion and ischaemia i.e. myocardial or cerebral infarction.

The severity of the clinical manifestation is dependent on the severity of the ischaemia and the amount of myocardium affected. Partial occlusion may lead to ischaemia without cell death, whereas complete occlusion will lead to extensive myocyte necrosis if not treated promptly. The lack of blood flow to the myocardium leads to dysfunction of normal oxidative metabolism, ending in the eventual death of the myocyte.

The magnitude of the myocardial infarction or more specifically the number of dead cardiomyocytes is a vital factor in determining outcome and long-term myocardial function (Takemura & Fujiwara 2004). Loss of cardiomyocytes during either the acute or chronic stage of myocardial infarction directly contributes to contractile dysfunction. The

ultimate size of an acute infarct, which can be determined within several hours of its onset (Reimer et al. 1993), is the most critical determinant of subsequent heart failure. Large myocardial infarctions lead to severe chronic heart failure due to unfavourable remodelling of the left ventricle that is characterized by ventricular dilation and reduced cardiac performance (Pfeffer et al. 1995). The survival of the cardiomyocyte is therefore the key factor in reducing mortality and morbidity after myocardial infarction. The time frame for irreversible damage is highly variable. In experimental models of myocardial infarction cell death begins after 15-40 minutes of total ischaemia (Reimer et al. 1977), (Connelly et al. 1982). In the presence of collateral flow and low oxygen requirement, however, the point of irreversibility can be extended for some hours (Schaper et al. 1987). A key question therefore is when does cell injury become irreversible? Then having identified this point the question to be asked is can the pathological changes occurring at this time be modulated so as to prevent further cell death?

1.2 Cardiac Protection

Research into the mechanisms of cell death has led to novel strategies to promote cell survival and reduce further necrosis (Gill et al. 2002). The time at which these interventions may be used, has a major influence on its extrapolation to the clinical setting. Therapies which can be given prior to ischaemia have the benefit of acting before serious damage has occurred, promoting protection and reducing the amount of cell death.

One of the most powerful protective treatments of this type is preconditioning (Murry et al. 1986). Preconditioning involves subjecting the heart to brief periods of sub-lethal ischaemia, rendering it more resistant to a subsequent period of lethal ischaemia (Sanada et al. 2004). First demonstrated in a canine model, two cycles of five minutes coronary occlusion with five minutes reperfusion, followed by a 40 minute sustained occlusion and subsequent reperfusion, resulted in smaller infarcts, equivalent to 25% of the infarct size obtained in the control group (Murry et al. 1986). It was shown that the reduction in infarct size was retained after four days of reperfusion. The caveat to this is that in the clinical setting prior knowledge of the exact time at which ischaemia will occur

is not possible (i.e. knowing when someone is going to have a myocardial infarction) and therefore the benefit of pre-treatment is lost. Therapies which are given at the time of ischaemia are more likely to have a practical role in clinical medicine. This has led to the development of treatments which can be given after the ischaemic insult in order to reduce further cell death and promote cell survival.

This transition from ischaemia to reperfusion is an important time point with respect to cell survival. Cells which are in the ischaemic region start to swell, leading to membrane rupture and induction of the inflammatory response. The restoration of blood flow (reperfusion) following ischaemia is the ultimate goal in treating ischaemic tissue. Prompt revascularisation of ischaemic tissue reduces the total infarction (Reimer et al. 1977), and improves morbidity and mortality (GUSTO 1993). Paradoxically, however, this process may lead to more deleterious effects, such as haemorrhagic infarction, as irreversibly damaged myocytes are torn apart, releasing injurious inflammatory cytokines. It has therefore been concluded that the development of strategies to reduce this so called "lethal reperfusion injury" is of vital importance.

1.3 Mechanisms of Injury

1.3.1 Ischaemic Injury

The mechanisms whereby ischaemia leads to infarction and cell death are complex. During the initial stages of ischaemia there is a rapid transformation in the metabolism of the heart in order that it may survive the ischaemic insult. The initiation of anaerobic glycolysis and an increase in glucose transport helps to maintain the intracellular levels of ATP (Young et al. 1997). As ischaemia progresses contractile function decreases; this is an adaptive mechanism to reduce oxygen demand and conserve the under perfused myocardium (Vandenberg et al. 1993).

1.3.1.1 Necrosis

Lack of oxygen leads to depressed mitochondrial metabolism which results in decreased production of ATP and the accumulation of fatty acid metabolites. As these metabolites increase various aspects of membrane function start to fail. Anaerobic metabolism causes the accumulation of lactate and CO_2 (Cross et al. 1995, Guth et al. 1987) leading to a fall in pH and intracellular acidosis. To compensate for the increase in H^+, the Na^+/H^+ exchange pump is activated leading to increased cytosolic Na^+ (Klein et al. 2000). Inhibition of the Na^+ K^+ – ATPase pump as a result of the depletion of intracellular ATP during ischaemia then leads to the activation of the $Na+/Ca^{2+}$ exchanger, but in reverse mode (Cross et al 1995). This leads to reduced intracellular Na^+ at the expense of an increase in intracellular and mitochondrial Ca^{2+}. The rise in calcium causes damage to contractile proteins as it overstimulates actin-myosin contractile components. Myocardial hypercontracture ensues, causing membrane destruction, swelling of the cell and eventual rupture (Barry et al. 1987). This type of cell death, necrosis, was previously regarded as the only mode of cell death. However, it is now thought that there are other modes of cell death each contributing to eventual cardiomyocyte death.

1.3.1.2 Apoptosis

Apoptosis (from the Greek words apo = from and ptosis = falling) is thought to be a second mode of cell death and involves cellular self-destruction to facilitate the controlled removal of cells; programmed cell death (Walker et al. 1988). In contrast to necrosis, which is a form of cell death that results from acute cellular injury i.e. non-programmed cell death in which, the energy deprived cells literally explode, apoptosis is carried out in a systematic, controlled fashion that generally confers advantages during an organism's life cycle. The apoptotic process facilitates the safe dismantling of the cell and occurs without inflammation and scar formation (Chowdhury et al. 2006). It is associated with cell shrinkage, intracellular degradation of its contents and activation of phagocytosis by neighbouring cells (Nadal-Ginard et al. 2003). The "decision" for apoptosis can come from the cell itself, from its surrounding tissue or from a cell that is part of the immune system. The process of apoptosis is a complex cascade of signalling and enzymatic regulation (Chowdhury et al. 2006). This regulation allows apoptotic

signals to either culminate in cell death, or be aborted should the cell no longer need to die. A major difference between apoptosis and necrosis is that apoptosis is an energy dependent process (Chiarugi et al. 2006). Apoptosis requires ATP whereas necrosis can occur without it. Particular interest in the role of apoptosis in ischaemia-reperfusion injury has come from observations made with cardio-protective agents which act on regulatory apoptotic pathways. Thus inhibition of apoptotic pathways could prevent further cell death and hopefully improve survival (Haunstetter et al. 2000).

1.3.1.3 Autophagy

Autophagy (the term is derived from ancient Greek and means to 'eat oneself') is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery. It is characterised by the accumulation of autophagic vacuoles within the dying cell. These vacuoles, or autophagosomes, are double-membrane enclosed vesicles that direct the dismantling of cytoplasmic components, protein aggregates and expired intracellular organelles via lysosomal action (Clark PG 1990). It has been proposed that autophagy, resulting in the total destruction of the cell, represents another form of programmed cell death, although conclusive evidence for such a process has yet to be obtained (Tsujimoto Y, Shimizu S 2005). Nevertheless, cells possessing autophagic features have been reported to occur in areas undergoing programmed cell death leading to the coining of the term autophagic cell death (also known as cytoplasmic cell death or type II cell death) (Tsujimoto Y, Shimizu S 2005) . This process, in which cells undergo partial autodigestion prolong cell survival for a short time under starvation. Hence, this survival mechanism may result in the generation of nutrients that are necessary for maintaining cell viability (Tsujimoto Y, Shimizu S 2005). The investigation of autophagic death is still in its infancy and the development of specific methods for examining autophagic death in more detail, will aid in the further characterisation of cell death under physiological and pathological conditions.

1.3.2 Reperfusion

Reperfusion of ischaemic myocardium in the patient can be achieved by various means. Thrombolytic agents, such as streptokinase or tissue plasminogen activator, may be employed to lyse the coronary thrombosis (GUSTO 1993). Alternatively, mechanical procedures can be employed to bring about reperfusion such as angioplasty or coronary bypass surgery. These techniques are now felt to be superior to thrombolysis (Keeley, Boura, & Grines 2003) and, consequently their use, especially in the form of primary angioplasty, is growing. Complete reperfusion of the ischaemic area is the ultimate goal of all methods, but it is not without its complications.

1.3.2.1 Reperfusion injury

Reperfusion, for example can lead to arrhythmias, stunning, microvascular damage and further cell death (Bolli et al. 1999, Brooks et al. 1995, Meissner et al. 1995). The re-supply of oxygen and metabolites to ischaemic tissue leads to a sudden revivification. Reoxygenation of mitochondria leads to recovery of oxidative energy production, which, in turn, can lead to the development of reactive oxygen species (ROS). The oxygen radicals are generated by injured myocytes and endothelial cells in the ischaemic zone, and become activated on reperfusion (Kloner et al. 2001, Hearse et al. 1975). ROS exacerbate membrane damage which leads to calcium loading (Zeitz et al. 2002). The accumulation of neutrophils in the microcirculation causes the release of inflammatory mediators which contribute to microvascular obstruction (Maxwell et al. 1997, Park et al. 1999, Ross et al. 1999). This paradoxical situation in which the need to reperfuse ischaemic tissue in order to avoid further cell damage, and the syndrome of reperfusion injury (Fliss et al. 1996) has led to a considerable amount of research into methods to prevent the further damage associated with reperfusion (Jonassen et al. 2001).

Defining lethal reperfusion injury as a separate entity has been controversial. Some authors suggest that the damage that occurs during reperfusion is a magnification of the cellular disruption that has occurred during the ischaemic period (Zhao et al. 2000). To counter this argument, the fact that agents given at the time of reperfusion can protect the myocardium and reduce infarct size (Lipsic et al. 2006, Forman et al. 2006) suggests

that this is a real phenomenon. Taken further, during this time of myocardial instability, an agent which could be given to protect the heart and reduce infarct size would have far reaching benefits for survival.

1.4 Cellular mechanisms of ischaemia-reperfusion injury (I/R)

The details relating to cellular damage during ischaemia and reperfusion have been well documented (Opie 2004). The mechanisms underlying cellular protection are also well described in the current literature and are fundamental to the exploration of new treatments (Chen Q. et al. 2007, Garcia-Dorado et al. 2006, Toledo-Pereyra et al. 2004). In order to understand the cellular mechanisms involved in ischaemia-reperfusion (I/R) injury and cardiac protection, particular attention needs to be focused on the complex cell signalling mechanisms associated with these processes (Hausenloy et al. 2006). This is because during I/R the cell can follow one of two paths, one leading to cell death whilst the other leads to survival.

1.4.1 Cell death

As discussed above cell death involves two principal components necrosis and apoptosis (Gottlieb & Engler 1999). The former being a non-regulated mechanism whereby cells are destroyed, whilst the latter is a programmed genetically-determined process (Chowdhury et al. 2006). The mitochondria are critically important in determining cell death and survival. It is their failure to produce ATP and maintain electrochemical equilibrium that ultimately leads to cell death. The mitochondria are, therefore, a crucial regulator of cell survival and death. Further discussion is therefore warranted to expand on the key role mitochondria play in cell death and their potential role in cell survival.

1.4.1.1 The role of mitochondria in cell death

Under normal conditions the primary function of the mitochondria in the myocardium is to provide ATP, through oxidative phosphorylation, to meet the energy demands of the beating heart (Mcfalls et al. 2003, Opie 2004 Chapter 3). This production is critical even in the resting state; any disruption to this balance, in the form of anoxia or ischaemia has an immediate effect on the ability of the heart to function (Rauch et al. 1994). This

critical role in energy production is not the only function that the mitochondria play with respect to cardiomyocyte function as within there organelles are also contained the apparatus to induce apoptotic and necrotic cell death. After prolonged myocardial ischaemia, a critical depletion of energy leads to the loss of ionic homeostasis with cell swelling, rupture, and necrotic death (Opie 2004). Over the past decade a distinctly different process of myocyte death has been observed that is programmed by stress-induced events within the mitochondria, apoptosis.

The precise molecular mechanisms that underlie apoptosis are under intense investigation. The process of apoptosis is controlled by a diverse range of cell signals that may arise either extracellularly (extrinsic pathway) or intracellularly (intrinsic pathway). Extracellular signals may include toxins, hormones, growth factors, nitric oxide or cytokines, and therefore must either cross the plasma membrane or be transduced to effect a response (Chowdhury et al 2006). Intracellular apoptotic signalling is initiated by a cell in response to stress, and may, ultimately result in cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration (e.g. by membrane damage) are all factors that can lead to the release by damaged cells of intracellular apoptotic signals (Opie 2004).

Before the process of apoptotic cell death can be initiated by the appropriate enzymes, apoptotic signals must be transmitted to the death pathway by way of regulatory proteins (Chowdhury et al. 2006). This step allows apoptotic signals to either culminate in cell death, or be aborted should it no longer be necessary for the cell to die. The principal mechanism through which this regulation is achieved is by way of the mitochondrion.

An important concept in the initiation of necrosis or apoptosis is the opening of a non-specific pore within the inner membrane of the mitochondria, the mitochondrial permeability transition pore (mPTP; Hausenloy, 2003). The opening of the mPTP leads to the loss of pH and ionic integrity as the mitochondrial membrane becomes permeable to smaller molecules (Lemasters et al. 1998). This leads to swelling and uncoupling of

oxidative phosphorylation (Hunter et al. 1976, Haworth et al. 1979). Consequently the mitochondria are unable to produce ATP as oxidative phosphorylation is uncoupled. If this process is left unchecked rupture of the outer mitochondrial membrane occurs followed by irreversible damage to the cell, and necrotic death (Halestrap 2006).

1.4.1.2 Mitochondria and Apoptosis

The breakdown in mitochondrial membrane selectivity sets in motion a cascade of events, which eventually leads to the breakdown of cellular integrity. Mitochondrial rupture not only prevents any further ATP production but also releases apoptotic signalling proteins into the cytosol (Halestrap 2006). Mitochondria release various pro-apoptotic proteins including cytochrome c, apoptosis-inducing factor, second mitochondria-derived activator of caspases/direct inhibitors of apoptosis protein (IAP)-binding protein (Smac/Diablo), and procaspases (2,3,8, and 9). Once released into the cytosol through the outer membrane, these proteins activate a family of proteases that precipitate the structural changes that occur within apoptotic myocytes (McFalls et al. 2003), and can ultimately lead to the death of the cell.

This change in mitochondrial membrane permeability, therefore, can lead to a situation in which cells die in both a necrotic and/or apoptotic manner. Whether the cell follows a necrotic or apoptotic route is thought to be dependent on the ability of the permeability transition pore to open and close (Halestrap et al. 2004). What determines mPTP opening and closing is an adequate supply of energy. Apoptosis is an ATP-dependent mechanism and if the mPTP does not close, the cell will progress towards necrotic cell death in order to maintain integrity. If, however, an ischaemic insult is followed by reperfusion and the mPTP is able to close, oxidative phosphorylation can re-couple and ATP levels are maintained, leading to a degree of apoptotic cell death (Halestrap 2006). This is evidenced by the apoptotic ring which is seen around a necrotic core of a coronary infarction (Crompton et al. 1999). The extent of mPTP opening, therefore, has a direct influence on cell survival. Too much mPTP opening and the cell will progress towards necrosis, too little and the cell will undergo apoptosis (Halestrap et al. 2004). Thus, if oxidative phosphorylation is re-coupled and ATP production reinitiated, recovery should therefore be possible.

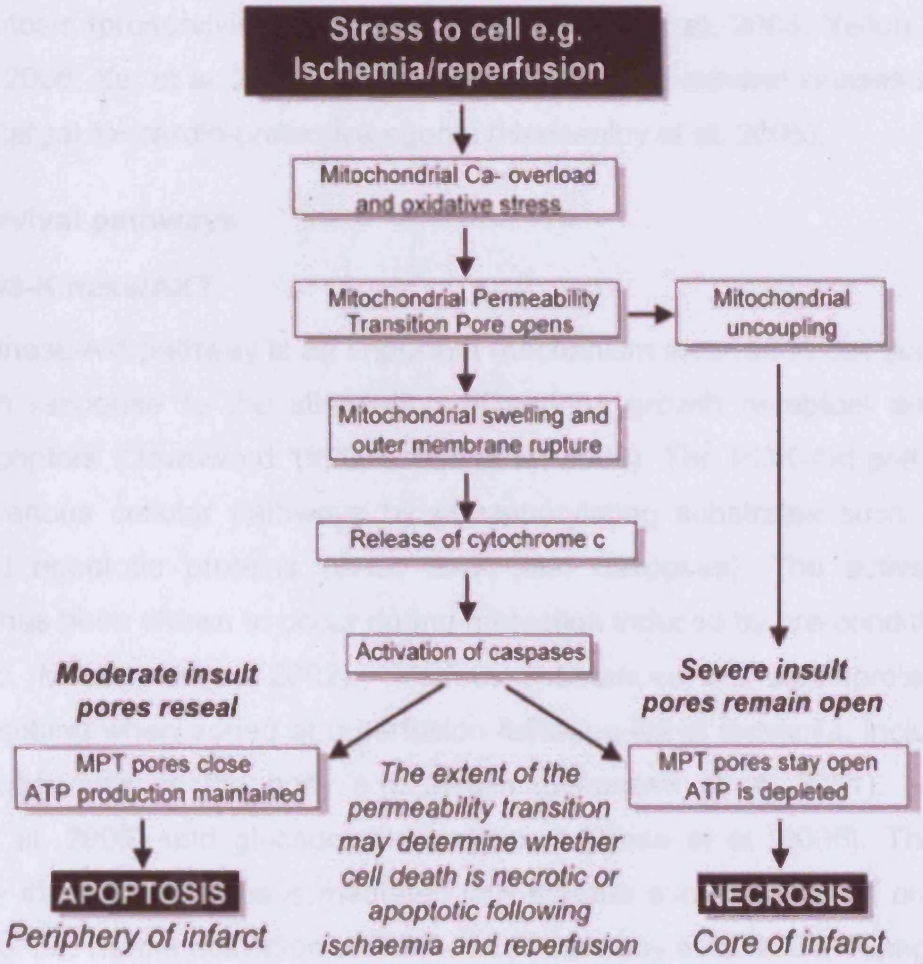

Figure 1-2 The effects of mPTP opening on ischaemic-reperfusion injury

Modulation of the mPTP consequently represents a key factor in attempting to protect the heart following myocardial infarction (taken from Halestrap 2006).

1.4.2 Cell survival

The pathways which promote cell survival and inhibit apoptosis have been extensively researched as potential mechanisms for inducing cellular protection around the period of ischaemia reperfusion (Hausenloy et al. 2004). As mentioned before one of the most potent cardioprotective treatments demonstrated is preconditioning (Murray et al. 1987), which is thought to act via pathways which promote cell survival (Hausenloy et al. 2006). Drugs given at reperfusion which reduce infarct size have been shown to stimulate growth factors which themselves act on downstream kinases to promote growth and

inhibit apoptosis (prosurvival) (Baines et al. 1999, Bell et al. 2003, Yellon et al. 2005, Zhao et al. 2006, Xu et al. 2005). The stimulation of these survival kinases seems to be a common target for cardio-protective agents (Hausenloy et al. 2005).

1.4.2.1 Survival pathways

1.4.2.1.1 PI3-Kinase/AKT

The PI-3 Kinase/Akt pathway is an important mechanism involved in cell survival, and is activated in response to the stimulation of various growth receptors and G-protein coupled receptors (Downward 1998, Cross et al. 2000). The PI3K-Akt pathway acts to modulate various cellular pathways by phosphorylating substrates such as GLUT-4, GSK-3 and apoptotic proteins (BAD, BAX, and caspases). The activation of PI3 kinase/Akt has been shown to occur during protection induced by pre-conditioning (Tong et al. 2000, Mocanu et al. 2002). Various substances are cardioprotective in the laboratory setting when added at reperfusion following lethal ischemia, including factors found endogenously in the body e.g. insulin (Jonassen et al. 2001), erythropoietin (Bullard et al. 2005) and glucagon-like peptide-1 (Bose et al. 2005). The protection afforded by these substances is mediated in a manner similar to that of pre- and post-conditioning, i.e. via the activation of PI3K-Akt (Hausenloy et al. 2004, Tsang et al. 2005, Yellon et al. 1999, Fujio et al 2000).

1.4.2.1.2 MAPK

The mitogen-activated protein (MAP) kinases are serine/threonine-specific kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis (Pearson et al. 2001). To date, various distinct groups of MAPKs have been identified in mammalian cells: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. The ERK pathway when activated during ischaemia-reperfusion injury has, like PI3K-Akt, been shown to mediate cellular protection (Darling et al. 2005, Yue et al. 2000, Shimizu et al. 1998, Hausenloy et al. 2005).

There is, therefore, evidence for increased activity of both PI3-K/Akt and MAP kinases during ischaemia/reperfusion. Consequently these cell signalling pathways were examined in the present study as potential targets in reducing myocardial injury.

1.4.2.2 RISK

The potential involvement of various cell signalling pathways in promoting cell survival and anti-apoptotic mechanisms has led to research into agents which may stimulate these endogenous protective pathways. A unifying term has been coined to cover these cell signalling mechanisms involved in tissue protection i.e. the Reperfusion Injury Salvage Kinase (RISK) pathway (Hausenloy & Yellon 2004). Two signalling cascades namely, P44/42 mitogen activated protein (MAP) kinase (also known as ERK) and phosphatidylinositol 3-kinase (PI3K-Akt) appear to be particularly important with respect to the RISK pathway.

The protection afforded by activation of the RISK pathway, involves anti-apoptotic mechanisms that induce cellular protection. The precise mechanisms, by which the components of the RISK pathway perform their anti-apoptotic effects, are, however, not fully elucidated, although a number of components involved in the mediation of protection have been identified. For example, Akt phosphorylates the Bcl-2-associated death promoter (BAD), which when unphosphorylated, holds one or more death-inhibitory proteins in an inactive state (Datta et al., 1997). Once phosphorylated BAD releases the inhibitory proteins, which can block apoptosis, thereby promoting cell survival. Subsequently BAD binds to a ubiquitous cytosolic protein called 14-3-3, preventing further apoptotic activity (Zha et al. 1996).

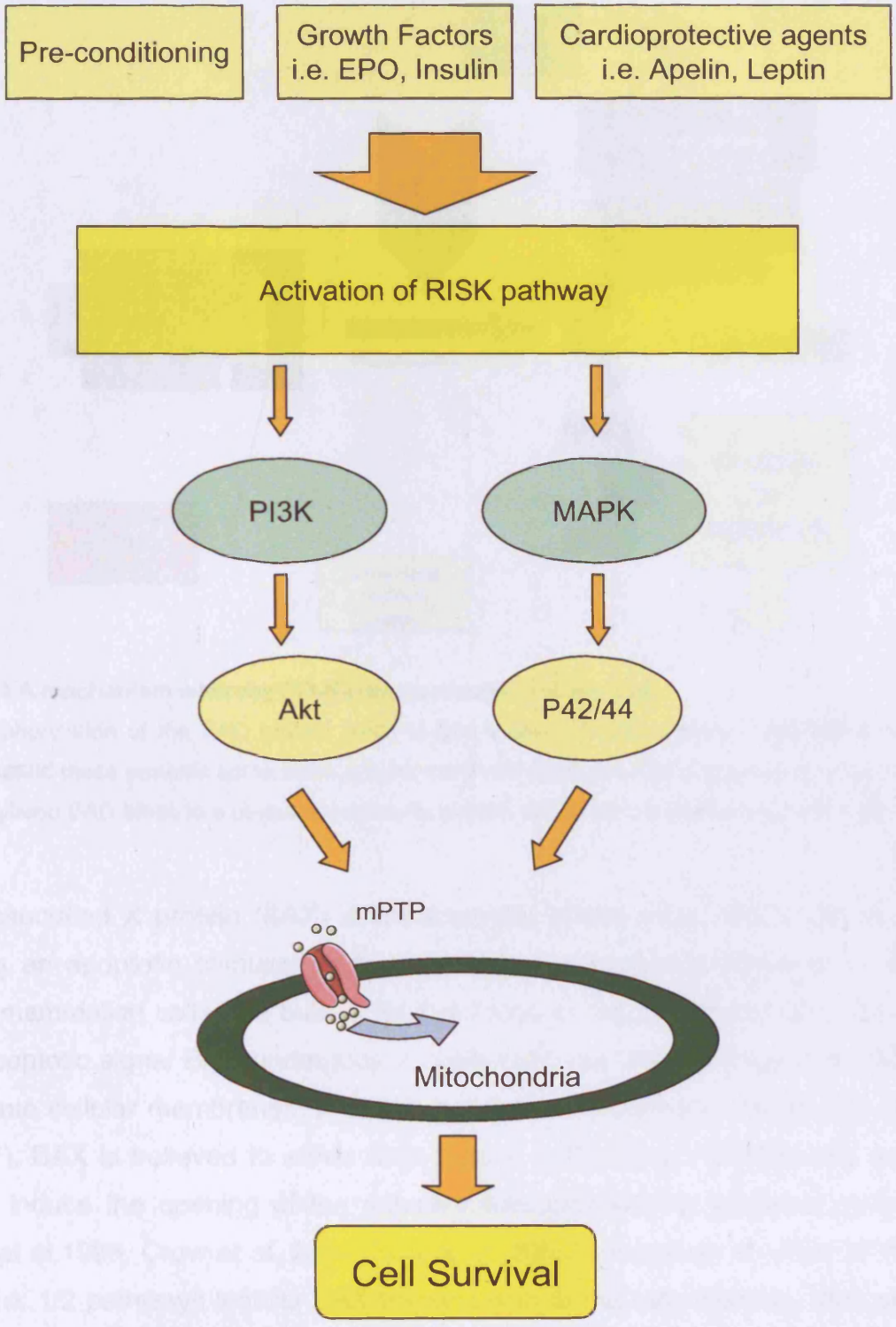

Figure 1-3 Activation of the RISK pathway

The activation of the pro-survival PI3K-Akt and p42/44 MAPK cascade, which constitutes the RISK pathway, protects the heart against lethal reperfusion-induced injury.

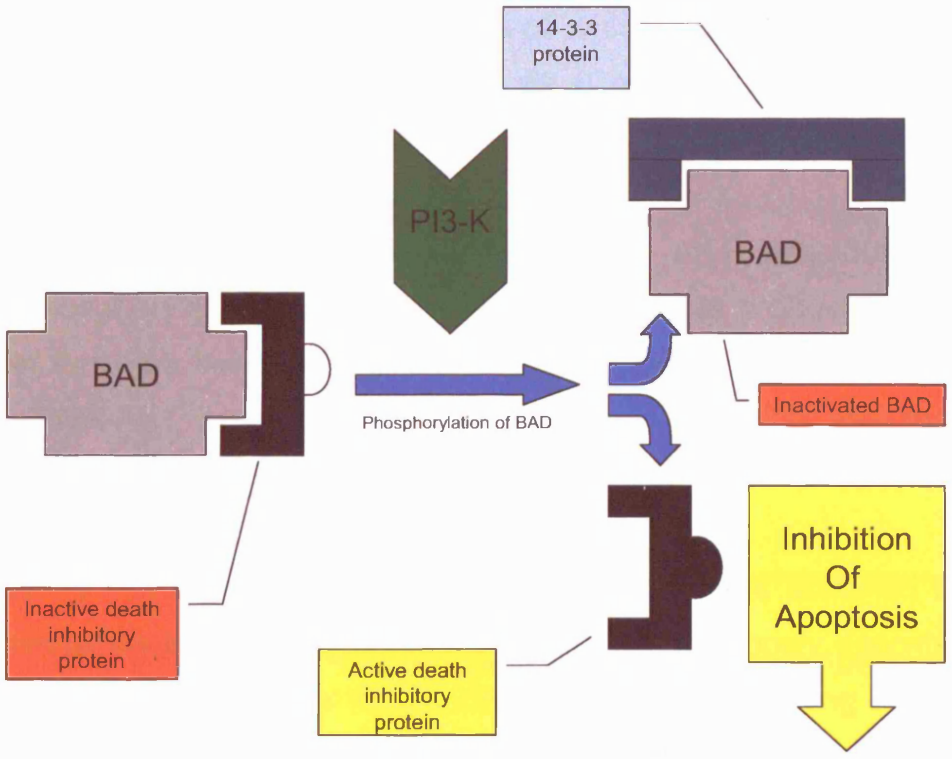

Figure 1-4 A mechanism whereby PI3-Kinase promotes cell survival.

The phosphorylation of the BAD protein leads to the release of one of more death inhibitory proteins. Once released these proteins act to block programmed cell death, thereby promoting survival. Meanwhile, phosphorylated BAD binds to a ubiquitous cytosolic protein called 14-3-3, preventing further BAD action.

Bcl-2 associated X protein (BAX) is pro-apoptotic (Datta et al. 1997) and is activated following an apoptotic stimulus such as ischaemia-reperfusion (Crow et al. 2004). In healthy mammalian cells, the bulk of BAX is found in the cytosol but upon the initiation of an apoptotic signal BAX undergoes a conformational change (Hou et al. 2005), and inserts into cellular membranes, primarily the outer mitochondrial membrane (Wolter et al. 1997). BAX is believed to either form a pore in the outer mitochondrial membrane itself or induce the opening of the mitochondrial permeability transition pore (mPTP) (Marzo et al.1998, Crow et al. 2004, Hou et al. 2005). Activation of either of the PI3K–Akt or Erk 1/2 pathways inhibits BAX translocation to the mitochondria, thus preventing apoptosis (Yamaguchi et al. 2001, Tsuruta et al. 2002, Weston et al. 2003).

BAX induced pore formation in the mitochondria leads to the release of apoptogens including cytochrome c (Crow et al. 2004). These apoptogens activate caspase 9, a

death protease, Akt-induced phosphorylation of which has been shown to decrease apoptosis by inhibiting protease activity directly (Cardone et al. 1998, Brunet et al. 1999).

Bell et al (Bell et al. 2003) have demonstrated that AKT activation results in the downstream phosphorylation of eNOS, which results in the production of nitric oxide. Nitric oxide in turn has been shown to prevent the opening of the mPTP. Therefore, activation of PI3K-AKT at the time of reperfusion protects the myocardium via an eNOS→NO→mPTP mechanism.

What is apparent is that many of the anti-apoptotic pathways activated by the RISK pathway converge on the mPTP (Kroemer et al. 1998), previously described as a key regulator of cell death and survival (see section 1.4.1.1). The opening of this pore is felt to determine the degree of cell death by apoptosis or necrosis (Halestrap 2005). The ability of the PI3K-Akt and ERK 1/2 kinase pathways during ischaemia-reperfusion injury to protect through actions on the mPTP was recently reported by Davidson et al. 2006. What is also of interest is that endogenous agents i.e. insulin, erythropoietin, leptin, may also act to stimulate the RISK pathway (Hausenloy et al. 2004). It can be hypothesised that manipulation of these agents may protect the heart during periods of anoxia via RISK pathway activation and mPTP opening. Over the last decade there has been great interest in the role adipose tissue plays in the pathogenesis of disease (Inui & Mequid 2003, Chan & Mantzoros 2005). The discovery of signalling peptides found in adipose tissue called adipocytokines, that act through these pathways has resulted in considerable investigation into the role played by these peptides in the development of disease.

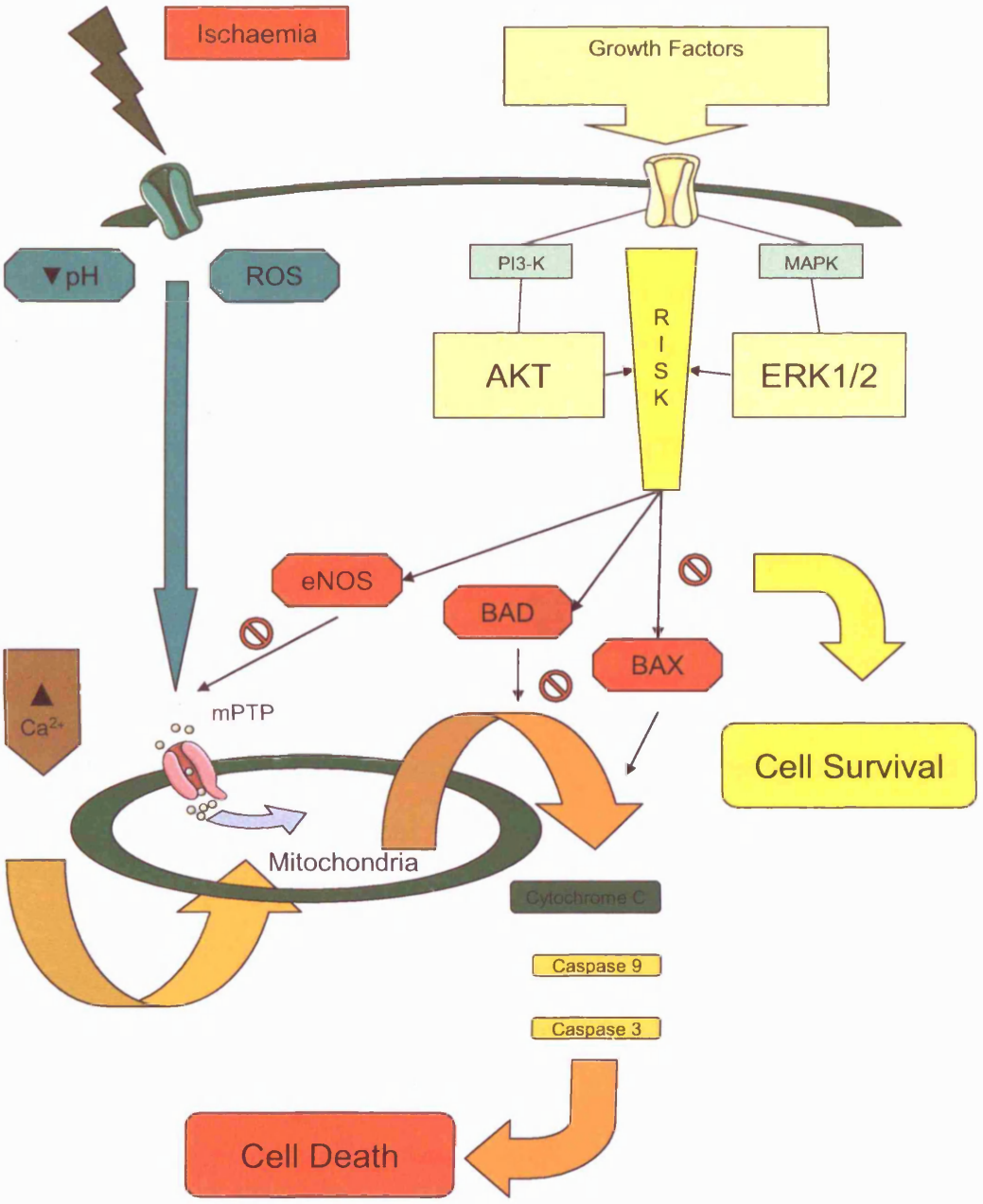

Figure 1-5 RISK pathway

From Hausenloy and Yellon 2004. The Figure portrays the important anti-apoptotic mechanisms that have been implicated in cellular survival associated with the recruitment of the cardioprotective kinase cascades. Signalling through the PI3K-Akt and ERK1/2 cascades results in phosphorylation and inactivation of the pro-apoptotic proteins BAD and BAX, one consequence of which is to prevent the release of mitochondrial cytochrome C in response to an apoptotic stimulus. Phosphorylation and activation of eNOS (endothelial nitric oxide synthase) resulting in the production of nitric oxide (NO) may also occur leading to inhibition of the opening of the mitochondrial permeability transition pore (mPTP).

1.5 Cardiometabolic disease

1.5.1 The 21st Century Obesidemic

Obesity is a major risk factor for hypertension, raised blood cholesterol, diabetes and impaired glucose tolerance. WHO figures estimate that around a third of patients with coronary heart disease and ischaemic stroke, and almost 60% of patients with hypertension in developed countries, have elevated body mass index (BMI) values. It is calculated that by 2050 there will be approximately 300 million cases of obesity-related type II diabetes (Grant 2005). More recently the INTERHEART case-control study estimated that 63% of heart attacks in Western Europe and 28% of heart attacks in Central and Eastern Europe are associated with abdominal obesity (a high waist to hip ratio), and those with abdominal obesity are at over twice the risk of a heart attack compared to those without (Yusuf et al. 2004). This study also found that abdominal obesity was a much more significant risk factor for heart attack than simple BMI (Yusuf et al. 2004). Obesity and diabetes are inextricably linked. Being overweight increases the risk of Type 2 diabetes and 80 per cent of people with Type 2 diabetes are overweight at the time of diagnosis (Astrup & Finner 2000).

Thus, the epidemic of obesity-related diabetes is a major challenge facing the world in the 21st Century. The increased number of people developing obesity-related type II diabetes will lead to a massive rise in the incidence of cardiovascular disease. The cardiovascular mortality rate has more than doubled in men and more than quadrupled in women, with diabetes compared with non-diabetics (Stamler et al. 1993) (Kannel & Wilson 1995). By 2025 the number of adults with diabetes will rise to 300 million (King et al. 1998) and 80% of patients with diabetes will die from cardiovascular causes (WHO 2005).

What is of most concern is the age at which people are developing type II diabetes. Hence, a disease that was unheard of in children in the 1990s is now occurring in children as young as eight years old. Recent statistics indicate that a child born in the United States in 2000 has a one in three chance of developing diabetes (Narayan et al.

2003). This dramatic increase in the incidence of obesity related type two diabetes has led to many theories as to why it has become so prevalent over the past twenty years.

1.5.1.1 Theory of thrift

The reasons for an increase in obesity-related diabetes are varied. Genetic and environmental factors have all been implicated in the aetiology of obesity. Polymorphisms in various genes controlling appetite, metabolism, and adipokine release predispose to obesity. Various genetic abnormalities that predispose to obesity have been identified such as Prader-Willi syndrome and leptin receptor mutations (Mutch & Clement 2006). While it is thought that a large proportion of the causative genes are still to be identified, much of obesity is likely to be the result of interactions between multiple genes and non-genetic factors (Mutch & Clement 2006). One of the major factors is the development of a Western lifestyle, as ultimately the condition requires the availability and consumption of sufficient calories.

In 1962 Neel (Neel 1962) put forward a theory in which he stated that the state of diabetes was beneficial in certain conditions. This theory was based on the idea that in times of plenty insulin resistance allows for energy to be stored for later use in times of famine. However, whilst insulin resistance might be advantageous during periods of nutritional fluctuation, potentially deleterious effects may develop when food becomes plentiful. A state of insulin resistance would alter the threshold at which insulin produces its metabolic actions after food intake, transforming the body's normal metabolism to an anabolic state leading to fat storage. This state of insulin resistance and hyperinsulinaemia, therefore, predisposes the body to the development of diabetes and obesity. The evolution of man in the 20th Century has led to a very different metabolic make-up. Man is now in a constant state of post-prandial hyperglycaemia and insulin sensitisation. This may explain why the adaptive mechanisms of protection have been lost and the development of obesity related cardiovascular disease is reaching epidemic proportions. The body's adaptation during this time to conserve energy and protect, ultimately leads to down regulation of these systems and the development of disease (Scott & Grant 2006). The innate mechanisms which the body has developed in order to

protect itself offer an intriguing insight into evolutionary metabolism. The study of these mechanisms has ultimately helped our understanding of the development of obesity related cardiovascular disease, leading to possible therapies to prevent it.

1.5.2 The Metabolic syndrome or Syndrome X

In 1988 Reaven noted that resistance to insulin-mediated glucose uptake was present in the majority of people with impaired glucose tolerance and type II diabetes (Reaven 1988). Furthermore, he suggested that the compensatory hyperinsulinaemia may lead to hypertension, and the relationship between hypertension, insulin resistance and hyperinsulinaemia might be causal. He raised the possibility that insulin resistance and hyperinsulinaemia were involved in the aetiology and clinical course of type 2 diabetes, hypertension and coronary heart disease. He termed the cluster Syndrome X (later called Reaven's syndrome and the metabolic syndrome) to describe the epidemiological association between insulin resistance and several other disease states. Since then other risk factors, including measures of obesity (body mass index BMI) have been added. What has become apparent is the importance of visceral adiposity in the development of cardiovascular and metabolic risk (Carey et al. 1996, Larson et al. 1996, Vanhala et al. 1998). The mechanisms linking visceral fat with a risk for disease states is currently under close scrutiny and their elucidation is paramount, if we are to understand, and treat the obesidemic which is upon us. Research has suggested that the visceral tissue itself expresses moieties which may lead to disease states. Apart from free fatty acids (FFA) (Kissbah 1996), interleukin-1, interleukin-6, TNF-α (Tumour necrosis factor), (Bullo et al. 2003) resistin, leptin and adiponectin have been implicated. These latter substances, collectively termed adipocytokines or adipokines, regulate a number of biological functions including appetite and energy balance, insulin sensitivity, lipid metabolism, blood pressure, and inflammation (Goralski & Sinal 2007). The physiological importance of adipocytokines has led to the hypothesis that changes in the synthesis and secretion of these compounds contribute to the development of obesity and obesity-related disease (Goralski & Sinal 2007). Following on from this it has been proposed that pharmacological manipulation of adipokine levels (Gary-Bobo et al. 2007; Choi et al. 2005) may provide novel therapeutic strategies to treat and prevent obesity,

type 2 diabetes, and cardiovascular disease. The following section will review the role adipokines play in the pathogenesis of disease and examine in more detail the actions of some of the key adipokines.

1.6 Adipocytokines

Adipose tissue is an anatomical term for loose connective tissue composed of adipocytes. Apart from its obvious role in insulation and cushioning the body, its function is to store energy in the form of fat (Krug & Ehrhart-Bornstein 2005). In addition to these functions, the cells from which adipose tissue is composed i.e. the adipocytes, display characteristics of endocrine cells and secrete a variety of adipocyte-specific hormones into the circulation (Okomoto et al. 2006). These adipokines or adipocytokines are a group of bioactive substances which include growth factors, complement factors and cytokines (Matsuzawa 2006). Adipocytokines have been shown to act in an endocrine, paracrine or autocrine fashion and influence processes such as energy storage, immunity, fluid homeostasis and glucose control (Boucher et al. 2005). More recently direct effects on the cardiovascular system have been found (Shibata et al 2005, Fruhbeck et al. 1999, Lembo et al. 2000, Correia et al. 2005).The importance of adipocytokines is underlined by the fact that adipose tissue is generally regarded as the largest endocrine organ in the body. The large size of this organ means that even small quantities of adipocytokines secreted by this tissue may have a strong influence on the body's homeostatic state (Matsuzawa 2006). Another notable feature is the fact that each adipocyte is connected to the vascular network, so that adipokines are released into the systemic circulation (Correia et al, 2004, Matsuzawa 2006).

The important endocrine function of adipose tissue is emphasized by the adverse metabolic consequences that can occur from both adipose tissue excess and deficiency (Inui & Mequid 2003, Chan & Mantzoros 2005). An excess of adipose tissue or obesity, particularly in the visceral compartment, is associated with insulin resistance, hyperglycaemia, dyslipidaemia, hypertension and prothrombotic and pro-inflammatory states (review Rader 2007, Guzik et al. 2006). It has become increasingly clear that adipose tissue is complex and constitutes a highly active metabolic and endocrine

organ, its role in the development of many chronic diseases possibly being pivotal (Matsuzawa 2006, Trujillo & Scherer 2006). The adipocyte is, therefore, becoming increasingly recognised as a key regulator of cardiometabolic disease.

1.6.1 Adipose tissue and inflammation

The majority of patients with type II diabetes are obese (Mokdad et al. 2000).The state of insulin resistance seen in obesity and type II diabetes is characterised by deranged adipocyte metabolism and alterations in fat deposition (Matsuzawa 2006). In both lean and obese type II diabetic's persistent elevation of free fatty acids is seen, with impaired FFA suppression with glucose loading (Reaven 1998). Insulin controls the release of FFA, stored as triglycerides, for use during fasting (Groop et al 1989). In type II diabetes the inhibition of lipolysis (breakdown of FFA to glucose) is impaired, leading to excessive FFA release. This chronic elevation of FFA causes insulin resistance in muscle and liver (Boden 1997 and 2002, Golay et al. 1987), and can impair insulin secretion. This sequence of events has been referred to as lipotoxicity (McGarry et al. 2002, Unger et al. 1995) and is believed to play an important role in β-cell (insulin producing cell) dysfunction (Bays et al. 2004) in the pancreas. The adipocyte is the storage medium for FFA and as such is implicated in the pathogenesis of insulin resistance in type II diabetes (Bays et al. 2004).

Adipocytokines which are produced by adipose tissue are altered in conditions such as diabetes and cardiovascular disease. Maladaptation of adipose tissue has therefore been postulated as the link between the developments of obesity-related type II diabetes and cardiovascular disease (Bays et al. 2004).The effect of fat-loading is to modify the levels of certain adipocytokines. In type II diabetes there is a reduction in the production of some factors that are normally synthesised by the adipocyte, e.g. adiponectin (Weyer et al. 2001, Hotta et al. 2000, Arita et al. 1999), whereas there is increased secretion of other adipocytokines, e.g. resistin, PAI-1 (plasminogen activator inhibitor), TNF-α, interleukins and leptin occurs. Some of these factors are known to be stimulatory or inhibitory proteins of the inflammatory system. TNF-α and IL-6 are examples of pro-inflammatory cytokines. TNF-α is over-expressed in models of human (Hotamisligil et al.

1995) and mouse obesity (Hotamisligil et al. 1993). It is also found to play a pivotal role in the insulin resistance of sepsis and cancer (McCall et al. 1992, Van der Poll et al. 1991). Additionally, it has been implicated in the development of atherosclerosis (Peraldi & Spiegelman 1998). IL-6 is an inflammatory cytokine which, like TNF-α, is highly expressed in adipocytes (Mohamed-Ali et al. 1997). It is the primary activator of acute phase proteins (Gabay et al. 1999) and has been implicated in the development of insulin resistance, both in skeletal muscle and liver (Kim et al. 2004), as well as pancreatic β cell apoptosis (Shimabukuro et al. 1998). In patients with type II diabetes increased levels of IL 6 are seen which correlate with the severity of glucose intolerance (Pradhan et al. 2001).

Examples of anti-inflammatory adipokines are adiponectin and IL-10. Adiponectin is produced solely by adipocytes and is known to increase tissue sensitivity to insulin (Berg et al. 2001), Combs et al. 2001), Yamauchi et al. 2001). Its anti- inflammatory properties result from its inhibition of phagocyte activity and TNFα production by macrophages (Pittas et al. 2004). Recent studies have shown a correlation between hypoadiponectinaemia and the development of diabetes (Daimon et al. 2003, Duncan et al. 2004) and cardiovascular disease (Rothenbacher et al. 2005).

A change in the balance of these cytokines, as obesity progresses could ultimately lead to the development of cardiovascular disease (Gorlaski & Sinal 2007). Evidence is available to link chronic inflammation, insulin resistance, type II diabetes and atherosclerosis (Pickup & Crook 1998, Festa et al. 2000, Tataranni & Ortega 2005). It is possible to hypothesise that change in adipokine expression over time leads to activation of the immune system via alterations in circulating inflammatory adipokines (Gorlaski & Sinal 2007). Maladaptation of this balance between pro-inflammatory and anti-inflammatory adipokines leads to a pathological state (Matsuzawa 2006). Suppression of protective mechanisms and over expression of damaging pathways by fat loading (Grant 2005), dysfunctional fat cells and obesity may provide the link between the development of both insulin resistance and cardiovascular disease. Adipocytokines are the signals within the adipose tissue which are orchestrating these

changes and therefore research into their actions is key to understanding the pathogenesis and, hopefully, treatment of obesity-related disease.

1.7 Adiponectin

1.7.1 Introduction

Adiponectin, also referred to as ACRP30, AdipoQ and GBP28 was identified by several groups (Kadowaki 2006), and is produced in adipocytes. It circulates at high concentrations (5-10μg ml^{-1}), accounting for 0.01% of plasma protein (Coombs et al. 2003; Whitehead et al. 2006; Xu et al. 2005). Interestingly, unlike other adipocytokines, adiponectin levels are inversely related to BMI (Arita et al. 1999). Adiponectin seems to have several beneficial and protective effects (Yamauchi et al. 2001; Yamauchi et al. 2003). Adiponectin has been shown to be insulin-sensitizing (Yamauchi et al. 2001), anti-inflammatory (Ouchi & Walsh 2007) and anti-atherogenic (Shimada et al. 2004; Yamauchi et al. 2003). It is reduced in the serum of both type II diabetic and obese individuals and is also decreased in patients with cardiovascular disease (Arita et al. 1999, Hotta et al. 2000). Adiponectin levels are a predictor of future risk of developing type 2 diabetes (Spranger et al. 2003) and myocardial infarction (Pischon 2004). Its beneficial actions make adiponectin a potential therapeutic option in the treatment of a number of disease states, characterised by metabolic abnormalities and vascular insufficiency.

1.7.2 AMPK

The cellular mechanisms whereby adiponectin acts has led to considerable interest in the roles played by the adipocytokines in the development of adipose-related disease. In mice adiponectin has been shown to lower glucose levels independently of insulin (Nawrocki et al. 2006). The administration of adiponectin reduces FFA, triglycerides (TG) and glucose (Fruebis et al. 2001), and leads to weight loss. The insulin sensitizing action of adiponectin was confirmed in adiponectin knock out mice (APN-KO) which exhibit diet-induced insulin resistance when fed a high fat/sucrose diet (Shibata et al. 2005). The mechanism through which adiponectin acts to regulate metabolism and insulin sensitivity is, in part, through AMP-activated protein kinase (AMPK), a stress

response kinase, which has been shown to be activated under certain circumstances in skeletal muscle, liver (Yamauchi et al. 2002) and adipocytes (Wu et al. 2003). AMPK is an evolutionarily conserved sensor of the energy status of a cell, and has a crucial role in controlling systemic energy balance by regulating food intake, body weight, and glucose and lipid homeostasis (Kahn et al., 2005, Greenberg & Obin 2006l).

1.7.3 Protective role

Evidence suggests that adiponectin acts as a protective agent against the effects of obesity and diabetes related endothelial dysfunction (Ouchi et al. 2004; Kobayashi et al. 2004). These effects seem to be mediated through AMPK which has been identified as a regulator of endothelial cell nitric oxide synthase (eNOS) activation (Morrow et al. 2003; Chen H. et al. 2003; Ouchi et al. 2004) and angiogenesis (Nagata et al. 2003). Adiponectin stimulates nitric oxide production in endothelial cells through AMPK-dependent phosphorylation and activation of eNOS (Ouchi et al. 2004, Chen H. et al. 2003). These effects suggest that adiponectin may play a critical role in endothelial function and vascular tone (Ritchie et al.2004). AMPK signalling has been implicated in the anti-apoptotic actions of adiponectin (Kobayashi et al. 2004) in human endothelial cells (Lin et al. 2004). Its angiogenic properties are suggested by the fact that adiponectin stimulates endothelial cell migration and differentiation, and blood vessel growth in mouse and rabbit models of angiogenesis (Ouchi et al 2004; Nagata et al. 2003).

The protective effects of adiponectin in the setting of ischaemic heart disease have been shown in various models of ischaemia-reperfusion (Shibata et al. 2005). Adiponectin was shown to inhibit apoptosis in cardiac myocytes exposed to hypoxia-reoxygenation (Shibata et al. 2005) and it was concluded that this protection occurred through AMPK as it was not seen in animals with a dominant negative AMPK mutation. APN-KO mice were shown to develop larger infarcts, whilst delivery of adiponectin led to reduced infarct size, myocardial apoptosis and TNFα production in both KO and wild type animals (Shibata et al 2005). These studies indicate that several important adiponectin-induced effects are mediated through the activation of AMPK.

The beneficial effects of adiponectin are enhanced by reports that it has pleiotropic effects on immune and vascular function (Kubota et al. 2002, Okamoto et al. 2006, Wolf et al. 2004, Yamamoto et al. 2005, Brakenhielm et al. 2004). These findings and those outlined above, therefore, strongly support the idea that adiponectin represents an excellent example of an adipocytokine with a potential therapeutic role in cardiovascular disease.

1.8 Leptin

1.8.1 Introduction

Leptin was the first adipocytokine discovered to have a role in the development of obesity. Leptin is produced mainly by adipocytes and plays key roles in regulating energy intake and energy expenditure (La Cava et al., 2004). Leptin itself was discovered in 1994 by Jeffrey M. Friedman and colleagues at the Rockefeller University through the study of mutant obese mice (Zhang Y et al. 1994). These mice were massively obese and hyperphagic. The Ob (Lep) gene (Ob for obese and Lep for leptin) is located on chromosome 7 in humans and is synthesised primarily in white adipose tissue (Koerner et al. 2005). Leptin itself interacts with six types of receptor i.e. LepRa, LepRb, LepRc, LepRd, LepRe and LepRf. Of these receptors LepRb is the only isoform that contains active intracellular signalling domains (Flier 2004). This receptor is present in a number of hypothalamic nuclei, including the ventral medial nucleus of the hypothalamus, known as the "satiety centre", where it exerts its effects on appetite regulation and energy balance (Harvey 2003, Grill & Kaplan 2002).

The activation of the leptin receptor leads to the activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Leshan et al. 2006). Apart from JAK/STAT signalling, leptin receptor activation also stimulates the PI3K and MAPK pathways (Harvey J 2003, Niswender et al 2004, Zhao T et al., 2005). These pathways have already been described in relation to cardioprotection and work carried out in this laboratory has shown that leptin, when given at reperfusion in a murine

ischaemia-reperfusion model, is cardioprotective via RISK pathway up regulation (Smith et al. 2006).

AMPK is another cell signalling target for leptin (Kahn et al. 2005) and is phosphorylated and activated in response to the energy deficit occurring during fasting or cellular stress, leading to stimulation of FFA oxidation. It is co-localized with STAT3 and hypothalamic peptides implicated in energy balance. Hypothalamic AMPK phosphorylation and activity are increased by fasting and decreased by leptin, insulin and various anorectics (Minokoshi et al. 2004).

1.8.2 Beneficial or detrimental?

Similar to adiponectin, leptin is produced mainly by adipocytes, however, unlike adiponectin, leptin is considered to be a pro-inflammatory cytokine (La Cava 2004). This could be detrimental in many animal models of inflammatory and autoimmune disease, but it might be protective in several infectious disease settings, such as during the acute phase of myocarditis (Takahashi et al 2006) or bacterial pneumonia (Mancuso et al 2002). With respect to the cardiovascular system little is known as to whether leptin serves a beneficial or detrimental role (Guzik et al. 2006).

Serum Leptin is elevated in coronary heart disease and hyperleptinaemia is associated with tachycardia, chronic heart failure (Leyva et al. 1998) and increased risk of myocardial infarction (Soderberg et al. 1999). As outlined above, recent studies have suggested a protective role for leptin, acute administration during cardiac ischaemia in mice leading to a reduction in infarct size (Smith et al. 2006). Leptin deficient ob/ob mice have also been shown to have impaired cardiomyocyte function (Dong et al. 2006) and in a cardiomyocyte model, prior treatment with leptin provided a significant protection against the detrimental effects of hypoxia (Erkasap et al. 2006).

The apparently conflicting findings as regards the positive and potentially detrimental effects of leptin have led to questions as to whether leptin may have a useful role in the treatment of human cardiovascular disease. There does seem to be a time-dependent

element as regards the actions of leptin i.e. acute administration being associated with protection at reperfusion, whilst chronic elevation is associated with disease states such as obesity (Guzik et al. 2006). What is apparent is that leptin is a multi-faceted cytokine, particularly in the cardiovascular system, and further research is needed to clarify its role as a protective peptide.

1.9 Apelin

1.9.1 Background

Apelin is the endogenous ligand for the G protein-coupled APJ receptor. The APJ receptor was first cloned from a human gene in 1993 and was shown to share close identity with the transmembrane portion of the AT_1 angiotensin receptor (O'Dowd et al. 1993). Despite its similarity to the AT_1 receptor, angiotensin was not found to activate cells expressing the APJ receptor. The APJ receptor remained an "orphan" receptor until 1998 when Tatemoto and co-workers (Tateomoto et al. 1998) purified a protein that bound to it.The term apelin was derived from APJ endogenous ligand and initially was isolated as a 36 amino acid peptide. The human gene for the APJ receptor is encoded on chromosome 11, whereas the apelin gene is found on the X chromosome (Kleinz 2005).

The apelin gene codes for a 77 amino acid prepropeptide which is thought to undergo post-translational modification leading to the production of shorter moieties (Tatemoto et al. 1998). Post-translational modification of a prepropeptide is commonly seen with biologically active peptides i.e. they are synthesised as inactive precursors that are subsequently activated under physiological conditions, thereby preventing enzymatic breakdown and preserving their biological activity; the signal sequence is cleaved off to form the more active protein or proprotein (Sykes et al. 1999, Schilling et al. 2003, Garden et al. 1999). Apelin 36 was the first of the apelin peptides identified, although subsequent studies have identified the shorter isoforms apelin-13, 15, 17 and 19 (Tatemoto et al 1998, Kawamata et al., 2001, Cayabyab et al., 2000, Hosoya et al., 2000). It is of interest that considerable sequence homology exists across different species, (Tatemoto et al., 1998, Habata et al. 1999) with a 23 amino acid segment occurring near the C terminal end of the peptide being completely conserved across rat, mouse, cattle and human apelin prepropeptide (Lee et al., 2000). Studies of shorter isoforms, in which the C-terminal fragments of apelin-36 are conserved, reveal a stronger activity then the longer isoform (Tatemoto et al. 1998).

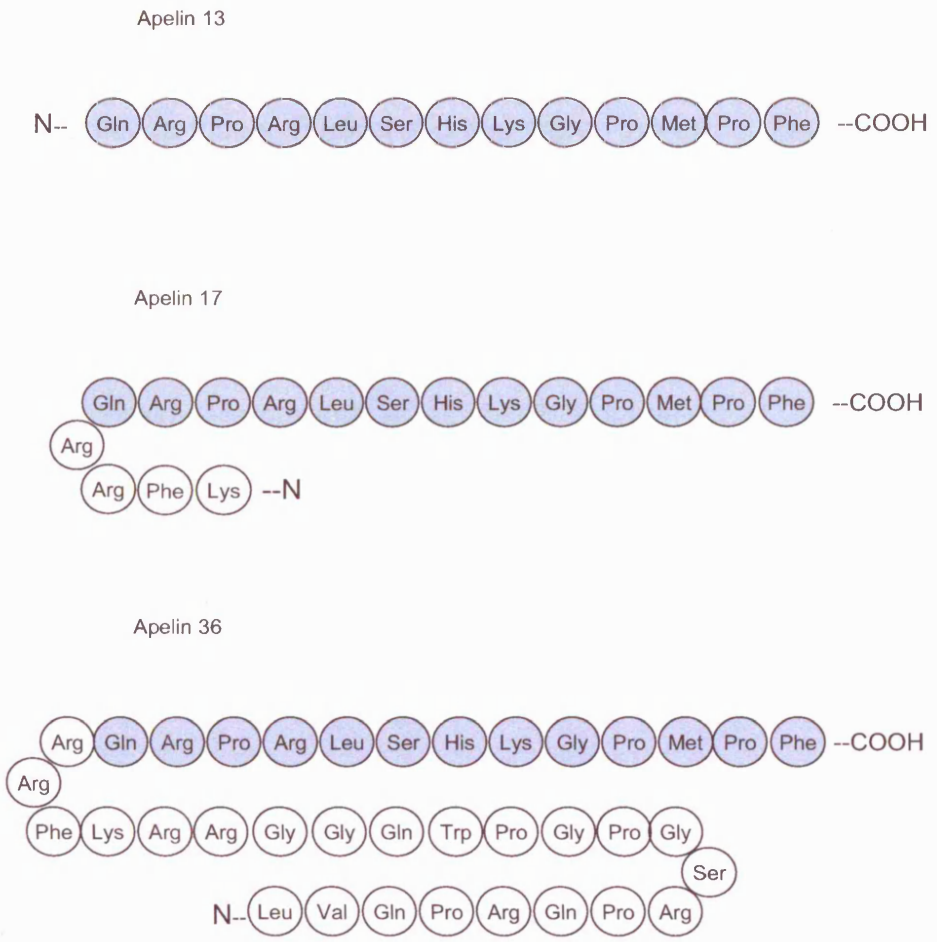

Figure 1-6 Apelin isoforms showing conservation of the C-terminal region.

The light blue circles indicate amino acids which are conserved throughout the endogenous apelin isoforms (taken from Kleinz and Davenport 2005).

It has therefore been suggested that the C-terminal region is important in apelin binding and biological activity (Habata et al. 1999, Tatemoto et al. 2001).This was evidenced by the fact that potency increases with a shortening of the peptide up to apelin-12. Apelin-12 is not synthesised in vivo, but is the most biologically potent isoform, whilst shortening the peptide further to apelin-11 renders it inactive (Tatemoto et al., 2001). A conclusion that has been drawn from these studies is that apelin-36 may function as a precursor with limited biological activity, post-translational modification of this peptide resulting in the formation of more potent shorter isoforms (Kleinz & Davenport 2005).

The similarities as regards the structure of the APJ and angiotensin (AT1) receptors (O'Dowd et al. 1993) prompted the suggestion that apelin, like angiotensin II, may play a role in cardiovascular function. Further studies were, therefore, carried out to elucidate the localisation and functions and signalling properties of the apelin-APJ system.

1.9.2 Distribution

Apelin and its receptor, and the mRNA transcripts that encode each, are expressed highly in both the brain (Matsumoto et al. 1996, Edinger et al. 1998, O'Carroll et al. 2000) and the periphery, particularly in regions of the cerebellum, hypothalamus, vascular endothelium, heart (Kleinz et al. 2004), lung, stomach and kidney (Hosoya et al. 2000, Susaki et al. 2005). Apelin is also produced and secreted by human and mouse isolated mature adipocytes (Boucher et al 2005), thereby defining apelin as an adipocytokine.

The presence of apelin in areas of the brain which are known to modulate fluid homeostasis and blood pressure has led to further investigation into apelin's role in these regulatory mechanisms (Reaux et al. 2002). Neurons synthesising apelin have been found in the hippocampus, striatum, cerebellum and paraventricular nucleus (O'Carroll et al. 2000). Cells in the paraventricular nucleus (PVN) also produce vasopressin which a hormone released when the body is low in water; it causes the kidneys to conserve water by concentrating the urine and reducing urine volume. Vasopressin is also a potent vasoconstrictor; its pharmacological analogue can be used in the treatment of shock (Barrett et al. 2007). The discovery of apelin receptors (APJ) in the human and rat heart and the surrounding vasculature (Katugampola et al. 2001), together with evidence of high levels of apelin expression in endothelial cells from human large conduit vessels, has furthered the case made for the involvement of the apelin-APJ system in cardiovascular regulation and fluid homeostasis.

1.9.3 Function

On the basis of APJ receptor structure and tissue distribution, potential functional roles for apelin were considered. The first evidence for the cardiovascular actions of apelin was provided by Lee et al 2000 (Lee et al. 2000), who reported a significant transient drop in systolic and diastolic blood pressure (− 10 mm Hg) after intravenous infusion of apelin-13 in anaesthetized male Wistar rats. In similar experiments, another group observed hypotensive actions for 3 different apelin peptides (apelin-12, apelin-13, and apelin-36), with a potency inversely related to the molecular weight of the peptides (Tatemoto et al 2001). The mechanism underlying this blood pressure lowering effect was found to be nitric oxide dependent. Thus, the hypotensive effect of the most potent apelin isoform, apelin-12, was dose-dependent and could be abolished by pre-treatment of rats with the nitric oxide (NO) synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; Tatemoto et al 2001). Further experiments in which intravenous administration of apelin-13 resulted in a significant drop in mean arterial blood pressure (MABP), coupled with an increase in heart rate, seemed to confirm that apelin had a potent hypotensive action (Reaux et al. 2001). A similar drop in MABP, combined with an increase in heart rate, was observed after intravenous infusion of apelin-12. Pharmacological ganglionic block abolished the compensatory increase in heart rate and a more significant drop in MABP was seen (Cheng et al 2003), suggesting the reciprocal heart rate change was most likely baroreflex mediated.

In view of the strong expression of the APJ receptor (Devic et al., 1999, Hosoya et al., 2000, O'Carroll et al. 2000) and preproapelin mRNA in the heart (Lee et al. 2000) it could be predicted that apelin would regulate certain cardiac functions (Masri et al., 2005). In isolated perfused rat hearts paced at a constant rate and contracting isovolumetrically, the peptide caused a dose-dependent increase in developed tension (Szokodi et al 2002). In anaesthetized rats, apelin caused an increase in left ventricular systolic pressure, dP/dt_{max}, and stroke volume (Berry et al. 2004). Since the increase in stroke volume was not accompanied by changes in end-diastolic ventricular volume, it was concluded that this provided evidence of a positive inotropic effect. Infusion of

apelin also improved ventricular diastolic relaxation as indicated by a reduction of $dP/dt_{min.}$

Conclusive evidence that apelin-induced cardiovascular actions are mediated by APJ was provided by a study showing that a decrease in MABP induced by intravenous infusion of (Pyr^1)apelin-13 into wild-type mice was not replicated in APJ-KO mice (Ishida et al 2004). In agreement with previous findings the hypotensive actions observed in wild-type mice were completely abolished by nitric oxide synthase inhibitor pretreatment, suggesting that the hypotensive effect of apelin involves an endothelium-dependent mechanism (Ishida et al. 2004). It has already been mentioned that the APJ receptor has similarities with the AT_1 receptor and that the hypertensive actions of angiotensin II were found to be more potent in APJ-deficient mice, suggesting that the apelin-APJ system may play a role in counteracting angiotensin II-induced vasoconstriction (Ishida et al. 2004; Lee et al 2006). Work in human tissue is scanty; the only functional study of apelin to date described the effects of apelin-13 on human endothelium-denuded saphenous veins. Studies in which the effects of increasing doses of apelin-13 (0.1 to 300 nM) on isometric contraction were assessed, indicated that apelin-13 potently contracted human saphenous veins (Katugampola et al. 2001).

The apparent contradictory effects of apelin on the vasculature have yet to be explained. In some cases the model used may explain the discrepancy of results. The use of denuded endothelium may explain the vasoconstrictor responses to apelin. Apelin has been shown to produce vasodilatation via the nitric oxide pathway (Ishida et al. 2004). In the absence of a functioning endothelium, apelin may directly activate APJ on vascular smooth muscle to cause vasoconstriction. Differences in the vasoactive response may not only be dependent on the type of apelin used, but also on the vascular bed it is applied to. In the review by Kleinz & Davenport attention was drawn to the wide variation in EC_{50}/IC_{50} (nM) values obtained with different apelin isoforms. In subsequent work carried out to investigate the mechanisms underlying apelin-mediated vasoconstriction, apelin was found to induce the phosphorylation of myosin light chains in vascular smooth muscle cells (VSMC) (Hashimoto et al. 2006). Thus, the theory was put forward

that apelin produces a biphasic effect on blood vessels involving endothelium-mediated vasodilatation and VSMC-dependent vasoconstriction. In the context of vascular disease, hypertension has been shown to be associated with endothelial dysfunction and reduced expression of apelin and APJ in heart and aorta (Zhong JC et al. 2007). Thus, it is possible to postulate a pathophysiological role for the apelin-APJ system in vascular disease (Ishida et al. 2004) in which apelin may function as a vasopressor in damaged vasculature (hypertension; atherosclerosis), but performs an antagonistic role to angiotensin under basal conditions.

1.9.4 Action

The APJ receptor mediates p44/42, Akt and p70S6K phosphorylation via protein kinase C (PKC) activation, the phosphorylation of all three kinases being sensitive to pertussis toxin (Masri et al. 2004; Masri et al. 2005).The actions of apelin have also been shown to reduce cAMP in cells transfected with human APJ receptor (Habata et al. 1999; Kawamata et al. 2001), an action that was inhibited again by pertussis toxin, suggesting that APJ receptor action is translated through an inhibitory G_i–protein complex (Reaux et al. 2001; Reaux et al. 2002; Medhurst et al., 2003).

1.9.4.1 Mitogenic action

The activation of the APJ receptor by apelin has revealed that it is capable of stimulating the growth of various cells types, including gastric cells, (Wang G et al. 2004) human umbilical vein endothelial cells (HUVECS) (Masri et al. 2004) and retinal endothelial cells (Kasai et al 2004). These actions, in part, occur through activation of both the PI3K and MAPK (ERK-1/2) pathways (Masri et al. 2002, Masri et al. 2004). In APJ-expressing human osteoblasts phosphorylation and activation of Akt, but not ERK-1/2, occurs in response to apelin (Xie et al. 2006). In a model of neuronal excitotoxic injury O'Donnell et al have obtained evidence that activation of neuronal APJ by apelin protects neurons. Furthermore, this protection was mediated by the stimulation of AKT and ERK-1/2 (O'Donnell et al. 2007). Together, these studies suggest that APJ signalling activates kinases (Akt, ERK-1/2) that are associated with cell survival.

1.9.4.2 Vascular Tone

As previously mentioned apelin peptides cause endothelium-dependent vasorelaxation by triggering the release of nitric oxide (NO), an effect that is almost completely abolished by the presence of the endothelial NO synthase (eNOS) inhibitor, NG-nitro-l-arginine methyl ester (L-NAME) (Tatemoto et al. 2001, Zhong et al. 2007). Work carried out in diabetic mice has shown that the beneficial effects of apelin on aortic vascular tone occur through the activation of Akt and eNOS (Zhong et al. 2007).

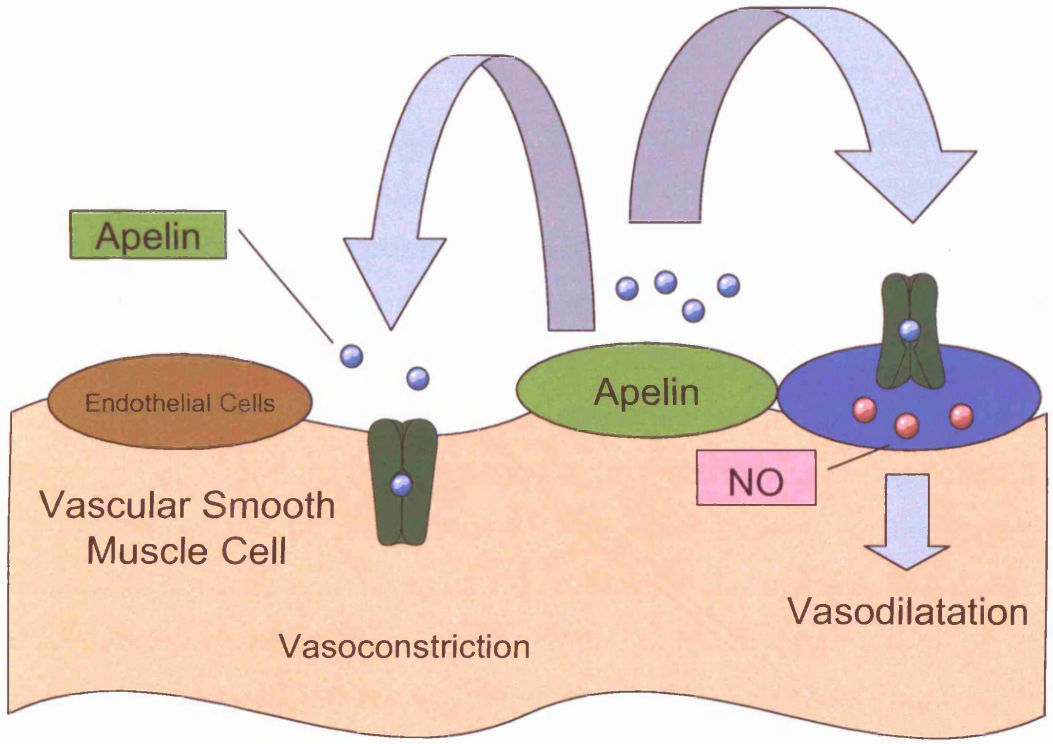

Figure 1-7 Vascular effects of apelin.

Apelin has been shown to produce vasodilatation via a nitric oxide (NO) pathway. In the absence of a functioning endothelium, apelin may directly activate APJ on vascular smooth muscle to cause vasoconstriction

1.9.4.3 Anti-apoptotic action

The anti-apoptotic effects of apelin have only recently become apparent. Inhibition by apelin of the release of cytochrome c, a critical protein in the apoptotic pathway, has been shown in an isolated cell model (Tang et al. 2006). Cytochrome *c* is released by the mitochondria in response to pro-apoptotic stimuli, as previously described. This release in turn activates caspase 9, a cysteine protease, stimulating the apoptotic cascade. Apelin was shown to inhibit the release of cytochrome c and activation of the apoptotic caspases (Tang et al. 2006). The use of inhibitors of JNK and PI3K led to the conclusion that this anti-apoptotic effect was mediated through these pathways.

1.9.5 Heart Failure

Interest in a protective role for apelin in clinical medicine is based on its unique properties. The triad of clinical sequelae that depict congestive cardiac failure i.e. fluid overload, vasoconstriction and pump failure are all potentially modifiable by apelin. Apelin's direct effect on cardiac contractility (Szokodi et al. 2002; Ashley et al. 2005) and its inhibitory action on vasopressin (Reaux et al. 2001; De Mota 2004) make it an ideal candidate for modulating the neurohumoral response which accompanies congestive cardiac failure (CCF). Apart from having actions on cardiovascular regulation, increasing evidence suggests a role for the apelin-APJ receptor system in the development of pathophysiological conditions relating to the cardiovascular system. The downregulation of cardiac APJ receptor expression seen in dilated cardiomyopathy (Foldes et al. 2003) may result in an attenuated cardiac response to apelin, leading to impaired contractility which is frequently seen in advanced heart failure. Decreased APJ receptor expression may also be a "down-regulation" phenomenon induced by an excess of ligand as apelin synthesis is up-regulated in the early stages of heart failure (Foldes et al. 2003). Analysis of cardiac gene expression in 11 heart failure patients before and after the implantation of a left ventricular assist device (LVAD) identified the APJ gene as being the most significantly and consistently up-regulated after the treatment (Chen M. et al. 2003). Apelin is not synthesised by adult cardiomyocytes, but in decompensated heart failure myocardial apelin synthesis is reactivated (Ashley et al. 2005). Taken together with the observed increases in the cardiac levels of the apelin peptide, these data

suggest that myocardial apelin synthesis is up-regulated in the early stages of heart failure, possibly reflecting an attempt by the myocardium to improve function (Chen et al. 2003). Subsequent down-regulation and desensitisation, which are common sequelae of chronic disease, leads to the loss of this amelioration by apelin.

1.9.6 Cardioprotection

The characterisation of the apelin-APJ system has allowed for conjecture as regards potential therapeutic strategies. This has followed the lines of the role apelin might play in blood pressure regulation and cardiac contractility. The effects of apelin in vivo have been studied and indicate a beneficial role in reducing left ventricular preload and afterload (Ashley et al. 2005). This finding taken together with apelin's observed propensity to decrease vasopressin output indicates that apelin may offer a unique alternative treatment for heart failure. The protective action of apelin on injured myocardium in vivo was first examined by Jia et al. 2005. Rat myocardial injury was induced by the administration of isoproterenol (ISO), a β-adrenergic agonist and well known inducer of myocardial hypertrophy. Apelin was subsequently administered and its effect on function and myocardial damage measured. The results suggested that apelin improved not only cardiac function but also myocardial injury in ISO-damaged hearts, myocardial damage being assessed by lactate dehydrogenase (LDH) leakage and the formation of lipid peroxides (elevated malondialdehyde formation). It was concluded that apelin may have cardioprotective properties and it could offer a new therapeutic target for cardiovascular disease (Jia et al 2005). This paper further supports the principle of the current study that apelin is cardioprotective.

1.9.7 Apelin as a cardiac biomarker

Further investigation into the possible clinical benefits of apelin has suggested a role for the peptide as a screening tool in atrial fibrillation (AF). Atrial fibrillation is a major independent risk factor for the subsequent development of left ventricular dysfunction, one third of affected patients going on to develop congestive cardiac failure within ten years (Wang et al, 2003). In a study in which an attempt was made to identify

biomarkers that might render individuals at risk of developing AF, it was found that in a homogenous group of patients with lone atrial fibrillation plasma apelin was significantly reduced compared with matched controls (Ellinor et al. 2006). This finding increased interest in the role abnormal biomarker profiles play in the screening, diagnosis, monitoring and prognosis of chronic heart failure (Tinenburg et al 1998), and indicated that apelin might be used as a discriminatory tool for the subsequent development of AF (Ellinor et al 2006).

The studies described have taken our knowledge regarding apelin from the point of its identification as a unique vasoactive peptide to the present stage where it is being suggested as a potential therapeutic tool. Further studies into the part played by apelin in the setting of myocardial infarction, i.e. ischaemia/reperfusion, are now urgently needed and form the basis of the investigation presented in this thesis.

2 AIMS

As discussed in chapter 1 the novel peptide apelin appears to have various functions, its actions on the cardiovascular system probably being the most important. The ability of apelin to stimulate kinases associated with the RISK pathway (Masri et al, 2004) and to act as an anti-apoptotic agent (Tang et al., 2007) and promoter of cell growth (Wang G et al., 2004; Masri et al. 2004, Kasai et al 2004) suggest that this peptide may play a role in tissue preservation as its features are characteristic of potential cardio-protective agents. Recently apelin was shown to act as a neuroprotective agent (O'Connell et al. 2007) and to reduce markers of ischaemic damage in ISO injured hearts (Jia et al 2005). Thus, it was hypothesised that apelin may protect the myocardium against ischaemia-reperfusion injury. This study, therefore, set out to examine if apelin is cardioprotective when given at reperfusion and whether any cardioprotective actions are mediated by the RISK pathway and modulation of the mPTP. Ultimately, the aim of the present study was to establish if apelin might find application as a therapeutic agent in the context of myocardial ischaemia/reperfusion injury.

3 METHODS

3.1 General

All the experimental procedures undertaken and described below were performed in the Laboratory of the Hatter Cardiovascular Institute and Biological Services Units, University College London Medical School, University College Hospitals. Animal experimentation was performed in accordance with the United Kingdom Home office 'Guidance on the operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationary Office, London.

3.2 Choice of animal model

The use of rodent models of myocardial ischaemia-reperfusion injury is well established. The characteristics of these models have been described by various groups, (Headrick et al. 2001) including our own, and have been proven to yield reproducible data (Awan et al 1999, Marber et al. 1995, Efthymiou et al. 2006, Smith et al., 2006., Sumeray & Yellon 1998). A murine model offers the advantage of being amenable to genetic manipulation and various transgenic knock-out and knock-in animals have been developed. This has permitted detailed examination of subcellular mechanisms which may have relevance to cardioprotection. In light of these facts a murine Langendorff model was chosen for this study.

Male Swiss White mice obtained from Charles River UK (Margate UK) were used initially to establish and characterise the ischaemic reperfusion model. C57 BL/6J mice were subsequently (Charles River) used to investigate the effects of ischaemic preconditioning and the administration of apelin as future investigations were planned in which genetically manipulated forms of this species where to be used.

3.3 Chemicals and drugs

All constituents of the Krebs Henseleit buffer were purchased from BDH Laboratory supplies (Merck Eurolab, Dorset England). Apelin–13, Apelin-36 and 2,3,5- Triphenyl-

Tetrazolium Chloride (TTC) were purchased from Sigma-Aldrich Company Ltd (Dorset, England). Apelin F13 was purchased from Phoenix pharmaceuticals USA.

3.4 Preparation of hearts for perfusion

3.4.1 Anticoagulation

Prior to removal of the heart, heparin was administered via an intra-peritoneal (i.p.) injection. This was in order to prevent the development of thrombus within the coronary vasculature or ventricular chambers. Animals were then killed by cervical dislocation which was carried out under the guidelines of Schedule 1 of the Scientific Procedures Act 1986.

3.4.2 Dissection

A bilateral transverse thoracosternotomy ("clam shell" incision) was used to expose the visceral surface of the thoracic cavity. The incision was made below the xiphoid sternum and extended laterally to the ends of the left and right costal margins. The anterior chest wall was reflected providing an optimal operating field. The thoracic organs were then removed en-bloc by transecting the descending aorta and inferior vena cava, followed by the ascending aorta and superior vena cava. The heart was dissected free from the lungs, thymus and fatty tissue and placed in Krebs-Henseleit buffer (at 4 ^{o}C). The ascending aorta and its root were then visualised and a 21 gauge flanged stainless steel murine cannula was inserted into the aorta (Figure 3-1). This was carried out under Krebs solution to avoid any risk of air embolism (Figure 3-2). The aorta was then secured with a 5-0 silk suture to the cannula, taking care that the flange sat above the aortic root, and the heart then transferred to the Langendorff perfusion apparatus. Once attached to the perfusion apparatus retrograde perfusion began. The time taken to the onset of Langendorff perfusion was kept to three minutes to reduce the potential effect of ischaemic preconditioning due to delayed perfusion (Minhaz et al. 1995, Awan et al. 1999).

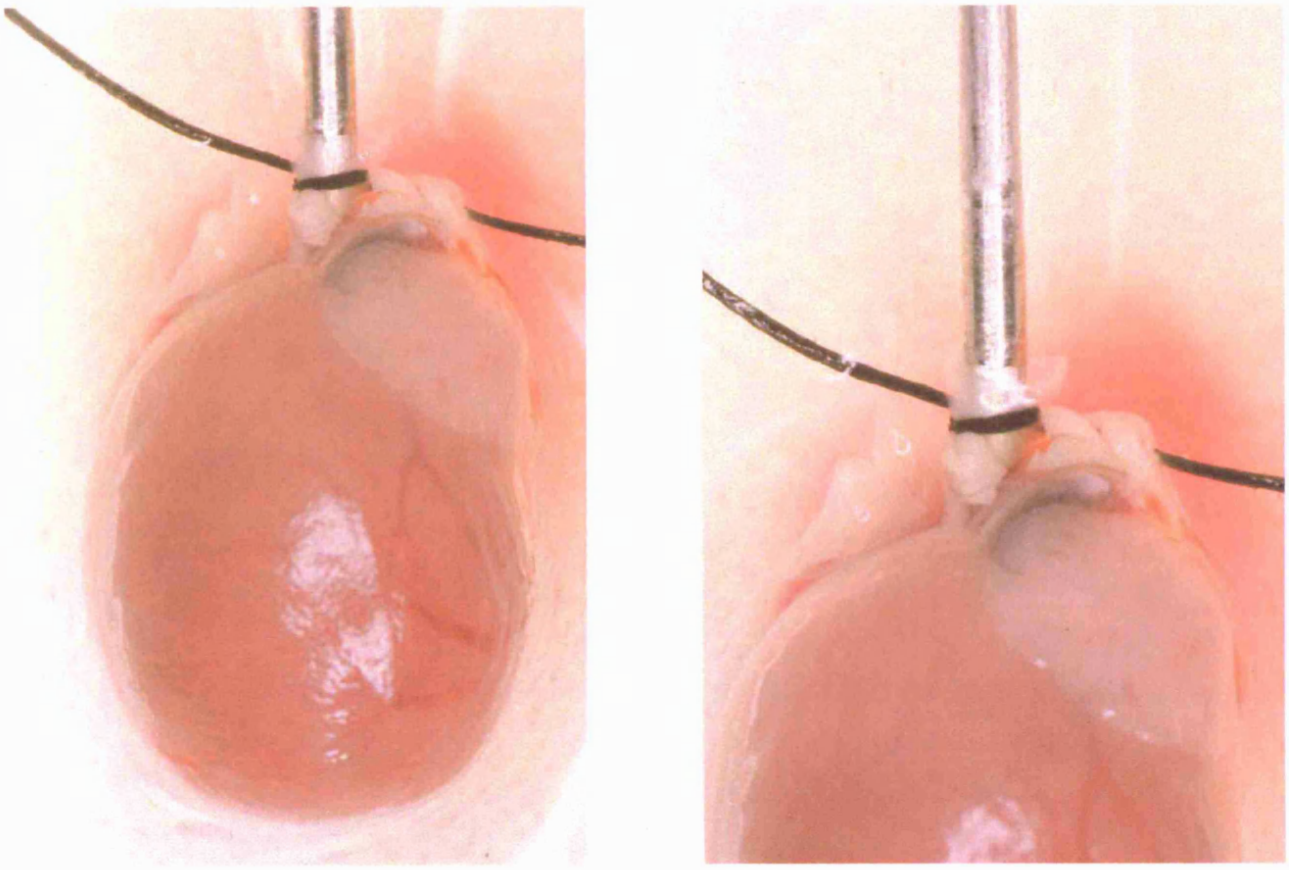

Figure 3-1 Aortic cannulation

This figure shows the placement of the murine cannula into the aorta and the application of the 5/0 silk suture. The cannula was inserted in such a manner that the flange sat above the aortic root to ensure perfusion of the coronary circulation.

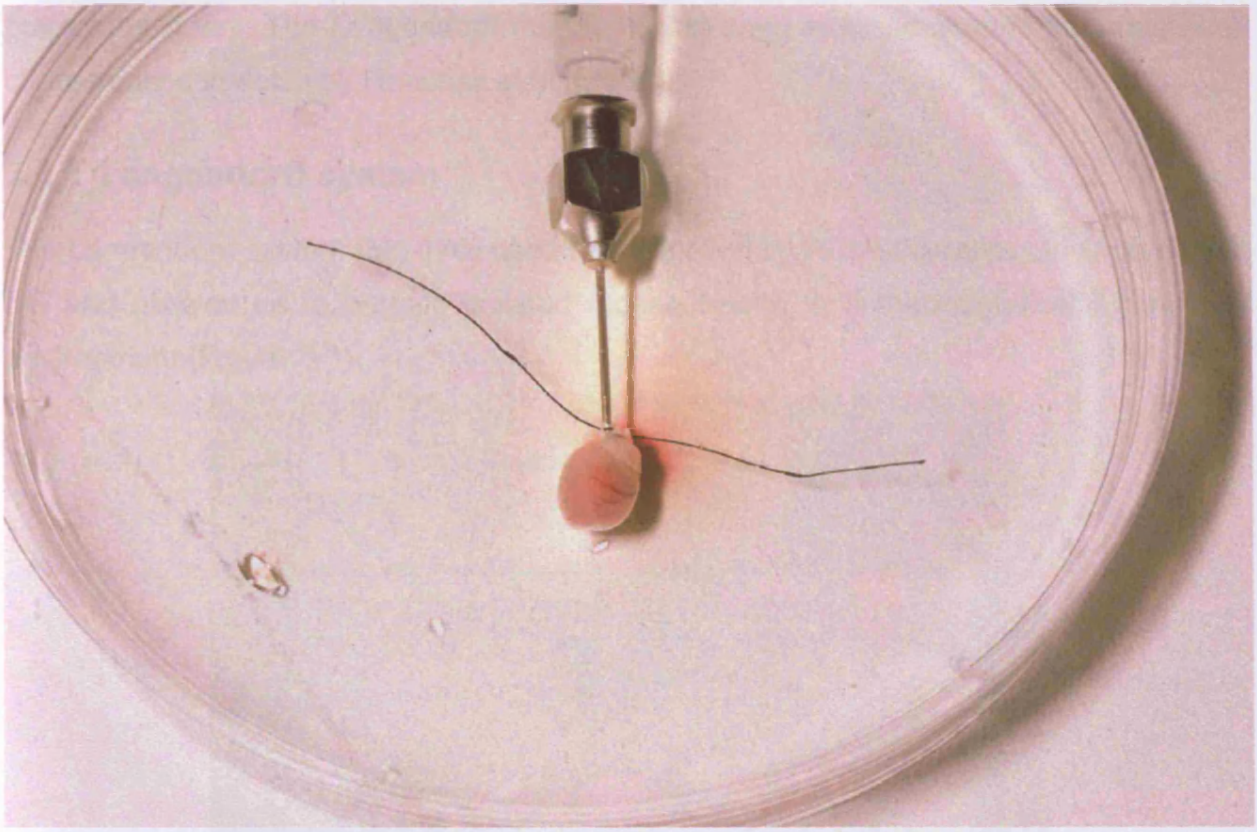

Figure 3-2 Cannulation under Krebs solution

Cannulation was carried out in ice-cold Krebs solution. This was to avoid any risk of air embolism and to reduce the potential effect of ischaemic preconditioning due to delayed perfusion. (Minhaz et al 1995, Awan et al 1999)

3.5 Langendorff perfusion

The methodology for the retrograde perfusion of isolated hearts was developed originally by Oscar Langendorff in 1895 and is recognised as a suitable experimental model for studying myocardial function and metabolism. (Langendorff, O. Untersuchungen am überlebenden Säugethierherzen. Pflügers Arch. 61: 291–332, 1895). The basic goal of the Langendorff model is to keep an isolated heart supplied with oxygen and metabolites via a single cannula placed into the ascending aorta. This constant retrograde perfusion keeps the aortic valve closed and allows oxygenated buffer to flow through the coronary vessels. The fluid then flows through the arterial branches to the arterioles, capillaries and coronary veins. It eventually passes via the coronary sinus into the right atrium. It leaves the heart via the excised ends of the

pulmonary artery. The Langendorff murine model used in these experiments has been characterised in detail by Headrick et al. (2001).

3.5.1 Langendorff system

The Langendorff system (ML 176) used was supplied by ADinstruments Ltd Oxfordshire, UK and allowed us to perfuse isolated mouse hearts in a thermostatically controlled environment (Figure 3-3).

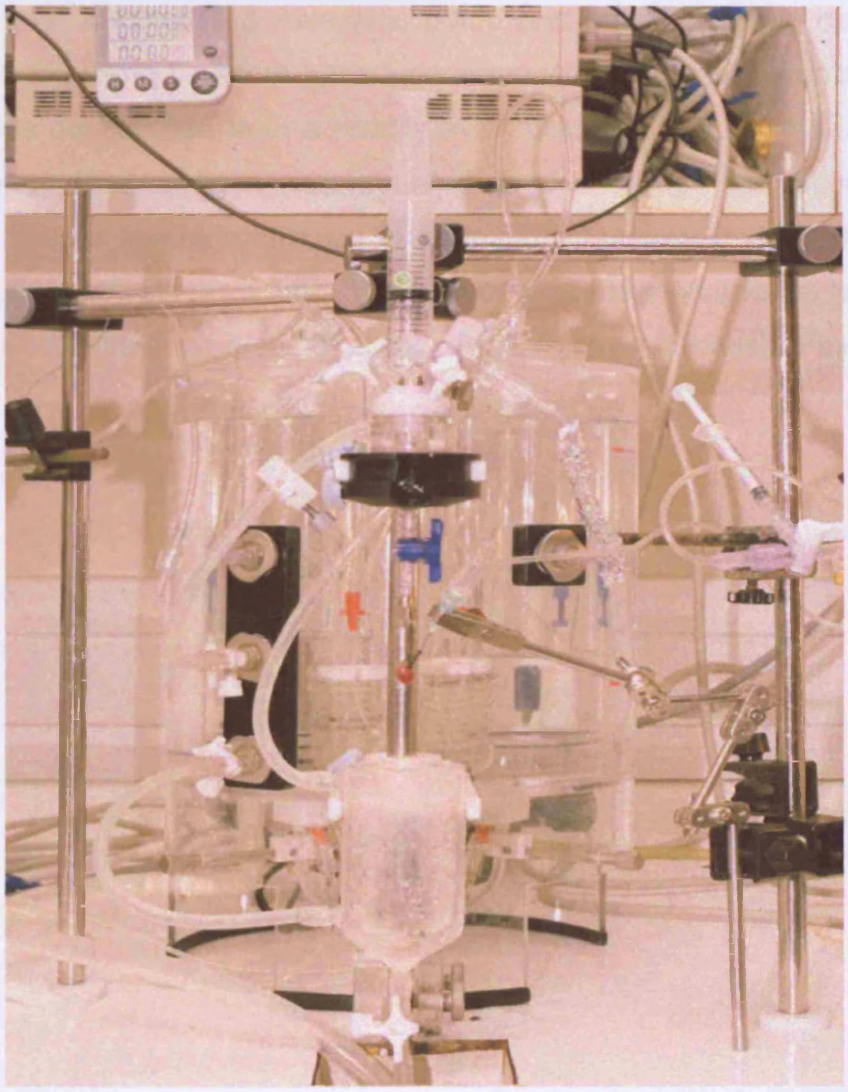

Figure 3-3 The Langendorff system

The perfusate reservoirs consisted of two self-contained compartments which were oxygenated individually with 95% O_2 and 5% CO_2 (BOC Gases, Manchester). Surrounding these two reservoirs was a thermostatically-controlled water jacket. This

allowed the perfusate temperature to be adjusted to reduce the loss of heat which occurs as fluid travels through the system, thus allowing the hearts to be maintained at 37°C. Heart temperature was monitored with a T-type thermocouple probe placed in the pulmonary vascular trunk. A separate thermostatically controlled water jacket was used to surround the heart and permitted fine tuning of the temperature during stabilisation, ischaemia and reperfusion. A constant myocardial temperature of 37°C (range 36.5-37.5°C) was maintained throughout the experimental protocol. Constant flow or pressure was achieved with a Minipuls 3 Peristaltic pump and the STH pump controller. Heart rate was measured using pressure transducer connected to an intra-ventricular balloon. The balloon was constructed from a readily available, pliable polyvinyl chloride plastic film (Saran wrap). Each balloon was mounted on a 21-gauge flanged stainless steel tube. Deflated balloons were connected to a pressure transducer (MLT 884) by a fluid-filled non compliant polyethylene tube. The balloon was inserted into the left ventricle via the left atrium and inflated to an end diastolic pressure of 5-10 mmHg (Figure 3-4).

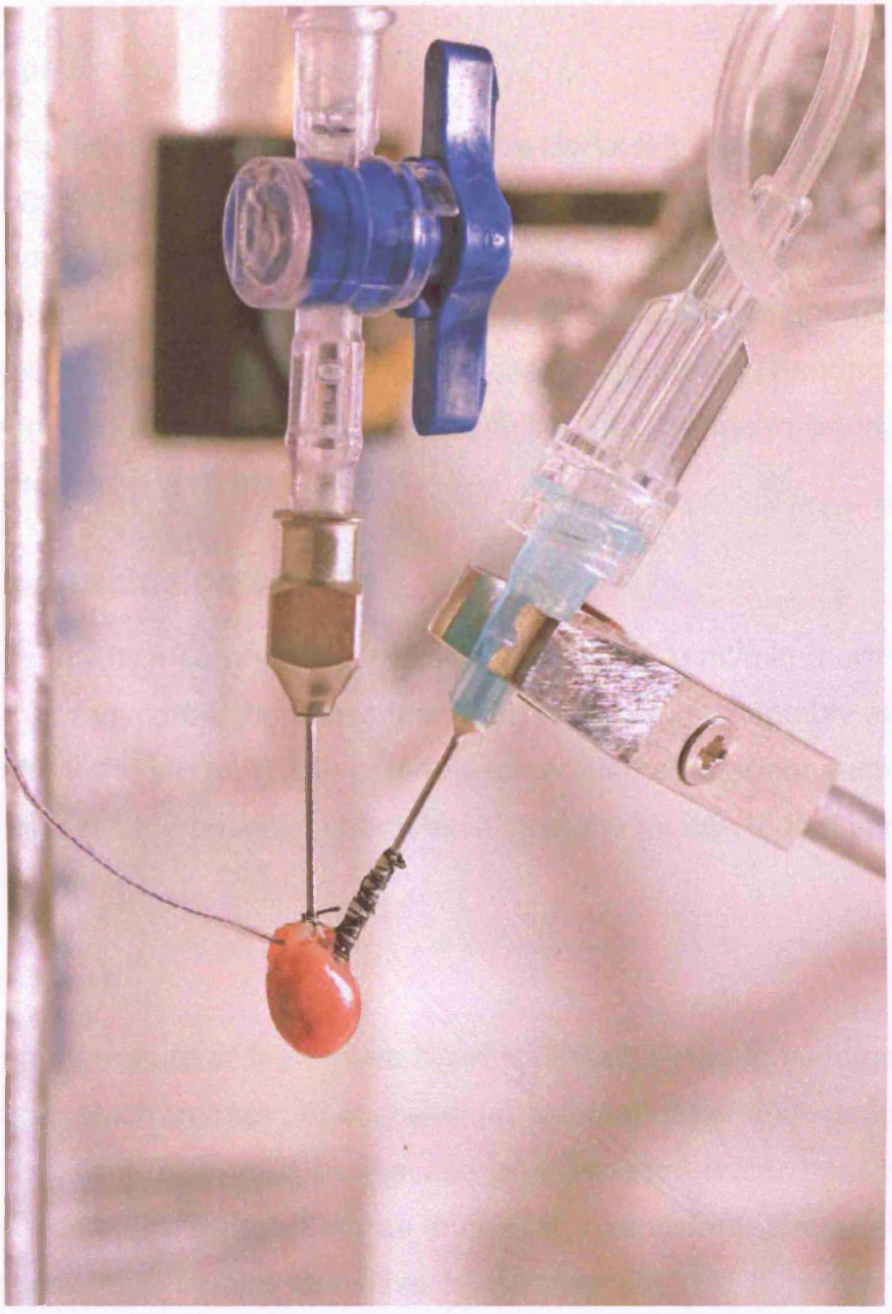

Figure 3-4 Left ventricular balloon transducer

This shows the placement of the transducer into the left ventricular cavity and the thermocouple into the right side of the heart.

3.5.2 Perfusion

The perfusion buffer used was modified Krebs-Henseleit buffer consisting of NaCl 118mM, $NaHCO_3$ 24 mM, KCl 4 mM, NaH_2PO_4 1 mM, $CaCl_2$ 2.5mM, $MgCl_2$ 1.2 mM, and glucose 10mM. The buffer was gassed with 95% O_2 and 5% CO_2 at 37°C in order to yield a pH of 7.4. Buffer was initially passed through a 5 µm filter to remove mircoparticulates before being placed in the reservoir chambers (Headrick et al. 2001). Hearts were perfused at a constant pressure, this being the mean arterial pressure of the conscious mouse i.e. 110 mmHg.

3.5.3 Inclusion/exclusion criteria

Stabilised hearts in which coronary flow was greater than 6.0 ml/min (normally due to an aortic tear), were bradycardic (bpm less than 300) or were unacceptably arrhythmogenic were excluded from the study. These criteria were arrived at after conducting a series of experiments to assess the baseline characteristics of the murine model and its response to ischaemia-reperfusion.

3.5.4 Temperature

Controlling the temperature of the mouse heart is difficult owing to its large surface area to volume ratio, which makes it susceptible to heat loss and rapid changes in temperature. This was minimised by the use of a jacketed water bath which allowed submersion of hearts in warmed Krebs buffer at a constant temperature of 37°C. Contractile function is extremely sensitive to temperature and hypothermia has been shown to reduce infarct size resulting from ischaemia-reperfusion (Hale & Kloner 1999). Temperature-dependent changes in heart rate can also explain some of the changes seen in contractile function. Model characterisation has shown that stepwise increments in contractile function occur as heart rate is increased up to 450 bpm, beyond which function declines (Headrick et al. 2001).

3.5.5 Standard Protocol

After hearts were mounted and the balloon and temperature probes inserted, an equilibration/stabilisation period followed in which hearts were assessed for their suitability for experimentation. The inclusion criteria described in 3.5.3 were applied during this period. No-flow ischaemia was then induced by switching off the perfusion pump for 35 minutes. Reperfusion was commenced by switching the pump back on so leading to re-flow throughout the Langendorff system, and was continued for 35 minutes.

3.6 Measurement of infarct size

3.6.1 TTC staining

For the histochemical determination of infarct size, hearts were injected with 5 ml of 1% Triphenyl-Tetrazolium chloride (TTC) solution (w/v) in phosphate buffer (Na_2HPO_4 45.1 mM, NaH_2PO_4 3.3 mM, pH 7.8, 37°C) at the end of reperfusion. The hearts were then immersed in a 10 ml Falcon tube containing 1% TTC (w/v) at 37°C for 10 minutes. TTC is reduced by NADH and dehydogenase enzymes and causes all tissue with retained enzymes and co-factors to stain a brick red colour. Infarcted areas do not react with the TTC and therefore do not stain (Ytrehus et al. 1994) (Fishbein et al. 1981). Following incubation hearts were dried, weighed and frozen at -20°C. The frozen hearts were then sliced parallel to the atrio-ventricular groove into 5-8 slices. These were then placed in 10% Formalin in order to enhance the contrast between infarcted and non-infarcted areas.

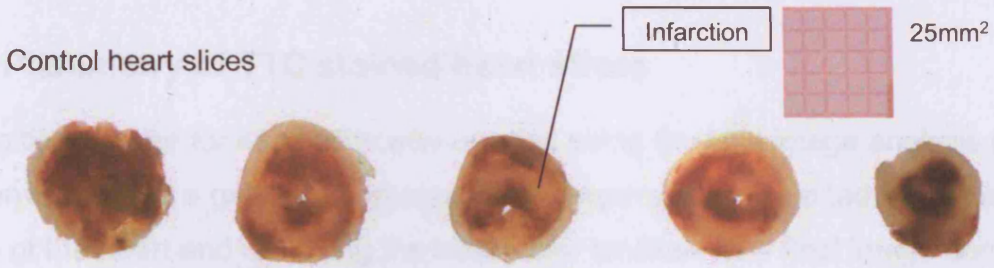

Figure 3-5 Sliced murine hearts.

Formalin allows for the enhancement of the contrast between infarcted and non-infarcted areas. The non-infarcted area stains red and the infarcted areas which have not reacted with TTC, and therefore do not stain, appear white.

3.6.2 Digitising TTC stained hearts

On analysis the sliced hearts were mounted between two plexiglass plates, separated by spacers. The mounted slices were then photographed using a high resolution megapixel camera. Individually photographed slices were then analysed with the National Institutes of Health freeshare image analysis programme (NIH version 1.63).

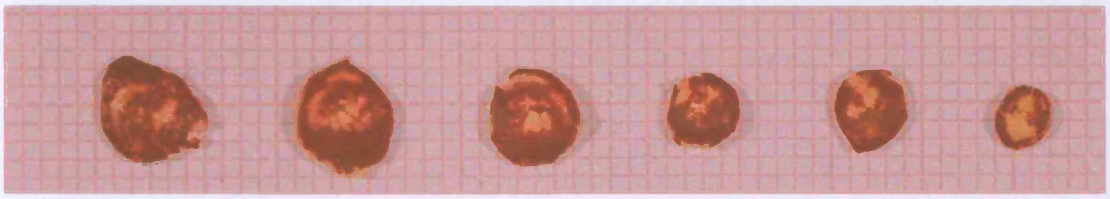

Figure 3-6 Sliced hearts prior to planimetry

Hearts are aligned and are mounted between two plexiglass plates. They are photographed and the images digitised.

3.6.3 Planimetry of TTC stained heart slices

The digitised image for each slice was opened using the NIH image analysis programme and converted into a grey-scale image. This image was then edited by demarcating the outline of the heart and removing the ventricular cavities. The final image corresponds to the area at risk and is then analysed using the infarct area macro. During these stages the images were saved onto the hard drive to provide a permanent record of the slices through the editing process. Calibration for each heart was carried out prior to analysis. A known area of graph paper i.e. 25 mm^2 was also converted into greyscale and the number of pixels for 25 mm^2 established. Subsequently this value was used as a reference when calculating the area for heart slices. The infarct area macro was then used to calculate the number of pixels which are white and therefore infarcted relative to the number which are grey and hence not infarcted. The results were then calculated as the total number of pixels infarcted against the total area of the slice, ultimately being expressed in mm^2 with the infarcted area expressed as a percentage of the area at risk. All analysis and measurements were carried out in a randomised blinded manner.

3.7 Isolated cardiomyocyte model

3.7.1 Introduction

Mitochondria are the key organelles responsible for cellular respiration and energy generation. Their production of ATP which powers virtually every biochemical reaction in the body is fundamental to the survival of cellular organisms. More recently it has become evident that they play an important role in apoptosis and the development of techniques for assessing mitochondrial function has allowed cellular-based modelling of ischaemia/reperfusion. Oxidative stress caused by the production of reactive oxygen species (ROS), is important in many disease processes and is part of the ischaemic/reperfusion cascade which leads to cellular death. Mitochondria are a major source of ROS and offer, therefore, a controlled model of mitochondrial stimulation and ROS release. ROS can trigger a transient increase in mitochondrial ROS production via the activation of the mitochondrial permeability transition pore, a phenomenon termed ROS-induced ROS release (RIRR) (Zorov et al. 2000). The mitochondrial permeability transition pore, or mPTP, is a protein pore that is formed in the membranes of mitochondria under ischaemic conditions such as myocardial infarction and stroke. An increase in the permeability of the mitochondrial membrane, referred to as mitochondrial permeability transition (mPT), results from opening of the permeability transition pore. Opening of the pore can lead to mitochondrial swelling and cell death. Using a model of oxidative stress involving mPTP opening in isolated cardiomyocytes would, therefore, simulate the events associated with reperfusion-induced cell injury (Jacobson & Duchen 2002) ; (Hausenloy, Duchen, & Yellon 2003), (Hausenloy & Yellon 2004).

3.7.2 Preparation of adult rat cardiomyocytes

Adult rat cardiomyocytes were used in preference to C57 Bl/6J mice cardiomyocytes as the latter can prove difficult to obtain in sufficient numbers due to their fragility. Ventricular cardiomyocytes were, therefore, isolated from adult male Sprague–Dawley rats (Charles River). Induction of anaesthesia was performed with sodium pentobarbital (55 mg/kg i.p) which was administered together with heparin sodium (300 IU) to reduce clotting. Hearts were rapidly excised, placed in ice-cold buffer, and mounted on a non-

recirculating perfusion apparatus. All solutions used were based on a modified calcium-free Krebs-Ringer-HEPES (KRH) buffer (in mM): 116.0 NaCl, 5.4 KCl, 0.4 $MgSO_4$, 20.0 Taurine, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 10 glucose (pH 7.4). The perfusate was bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. The hearts were first perfused with KRH buffer. After 5 min, the hearts were then perfused with KRH buffer containing 1 mg/ml collagenase (Worthington type II) and 44 μM calcium for 10–15 min. After perfusion, the hearts were removed from the perfusion apparatus and the atria were trimmed away. The ventricles were sliced into longitudinal strips and underwent further digestion with collagenase in a process which involved mechanical agitation of the strips for 10 minutes at 37°C after which the solution containing cellular debris was discarded. Fresh KRH Buffer (15ml) containing 1 mg/ml collagenase and 44μM calcium was then added and the suspension agitated for a further 20 minutes whereupon the cells in solution were transferred to a fresh Falcon tube and centrifuged. The cells were then re-suspended in KRH Buffer (44μM calcium) and seeded onto sterilized laminin-coated 25-mm-diameter round coverslips and incubated at 37°C in an atmosphere of 95% air-5% CO_2 in M-199 medium (M7653, Sigma) containing 10% fetal calf serum and 1% penicillin-streptomycin (Sigma). After approximately one hour the cells were washed with 1 ml of plating medium (PM) which was then replaced with a further 1ml of PM.

3.7.3 Model for induction and detection of the mPT in intact cells

Isolated adult ventricular myocytes were incubated with the fluorescent dye Tetramethylrhodamine methyl ester (TMRM) (3 μM) for 15 min at 37°C, washed, and visualized using confocal fluorescence microscopy. TMRM is a cell-permeable fluorescent dye that accumulates selectively into mitochondria according to the membrane potential. Laser scanning confocal microscopy (LSCM) was used to generate mitochondrial ROS within a defined region of a cardiomyocyte.

3.7.3.1 Confocal fluorescence imaging

The coverslip containing the myocytes was placed in a chamber and mounted on the stage of a Zeiss 510 CLSM confocal microscope equipped with x40 oil immersion, quartz objective lens (numerical aperture 1.3). For TMRM fluorescence, the cells were illuminated by use of the 543-nm emission line of a HeNe laser.

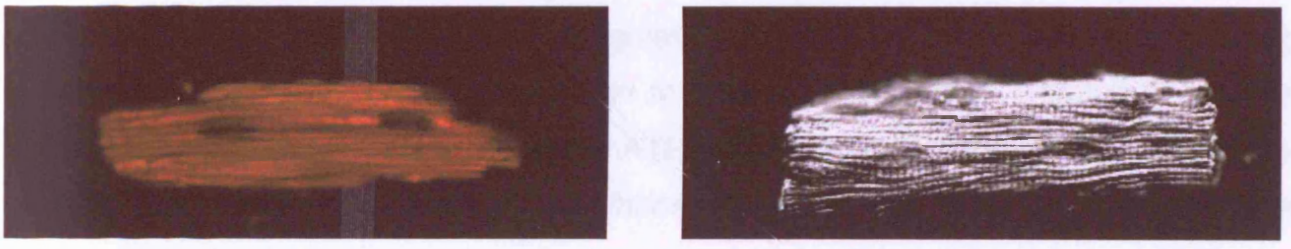

Figure 3-7 Isolated cardiomyocytes imaged under confocal microscopy.
Two images taken before laser stimulation.

For all photosensitization experiments, the settings for the confocal imaging system were identical to ensure comparability between experiments. TMRM fluorescence was collected using a 585-nm long-pass filter and images analyzed by use of Zeiss software (LSM 2.8).

Laser photoexcitation of TMRM generates sufficient levels of ROS from within the mitochondria to activate the mPTP. Reperfusion of ischaemic myocardium also involves excess production of ROS from within the mitochondria. Mitochondrial permeability transition increases as the pore forms. Increasing membrane permeability causes mitochondria to become depolarized i.e. the mitochondrial membrane potential or difference in voltage between the inside and outside of the mitochondrial membrane (known as $\Delta\psi$), is lost. TMRM accumulates in mitochondria because of the negative potential of the mitochondrial matrix (about -150 mV relative to the cytoplasm) (Duchen et al. 1998). Depolarization leads to a redistribution of TMRM from the mitochondria to the cytoplasm with a resulting decrease in quenching (dequenching) and increase in the fluorescence generated by the whole cell. Mitochondrial membrane depolarization, therefore, results in the loss of dye to the cytosol where the signal increases (autoquenching) (Duchen & Biscoe 1992). Laser-induced oxidative stress was applied until the mPTP had been provoked (indicated by the collapse of the mitochondrial membrane potential $\Delta\psi$) and continued until irreversible cellular damage had occurred, as indicated by myocyte shortening to a 'hyper-contracted' state with the loss of striations and a change from a rectangular to a more rounded cellular morphology (Silverman et al. 1994). The times taken to induce mitochondrial membrane potential collapse and hypercontracture (signalling ATP depletion) were measured and the values obtained under control (untreated) conditions compared with those observed following drug treatment.

3.8 Western Blotting

3.8.1 Tissue preparation

Mouse hearts were isolated and Langendorff perfused as previously described (Chapter 3.4) except that the period of reperfusion was shortened. Thus, hearts underwent 30 minutes of stabilization followed by 35 minutes of global ischaemia and 5 or 10 minutes of reperfusion. After the termination of reperfusion, the hearts were removed from the Langendorff apparatus and immediately freeze-crushed in liquid nitrogen using a pre-chilled Wollenberger clamp. The hearts were then stored at –80°C for later analysis.

3.8.2 Protein extraction

Each heart was homogenized on ice in 0.6ml of suspension buffer using a Polytron T25 homogeniser. The suspension buffer consisted of NaCl 100 mM, TRIS 10 mM (pH 7.6), EDTA 1 mM (pH 8.0), sodium pyrophosphate 2 mM, sodium fluoride 2 mM, glycerophosphate 2 mM; 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride (AEBSF HCl) 0.1 mg/ml, and Sigma proteases inhibitor cocktail. The homogenate was then centrifuged for 10 minutes at 10,000 rpm at 4°C and three aliquots of 160 µl of the protein-rich supernatant transferred to lockable 2ml Eppendorff tubes. Two of these aliquots were frozen at -80°C for future analysis. Two x 5 µl aliquots were removed from the final aliquot and used for protein quantification, whilst the remaining 150µl went for SDS gel electrophoresis.

The 150 µl samples were diluted with an equal volume of sample buffer consisting of 100mM TRIS (pH 6.8), 10 % SDS, bromophenol blue 0.2%, glycerol 20% and 200mM dithiothreitol (DTT) and then placed in a heating block for 10 minutes at 100^{0}C to denature the proteins. The denatured samples were stored at -80^{0}C for subsequent electrophoresis.

3.8.3 Protein Estimation

The protein contents of extracted tissue samples were estimated using a Bicinchoninic acid based (BCA™) protein assay reagent system (Pierce, Rockford, USA). The assay relies on the reduction of ionised copper and the formation of a BCA-Cu^{+} complex. In this assay the BCA-Cu^{+} complex turns purple after 30 minutes of incubation, the optical density then being measured at a wavelength of 562 nm using a spectrophotometer (Janway model 6405 UV/Vis, Dunmow, UK). Using this assay the relationship between protein content and the absorbance at 562 nm is linear over a wide concentration range (20 - 2000 µg/ml). Duplicate samples of increasing concentrations of bovine serum albumin (BSA) in suspension buffer 0, 20, 40, 60, 80, µg were used to generate the standard curve. Duplicate extracted 5µl protein samples were then quantified using this method, and a mean value for each sample recorded. This was compared against the standard curve to provide an estimate of the protein concentration (µg/l) and to ensure that equal protein loading onto polyacrylamide gels occurred.

3.8.4 Polyacrylamide gel electrophoresis

3.8.4.1 Gel Electrophoresis

The constituent proteins of sample extracts were separated according to molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The samples were loaded side by side into "wells" formed in the gel, the proteins travelling in one dimension along the gel.

3.8.4.2 Gel Preparation

SDS gels were formed between two glass plates that had been thoroughly cleaned with 70% ethanol. The running gel consisted of 24 ml of dH_2O, 18ml running gel base (1.5M Tris, 0.4% SDS in distilled H_2O, pH 8.8), 30ml 30% acrylamide, 80 µL TEMED (NNN-tetraethylethalinediamine) and 400 µL 10% ammonium persulphate (APS), and after pouring were left to set for 30 minutes.

Plastic combs were used to create the wells into which protein samples were loaded. A stacking gel consisting of 7 ml distilled water, 3ml stacking gel base (0.5 M TRIS, 0.4% SDS in dH_2O, pH 6.8 with HCL acid), 2ml 30% acrylamide, 20 µl 8% bromophenol blue, 24 µl TMED and 120 µl 10% APS was pipetted above the running gel to fill the area around the combs. It took 10 minutes for the stacking gel to set and after this time the combs were removed and the gels placed in the electrophoresis apparatus. Running buffer was then poured into the top half of the rig (comprising glycine 14.42 g/l, SDS 1.0 g/l, Tris 3.0 g/l, distilled H_2O 1.0 L).

The first lane was loaded with a standard reference mixture of proteins (Precision plus protein dual colour standards, Bio Rad) with defined molecular weights. Subsequent wells were then loaded with 60 µg of cardiac protein extracts. The gel was then placed into a vertical electrophoresis tank connected to a water cooling circuit and the tank topped up with running buffer. The gels were then run at 200 V for 3-4 hours. The electric current applied across the gel, caused the negatively-charged proteins to migrate down the gel. Depending on their size, each protein moved differently through the gel matrix: short proteins pass more easily through the pores in the gel, while larger ones have more difficulty and remain closer to the point of origin. Satisfactory electrophoresis was confirmed by migration of the bromophenol blue maker.

3.8.4.3 Protein Transfer

The gels were placed in a protein transfer tank containing transfer buffer: transfer buffer stock solution (glycine 144.2 g, Tris 30.3 g, distilled water 1 litre) 100mls, methanol 200ml and 700ml of distilled water. In order to render the proteins accessible to antibody detection it is necessary to transfer them from the gel onto a nitrocellulose membrane (Hybond ECL membrane (Amersham)). To achieve this, membranes were placed on top of the gels, through which a current was applied. The charged proteins then moved from within the gel onto the membrane whilst maintaining the position they had on the gel. Transfer was allowed to occur overnight (12-16 hours) whilst maintaining the current at 140mA.

3.8.4.4 Antibody Probing

Following protein transfer nitrocellulose membranes were placed in plastic containers on a mechanical shaker and washed with a washing buffer (50 ml 10 x TBS, 450 ml deionised water and 0.5 ml Tween-20 (Sigma). To prevent non-specific interactions between the proteins on the membrane and the antibodies used for detection of the target protein, a blocking buffer was applied (TBS and Tween with 5% Marvel (dried skimmed milk powder)) and the membranes rocked gently on a rocking platform for 5 minutes. Membranes were then washed three times with TBS+Tween.

The primary antibody solution (5% BSA in TBS+Tween plus the antibody at a 1:250 dilution) was applied to the membranes which were placed on the rocking platform for at least 3 hours. The primary antibody was then removed and the membranes washed three times for five minutes with TBS+Tween.

Secondary antibody solution was then used to detect the primary antibody and allowed to wash over the membranes for at least 1 hour. The secondary antibody used was affinity purified goat anti-rabbit IgG (H&L) conjugated to horseradish peroxidase (Cell Signalling); for β-Actin a rabbit polyclonal to mouse IgG (H&L) conjugated to horseradish peroxidase (Abcam Ltd. Cambridge) was employed. β-Actin is one of the most abundant proteins in eukaryotic cells and was used as an internal standard for the correction of sample protein densitometry readings i.e. differences regarding gel protein loading. A final set of three five minute washes was then performed before applying an enhanced chemi-luminescent (ECL) Western blotting detection reagent. This allowed visualisation of the protein bands on exposure of the membrane to Kodak Omat XR photographic film. The film was developed using a Kodak XOMAT 1000 Film Developer (Serial No. 2225; Kodak House, Hemel Hempstead, HP11JU).

3.8.4.5 Stripping and re-probing

To allow analysis of phosphorylated, total and β actin proteins, membranes were stripped of the previous antibody before re-probing. The membranes were stripped using a 0.2M solution of NaOH which was poured onto the membrane whilst on the rocking platform and then washed off using distilled water after five minutes. Membranes were not stripped more than twice to prevent excessive loss of bound proteins which

can occur as a result of the stripping process and lead to a slight degradation in signal quality on probing and development. After washing with three-four changes of distilled H_2O the process described above in Antibody Probing 2.8.4.4 was followed.

3.8.4.6 Densitometry of protein bands

The quantification of protein bands was carried out using The National Institutes of Health (NIH) Shareware programme; NIH Image (version 1.63).The photographic films were scanned onto a computer using a flatbed document scanner. These images were converted to greyscale images and using a specific gel-plotting programme the relative densities for the individual protein bands calculated.

3.8.5 Statistical Analysis

All results are presented as group means ± standard error of the mean (SEM). For comparison between two groups, data were compared with Student's unpaired t-test. For comparison between more than two groups, factorial one way analysis of variance (ANOVA) was employed. Where a significant F-value was obtained, the Fishers protected least significance difference (PLSD) post hoc test was applied for between group comparisons. Results were considered significant when $P \leq 0.05$. All statistical analysis was carried out on a Power Macintosh computer, using Statview statistical software (Version 4.5, Abacus Concepts Inc.).

4 MODEL CHARACTERISATION

4.1 Ischaemic reperfusion model

The use of an accurate reproducible model is fundamental to the investigation of the mechanisms involved in ischaemic reperfusion injury. In order to validate the methodology a series of characterisation experiments were carried out to establish the optimal durations for ischaemia and reperfusion. As infarct size was to be used as the primary end-point in all studies the protocol used needed to be easily reproducible thus allowing accurate assessment of changes in infarct size. From this we were able to ascertain the optimal ischaemia/reperfusion period for consistent and accurate results within a practical time-frame.

4.2 The effects of global ischaemia

A murine model was chosen for these studies because of the possibility of using transgenic mice for further investigations. Male Swiss White mice aged between 12 -16 weeks and weighed between 20-30g were used in all experiments. The challenging nature of the murine Langendorff model meant in order to reduce the losses of a more expensive mouse strain i.e. C57 Bl/6J, Swiss White mice were employed in the initial learning phase of the technique.

Due to the technically demanding nature of using a mouse Langendorff model, global ischaemia was applied as opposed to regional ischaemia In larger rodent and animal models regional ischaemia is used due to the obvious ease of identifying and ligating an arterial vessel. This is not possible with any degree of accuracy when using an isolated, contracting murine heart. Initially, the effect of varying global ischaemic time was investigated, so that a time period which yielded reproducible infarct sizes could be established. The infarct size also had to be sufficiently large that the effects of a mediator of reperfusion injury could be accurately assessed.

Three ischaemic time periods were studied, with the addition of a sham treatment in which hearts were followed through the total time course of the experiment without undergoing infarction. The experimental protocol consisted of a stabilisation period of 30

minutes 35, 40 or 60 minutes ischaemia and 35 minutes reperfusion. This protocol was in keeping with previous characterisation studies from this laboratory (Sumeray & Yellon 1998), Figure 4-1. The operative mortality rate in the Langendorff model was 15% and was evenly distributed between the groups.

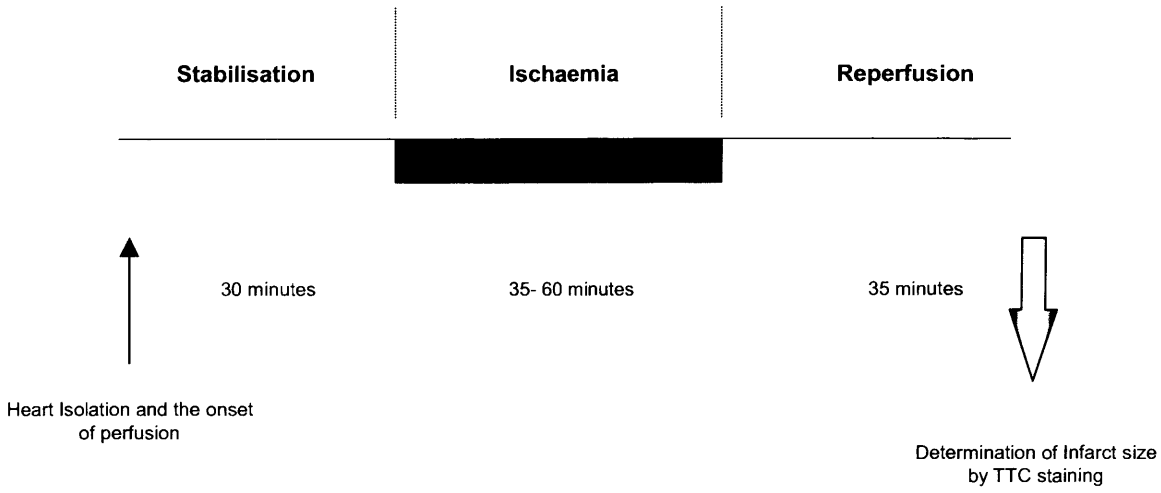

Figure 4-1 Protocol for global ischaemia and reperfusion.

The timeline illustrates the experimental protocol used for the characterisation of the effects of global ischaemia and reperfusion on infarct size in isolated mouse hearts. Hearts were randomised to 35 minutes, 40 minutes and 60 minutes of ischaemia after 30 minutes of stabilisation, followed by 35 minutes reperfusion.

Infarct size at 35 minutes of index ischaemia was 35.91% ± 2.52 (mean ± SEM) , whilst at 40 minutes and 60 minutes infarct sizes were 49.63% ± 4.38 and 73.18% ± 2.72, respectively (n=5-7), see Figure 4-1.

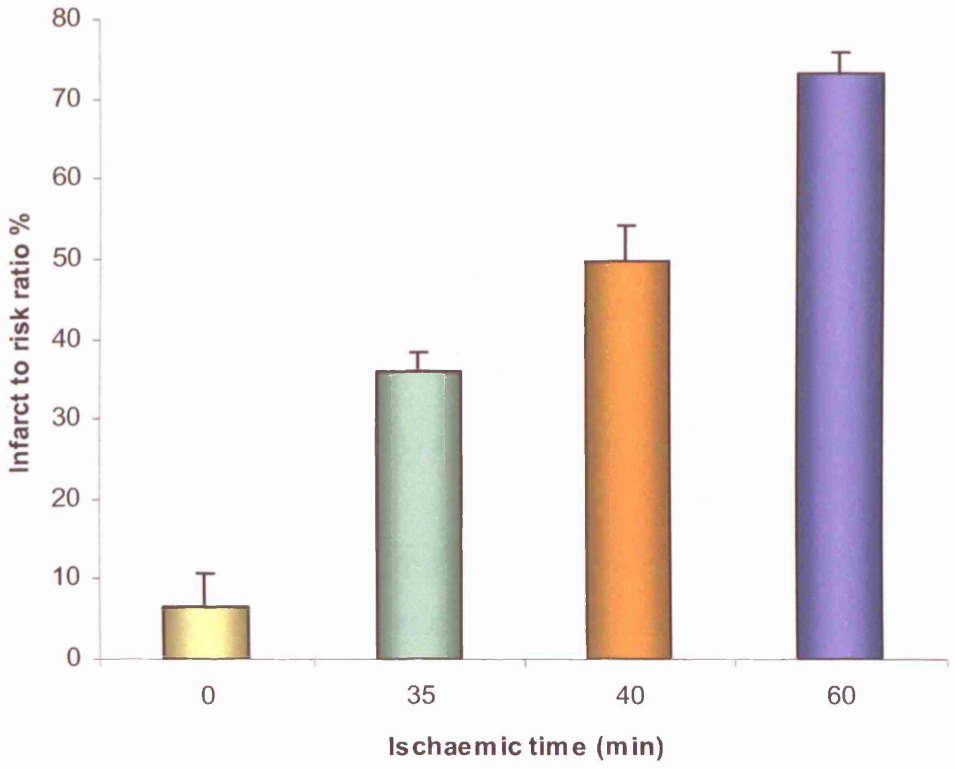

Figure 4-2 Effects of increasing index ischaemia time on infarct size

Infarct size, as a percentage of the risk zone (I/R %), in isolated mouse hearts following 35, 40 and 60 minutes of index ischaemia (n=5-7).

The sham protocol Figure 4-3 in which hearts were taken through the complete experimental period without the induction of ischaemia (0 minutes ischaemia) revealed an infarct size of 6.5% ± 4.14 (n=4)(see Figure 4-2)

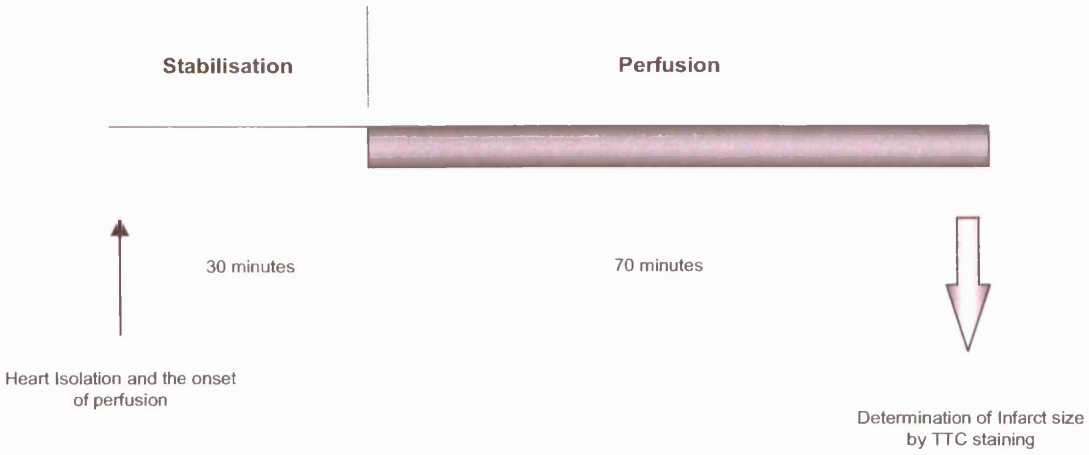

Figure 4-3 Sham protocol

The timeline illustrates the protocol used to assess the effects of continuous perfusion on infarct size.

These data demonstrated one of the fundamental properties of the model employed i.e. that hearts subjected to a Langendorff protocol are in a slow but constant state of deterioration. The reasons for this deterioration are multifactorial, but it is primarily due to a deficiency of key blood-borne elements and progressive tissue oedema (Headrick et al. 2001).

4.3 Mouse strain and myocardial ischaemic injury

The initial characterisation experiments utilised the Swiss White strain of mouse. Subsequent studies were however carried out using C57 Bl/6J inbred mouse. The C57 Bl/6J is used as the basis for many genetic variants and it was felt that given the potential influence of genetic and environmental factors on infarct size it too should be characterised before use. Hence isolated hearts from both types of animal (i.e. Swiss white and C57 Bl/6J) were compared with respect to susceptibility to infarction following the standardised ischaemia/reperfusion regime.

Five adult male mice aged 2-3 months from each group were subjected to 35 minutes global ischaemia and 35 minutes reperfusion prior to infarct size determination (for protocol see Figure 4-1.) Significantly, Swiss White and C57 Bl/6J appeared to posses different sensitivities to lethal ischaemic injury. Hearts from Swiss White mice had a mean infarct size of 35.91% ± 2.52, whereas the C57 Bl/6J strain had a significantly

larger mean infarct size of 52.03% ± 4.38 ($p<0.01$ n=5) see Figure 4-4. Differences between strains of rodents as regards infarct size have been reported previously (Baker et al. 2000), however possible mechanisms underlying these differences were not discussed. In the light of this, further studies were carried out into the effects of pre - conditioning in the two strains

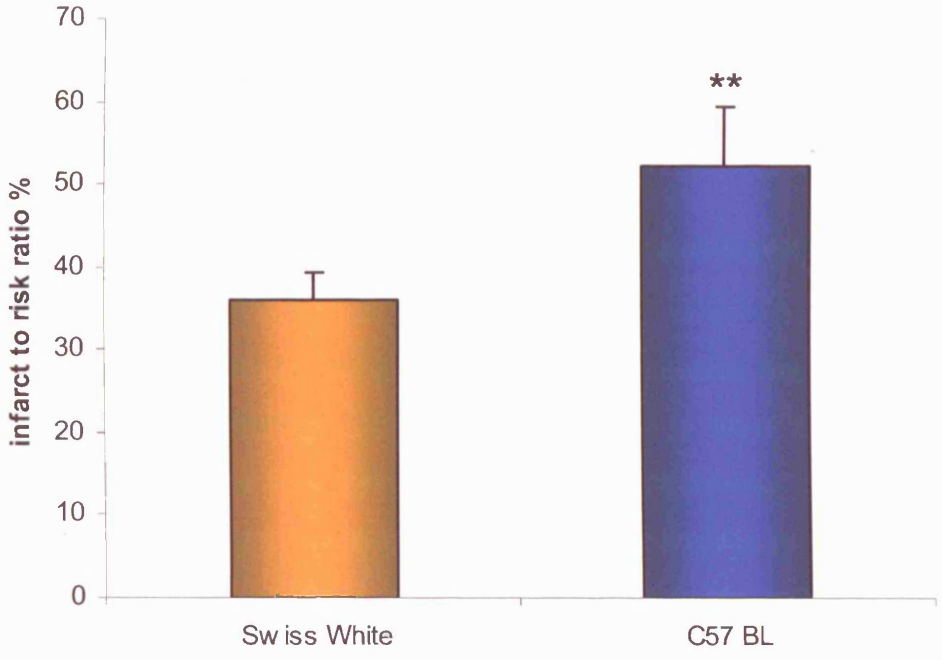

Figure 4-4 Susceptibility of mice strains to infarct size.

Hearts from C57 Bl/6J and Swiss White were subjected to an identical standardised ischaemia/reperfusion regime as previously described. In brief, hearts were perfused on the Langendorff rig and allowed to stabilise for 30 minutes. The hearts were then subjected to a 35 minute, normothermic, global ischaemic insult prior to reperfusion for 35 minutes. At this point the experiment was terminated and the hearts stained with TTC to determine infarct size (**$p<0.01$ n=5).

4.4 The effects of preconditioning

Ischaemic preconditioning of the myocardium is one of the most powerful protective strategies that can be applied in mammalian species (Bell & Yellon 1998). Therefore, in order to fully characterise hearts from both the Swiss White and C57 Bl/6J strains, their susceptibility to preconditioning was examined. The preconditioning protocol hearts were subjected to consisted of 4 cycles of 5 minutes of ischaemia and 5 minutes of reperfusion whilst control treated hearts underwent a time-matched perfusion period Figure 4-6 (Sumeray & Yellon 1998).

The results are summarised in Figure 4-7 and Figure 4-8. Significant protection was observed in both experimental groups. Preconditioning in hearts from Swiss White mice resulted in a 39.9% reduction in infarct size (49.52 ± 7.11 and 29.77 ± 3.32 in controls and preconditioned hearts, respectively, $p<0.05$).

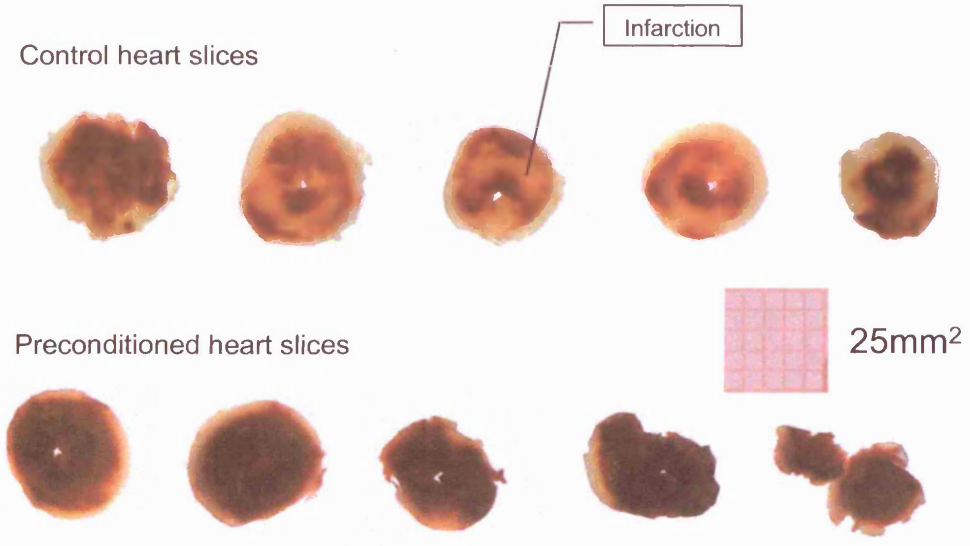

Figure 4-5 Swiss White heart slices.

Comparison of control vs. preconditioned hearts after slicing and TTC staining. The slices show the areas of white, non- stained, infarcted tissue, and red stained, non-infarcted tissue.

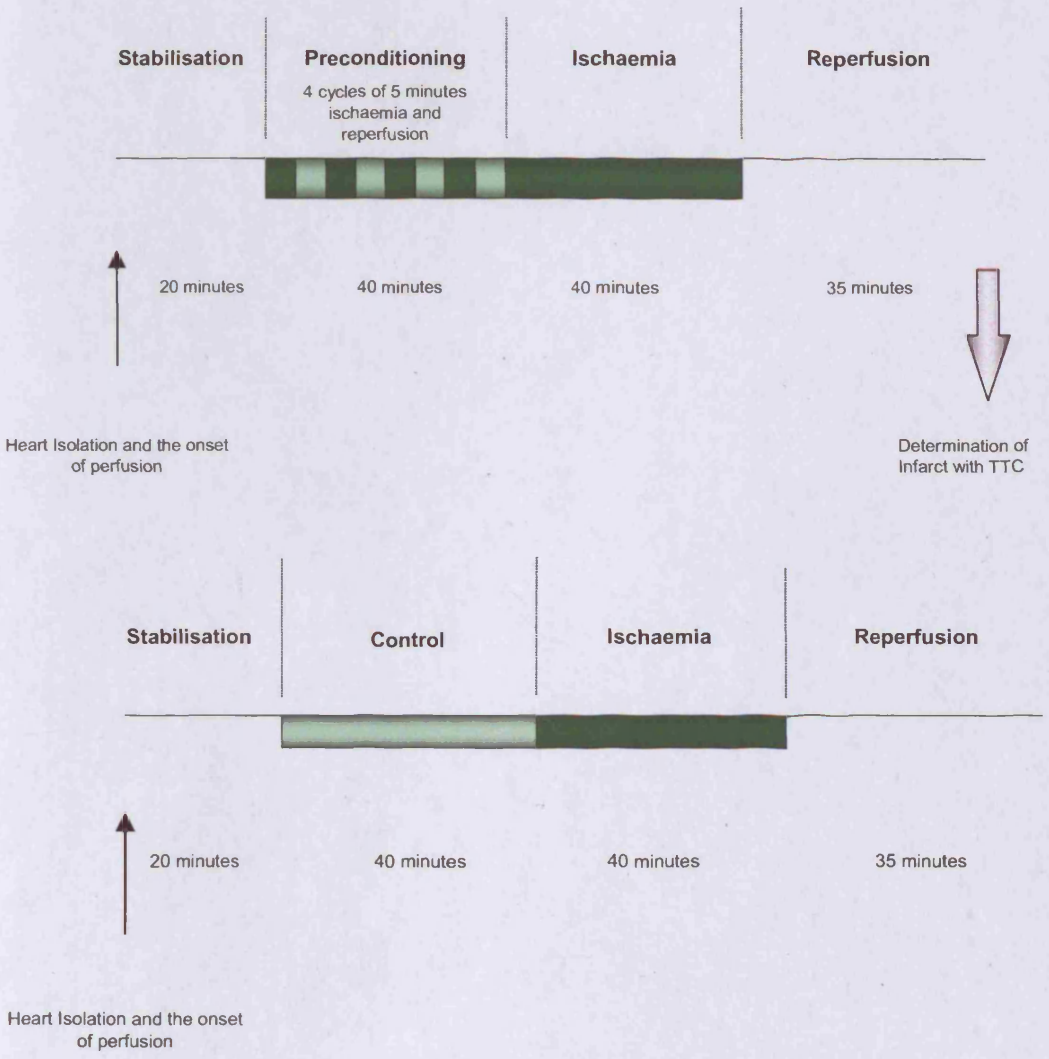

Figure 4-6 Preconditioning and control protocol

The timeline illustrates the control and preconditioning protocols used in hearts from Swiss White and C57 BL/6J mice. Hearts were randomly assigned to control or ischaemic preconditioning (IPC) groups. Ischaemic preconditioning consisted of 4 cycles of 5 minute ischaemia and 5 minutes reperfusion. In controls, in place of the ischaemia preconditioning protocol, the hearts were instead perfused for a further 40 minutes prior to index ischaemia. All hearts were reperfused for 35 minutes prior to determination of infarct size by TTC staining.

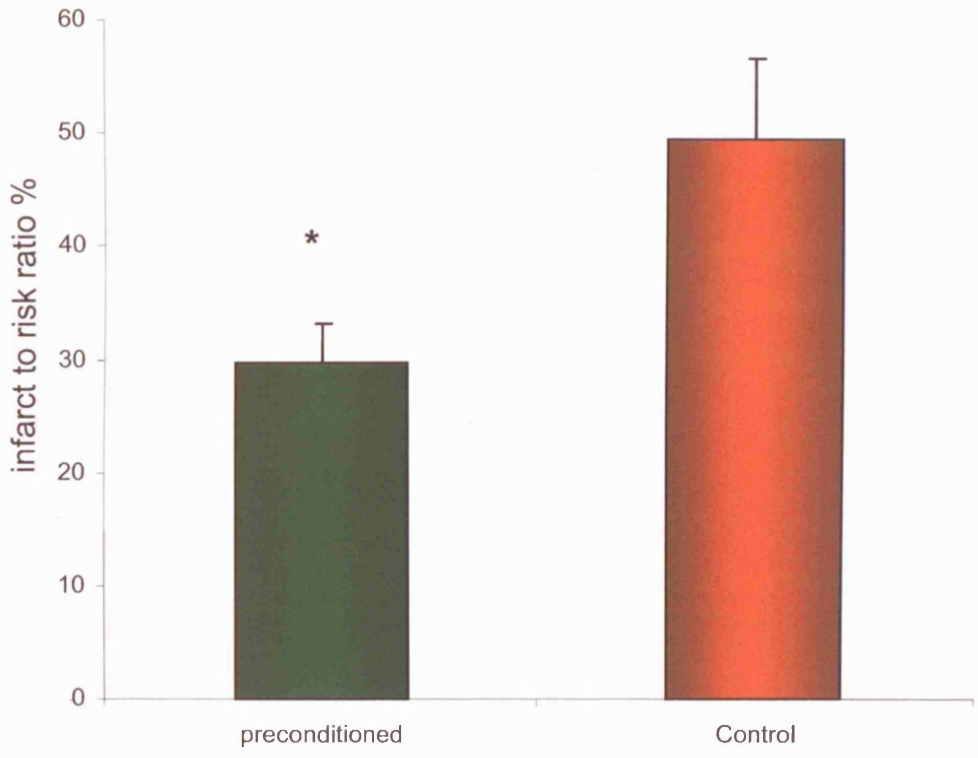

Figure 4-7 Preconditioning of hearts from Swiss White mice

Preconditioning in hearts from Swiss White mice resulted in a 39.9% reduction in infarct size (*p<0.05 n=5).

Significant protection was also observed with hearts from C57 Bl/6J mice, a 59.9% reduction in infarct size being observed (52.02% ± 4.38 to 20.85% ± 1.55 in controls and preconditioned respectively, p<0.001) Figure 4-8 . These experiments revealed that hearts from Swiss White mice were more resistant to ischaemia then hearts from the C57 Bl/6J, Figure 4 6.

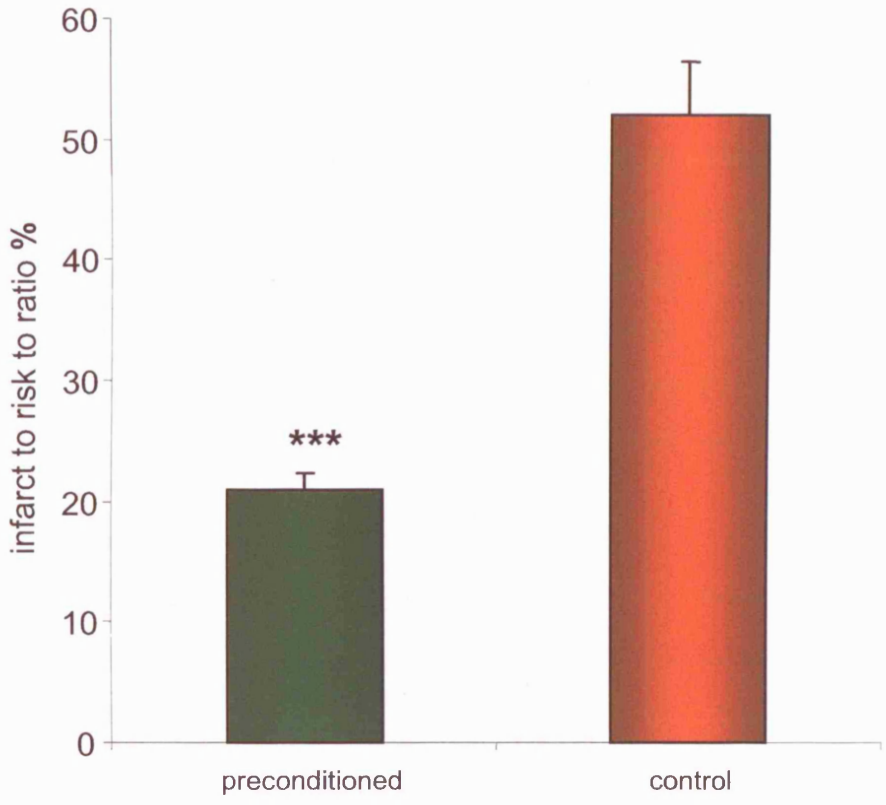

Figure 4-8 The effects of preconditioning on hearts from C57 BL mice

The graph shows the effects of preconditioning on infarct size using C57 Bl/6J strain of mouse when compared to control (***p<0.001 n=5).

4.5 Determination of the optimal reperfusion period

The final set of characterisation experiments entailed an examination of the effects of varying the reperfusion time on infarct size. Previous studies in mouse hearts have shown that 30 minutes of reperfusion is adequate for accurate determination of infarct size by TTC following an injurious 30 minute ischaemic insult. Prolonging the reperfusion period was found to have minimal influence upon measurable infarction (Marber et al. 1995; Sumeray et al. 1998). However, in animal models other than mouse, a minimum reperfusion reperfusion period of 2 hours was found to be required for accurate assessment of infarct size (Ytrehus et al. 2000). This probably relates to the fact that in some models longer periods of time are necessary for efficient washing out

of dehydrogenase enzymes from necrotic cells with incompetent sarcolemmal membranes. The apparent discrepancy between the required reperfusion times in the mouse compared to other animal models may also, in part, be explained by the comparatively high flow rate per unit mass of myocardium in the mouse (25 ml/g heart weight/minute in mouse versus 4 ml/g heart weight/minute in rabbit heart). Therefore to ensure that under our conditions infarct size was not influenced by reperfusion time and that a short reperfusion time is adequate for the purposes of subsequent experiments, hearts were reperfused for 35 minutes or 120 minutes. Isolated hearts from adult male C57 Bl/6J mice were used and subjected to the protocols shown in Figure 4-9.

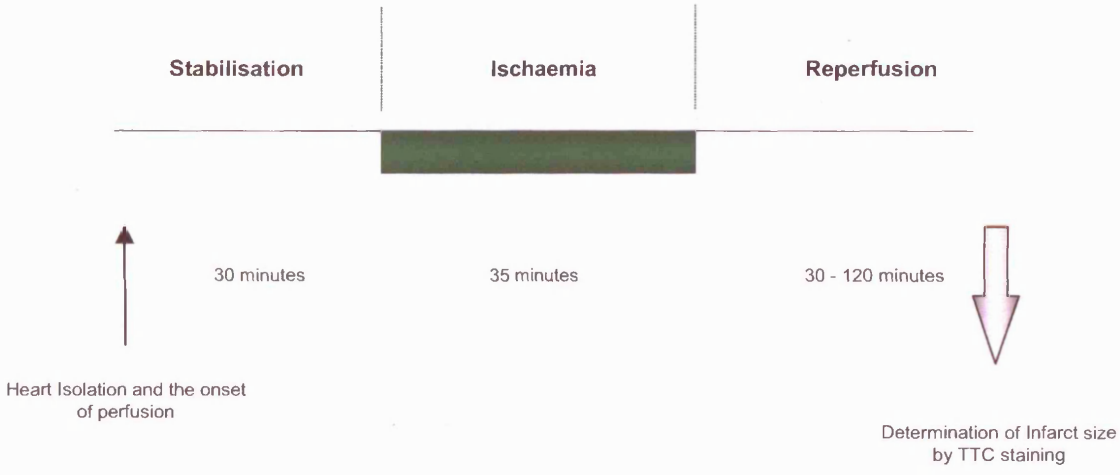

Figure 4-9 Perfusion protocol with increasing reperfusion time

The timeline illustrates the experimental protocol used to investigate the effects of increasing reperfusion time on infarct size. Hearts were randomised to either the shorter or longer reperfusion period after 30 minutes of stabilisation, and 35 minutes of normothermic ischaemia.

The infarct data is summarised in Figure 4-10. Prolonging the reperfusion period had no influence upon infarct size, infarct size at 35 minutes being 52.03% ± 4.38 and at120 minutes 50.7% ± 7.13 ($p=0.868$). The absence of any statistical difference between the results obtained for the two reperfusion times suggests that the injury sustained during ischaemia/reperfusion occurs early on and is resolved by 35 minutes reperfusion. Consequently, subsequent studies did not employ the longer reperfusion protocol, 35 minutes reperfusion being used as standard.

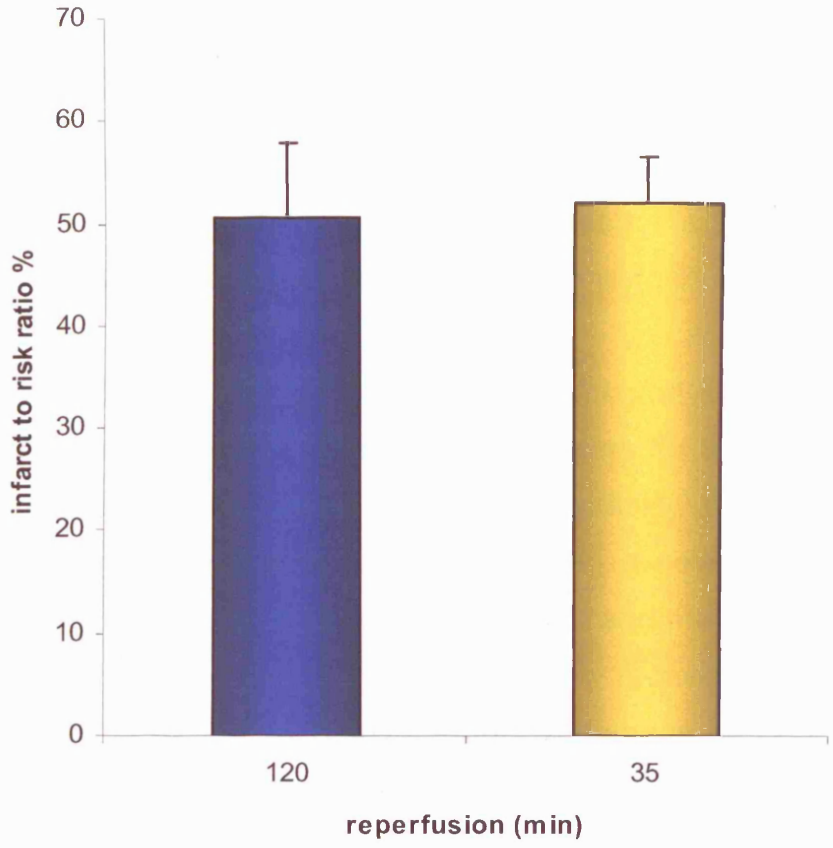

Figure 4-10 the effects of reperfusion time on infarct size

No significant difference in infarct size was observed between hearts reperfused for 35 or 120 minutes (p=0.868, n=6-9).

4.6 Discussion

4.6.1 Characterisation of the study model

Characterisation of the ischaemia/reperfusion model enabled an accurate and reproducible protocol to be established. Hence, the times for ischaemia and reperfusion finally selected corresponded to the time points found to yield the most consistent data.

Functional data (heart rate and left ventricular developed pressure) for recovery, although recorded, were not found to be valid parameters for the establishment of the optimal duration for ischaemia, owing to the susceptibility of the mouse heart to "stunning". Post-ischaemic dysfunction occurs following a severe but brief ischaemic insult, despite the absence of irreversible tissue damage and the restoration of adequate blood flow, and is thought to occur due to the slow recovery of intracellular ATP concentrations (Kloner et al. 2001). Since permanent damage has not occurred to the myocardium contractile function will be restored with time. This recovery, however, may take hours or even days (Brunwald and Kloner 1982), therefore diminishing the reliability of post-ischaemic functional analysis in the context of the Langendorff perfused heart.

4.6.2 Final Protocol

As hearts from C57 Bl/6J mice exhibited larger infarcts than hearts from Swiss White animals, coupled with the fact that future investigations are planned in genetic variants of the C57 Bl/6J mouse, all apelin studies were conducted using this breed. The standard protocol adopted for infarct studies involved subjecting hearts to 30 minutes stabilisation followed by 35 minutes global ischaemia and 35 minutes reperfusion.

5 EFFECTS OF APELIN ON REPERFUSION INJURY

5.1 Aims

Apelin has been shown to have a direct effect on cardiac contractility in the isolated rat heart model (Szokodi et al. 2002). Apelin also improves cardiac function and reduces myocardial injury in hearts treated with isoproterenol (Jia et al. 2006). In addition, apelin has been shown to activate PI3K/AKT and P42/44/ERK (Masri et al. 2002, Masri et al. 2004, El Massari et al. 2004, Wang G et al. 2004, Llorens-Cortes et al. 2005), key kinases involved in anti-apoptotic processes and cell growth. The actions of apelin, however, have not been investigated with respect to myocardial ischaemia/reperfusion. Based on these findings it was hypothesised, therefore, that apelin may prove to be cardioprotective via the induction of the RISK pathway.

5.1.1 Apelin isoforms

In order to assess the potency of apelin-13 in our experimental system experiments were conducted in which the effects of varying concentrations of the peptide on the primary end-point of infarct size were examined. Consequently, a concentration response curve was constructed to identify the most appropriate dose for subsequent studies. Thus, using this dose possible mechanisms underlying protection against myocardial injury were investigated using inhibitors of RISK pathway signalling and Western blot analysis. Apelin is found in various isoforms, which are cleaved from a 77 amino acid preproapelin peptide. Of these smaller isoforms, i.e. apelins 11, 12, 13, and 36, the latter two have been studied in most detail. Apelin 36 is the commonest isoform and previous studies have indicated that it differs from the smaller peptide, apelin-13, in its biological potency (Hosoya et al. 2000). The decision was, therefore, made to investigate if differences also existed between apelin-13 and apelin-36 in the context of I/R injury.

Potential, differences were additionally investigated by examining the effects of the apelin analogue apelin-13 (F13). Work carried out by Lee et al. (2005) focused on the influence that modification of the carboxyl- terminal phenylalanine group of apelin-13 had on its physiological activity.

Figure 5-1 Sequence of apelin-13 and 36 and the analogue apelin-13(F13).

The light blue circles indicate the conservation of sequences seen across species and isoforms. The light green circle indicates the substitution reported to antagonise the hypotensive effects of apelin-13

Previously it had been shown that a similar modification to angiotensin II yielded the angiotensin II antagonist Saralasin (Pals et al. 1986). Apelin-13(F13) was, therefore, synthesised by substituting alanine for phenylalanine on the carboxyl-terminal of the peptide. This modification was found to result in blunting of the hypotensive response to apelin-13, indicating that apelin-13 (F13) functioned as a pharmacological antagonist for apelin-13 (Lee et al. 2005). In light of these previous findings, experiments were carried out to investigate the hypothesis that apelin-13 (F13) may antagonise any cardioprotective effects induced by apelin-13.

5.2 Methods

5.2.1 Langendorff Model

5.2.1.1 Apelin-13 concentration response

The concentrations of apelin used in various cellular and animal models have been wide ranging (Tatemoto et al. 1998, Reaux et al. 2001, Tatemoto et al. 2001, Xie et al. 2006). No experiments using the mouse isolated Langendorff model, however, have been carried out previously, so in order to establish the optimal apelin-13 concentration for experimental use, varying concentrations of peptide were given at reperfusion and the effects on infarct size assessed. A standard control protocol was used as described in Chapter 4.2 for comparison with the apelin-13 test group. Thus 30 minutes of stabilisation was followed by 35 minutes of ischaemia whereupon apelin was administered at the point of reperfusion which was continued for 35 minutes (see Figure 5-2). Apelin concentrations of 10 nM, 50 nM, 100 nM and 1000 nM were tested.

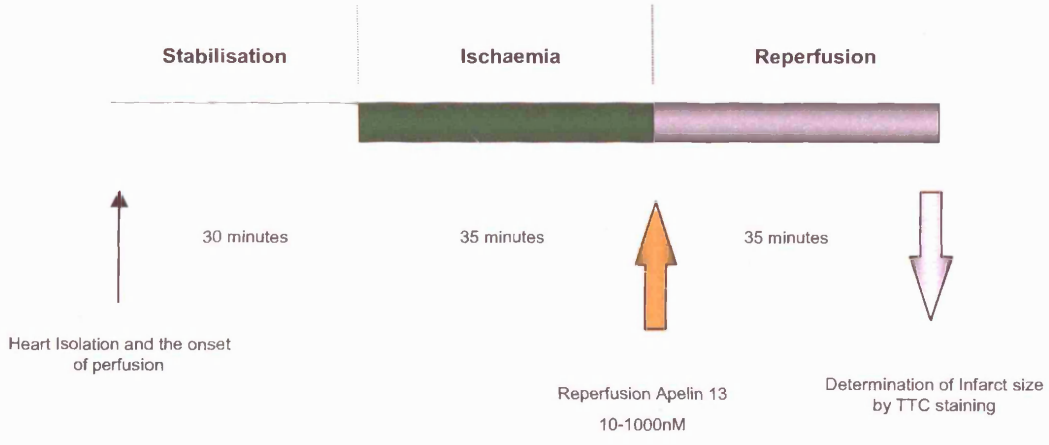

Figure 5-2 Protocol for apelin dose response

The timeline shows the experimental protocol used for the assessment of the dose response to apelin-13 (10-1000nM). Apelin was given at the point of reperfusion after 35 minutes of ischaemia.

5.2.1.2 Inhibition of PI3K/AKT and p42/44 MAPK

Once the optimal cardioprotective dose for apelin had been established, potential mechanisms underlying the apelin-induced reduction in infarct size were investigated. Thus, specific inhibitors of kinases suspected of being involved were employed.

LY294002 (15μM) a specific PI3K/AKT inhibitor, and UO126 (10μM), an inhibitor of the p42/44/ERK pathway, were utilised in order to see whether the level of protection was affected. The dose of each inhibitor was selected on the basis of previous studies (Smith et al. 2007, Hausenloy et al. 2004, Mocanu et al.2002). Each inhibitor was used with or without apelin and the effects on infarct size assessed. The protocol used was identical to that for the apelin concentration experiments, with changes only being made to the composition of the reperfusion buffer (see Figure 5-3).

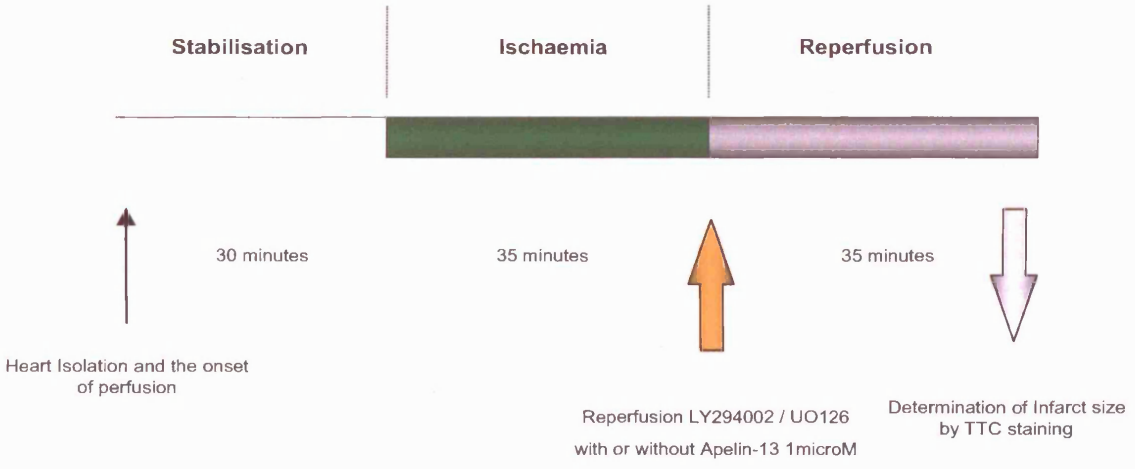

Figure 5-3 Protocol investigating the effects of inhibitors of the RISK pathway on infarct size
The timeline illustrates the experimental protocol for investigating the effects of the inhibitors LY294002 (15μM) and UO126 (10μM) with or without apelin-13 (1μM) on infarct size. All hearts were Langendorff perfused for a 30 minute stabilisation period prior to index ischaemia. Hearts were then subjected to 35 minutes ischaemia. After index ischaemia hearts were randomly assigned to 35 minutes reperfusion with LY294002 (15μM) or UO126 (10μM) with or without apelin-13 (1μM), prior to determination of infarct size by TTC staining.

Both LY294002 (15μM) and UO126 (10μM) were dissolved in dimethylsulphoxide (DMSO; final concentration 0.02%) prior to use and to exclude any possible effect of the vehicle alone, a further experimental group was examined in which DMSO (0.02%) was applied.

5.2.1.3 Apelin-36

In order to study the effects of apelin-36 a protocol identical to that used in apelin-13 concentration response experiments was used. Mouse hearts underwent a standard I/R protocol with or without apelin-36 (1μM).

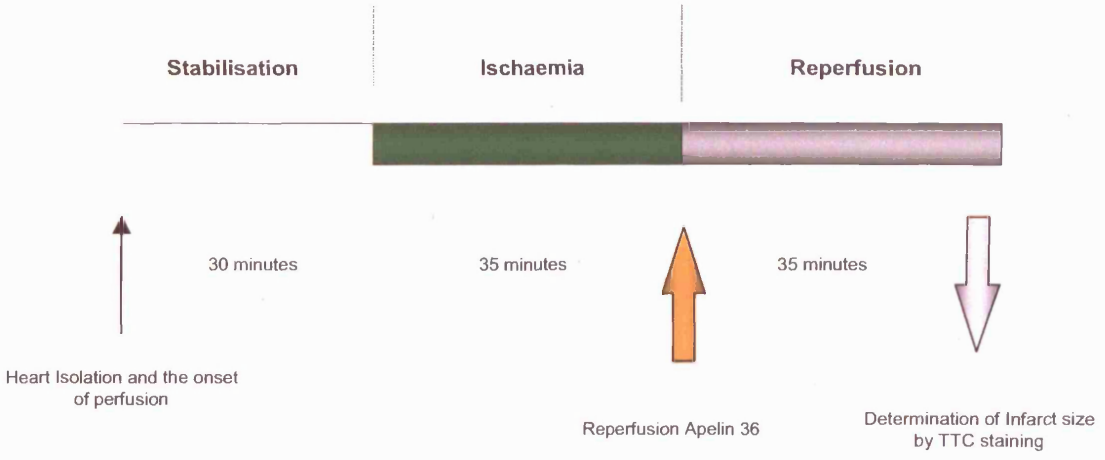

Figure 5-4 Protocol for reperfusion with apelin-36

The timeline illustrates the experimental protocol for investigating the effects of apelin-36 on ischaemia-reperfusion injury. All hearts were Langendorff perfused for a 30 minute stabilisation period prior to index ischaemia. Hearts were then subjected to 35 minutes ischaemia. After index ischaemia hearts were subjected to 35 minutes reperfusion with or without apelin-36 (1μM), prior to determination of infarct size by TTC staining.

To investigate the effects of apelin-F13 (1μM) hearts were perfused with the antagonist prior to perfusion with apelin-13 (1μM). The modifications made to the standard protocol are shown in Figure 5-5. Apelin-13 (F13A) (1μM) was given prior to the onset of ischaemia in order to ensure that full occupancy of the APJ receptor occurred prior to ischaemia with subsequent reperfusion with apelin-13 (1μM), therefore, being antagonised.

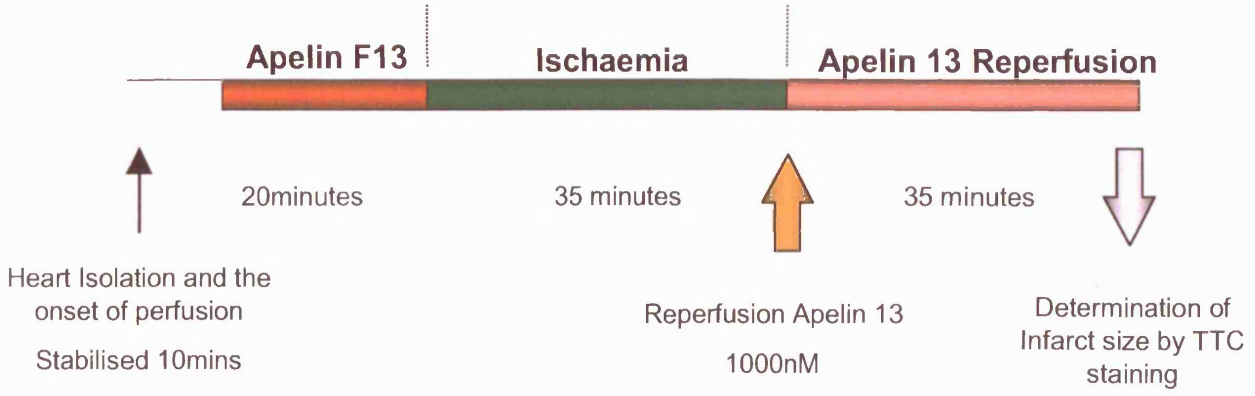

Figure 5-5 Perfusion with apelin-F13 prior to reperfusion with apelin-13

The timeline illustrates the experimental protocol used to investigate the effects of perfusion with apelin-13(F13) (1μM) prior to ischaemia and reperfusion with apelin-13 (1μM). 10 minutes of stabilisation was followed by perfusion with apelin-13 (F13) (1μM). This continued for 20 minutes before the induction of global ischaemia for 35 minutes. Apelin-13 (1μM) was then administered at reperfusion and throughout the whole of this period and infarct size determined by TTC staining. In controls, in place of apelin, hearts were perfused with normal Krebs buffer.

5.2.2 Western Blot Analysis

The effects of apelin-13 on the activation of the protective kinases PI3-K-Akt and p42/44ERK were studied. To establish the point at which maximal phosphorylation of these kinases occurred hearts were reperfused for 5 or 10 minutes before being frozen for subsequent analysis (Xie et al. 2006). The protocol employed is shown in Figure 5-6. Once collected cardiac samples were subjected to Western blot analysis, as described in Chapter 3.8.

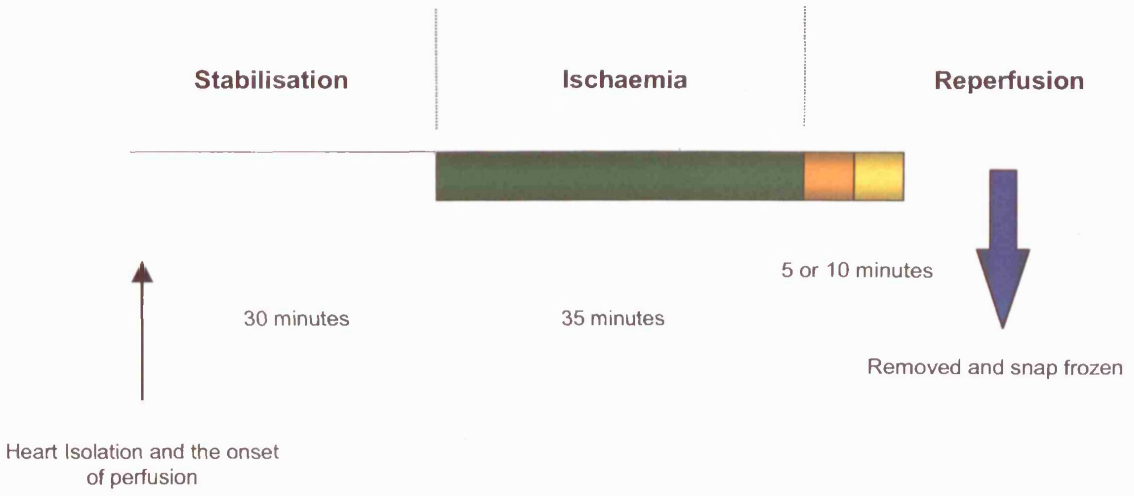

Figure 5-6 Protocol for the collection of samples for Western blot analysis

The timeline shows the protocol used for the collection of samples for Western blot analysis. Hearts were subjected to either a 5 minute or 10 minute reperfusion period.

5.3 Results

5.3.1 The effect of apelin administered at reperfusion on infarct size

5.3.1.1 Concentration response to apelin-13

The concentration dependent effect of apelin-13 (10nM, 50nM, 100nM and 1000nM), administered at reperfusion, on infarct size is shown in Figure 5-7 .

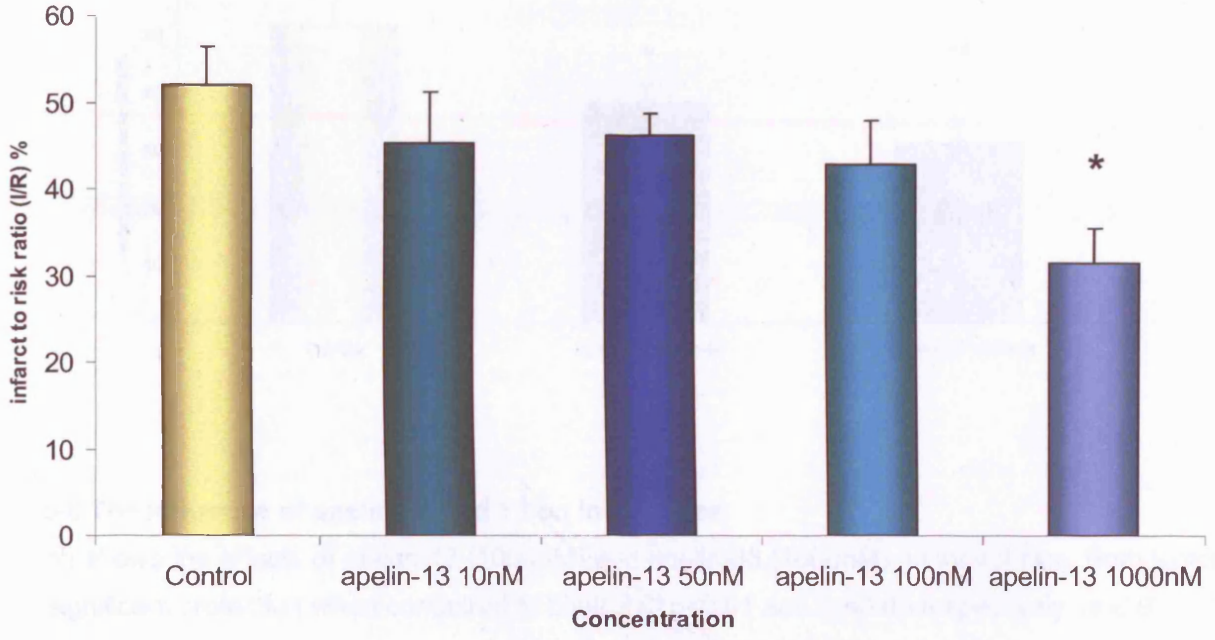

Figure 5-7 Dose response to apelin-13 (10-1000nM)

Infarct size, as a percentage of the risk zone (% I/R), in isolated mouse hearts perfused with apelin-13 (10 nM-1000nM) during reperfusion (35 min) (*$p<0.05$, n=8-10).

It can be seen that at the lower concentrations apelin did not statistically alter infarct size compared to control, although a slight trend towards reduced infarction occurred as the peptide concentration was increased. At the highest concentration, i.e. 1000nM (1μM), apelin afforded significant protection with an infarct size of 31.4% ± 4.03 for apelin-13 versus 52.93% ± 4.79 for control *$p<0.05$. The effect of apelin-13, therefore, was to reduce infarct size by 40% compared with control. Given this result apelin-13 was used at a concentration of 1μM for all subsequent experiments.

5.3.1.2 The effect of apelin-36 on infarct size

The results of experiments with apelin-36 are shown in Figure 5-8 and reveal that apelin-36, like apelin-13, also reduced infarct size compared to control, although not to the extents seen with the shorter isoform (apelin-36, 38.4%± 3.02, $p<0.05$, apelin-13, 31.4± 4.03, vs. control 52.93% ± 4.79, $p<0.01$).

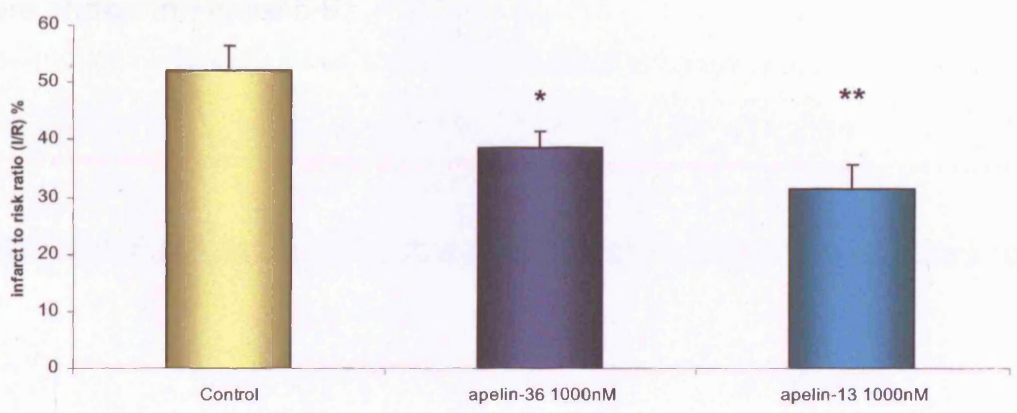

Figure 5-8 The influence of apelin-36 and 13 on infarct size.

The graph shows the effects of apelin-13 (1000nM) and apelin-36 (1000nM) on infarct size. Both isoforms provide significant protection when compared to control (**$p<0.01$ and *$p<0.05$ respectively, n=6-9)

Mean infarct sizes obtained on application of apelin-36 and apelin-13, were, however, not significantly different (38.4% ± 3.02 vs. 31.40% ± 4.03 $p=0.17$).

5.3.1.3 The effects of inhibitors of PI3K and ERK on apelin-13 induced protection

In order that the mechanisms underlying the protective effects of apelin-13 might be investigated, inhibitors of PI3-K and ERK were used with the aim of establishing if the RISK pathway played a role. LY294002 (15 μM) and UO126 (10μM) were administered separately or concomitantly with apelin-13 during reperfusion. Both inhibitors were dissolved in DMSO (0.02% final concentration), so a separate group consisting of hearts perfused with the vehicle was examined. The data obtained with the RISK pathway inhibitors are shown in Figure 5-9.

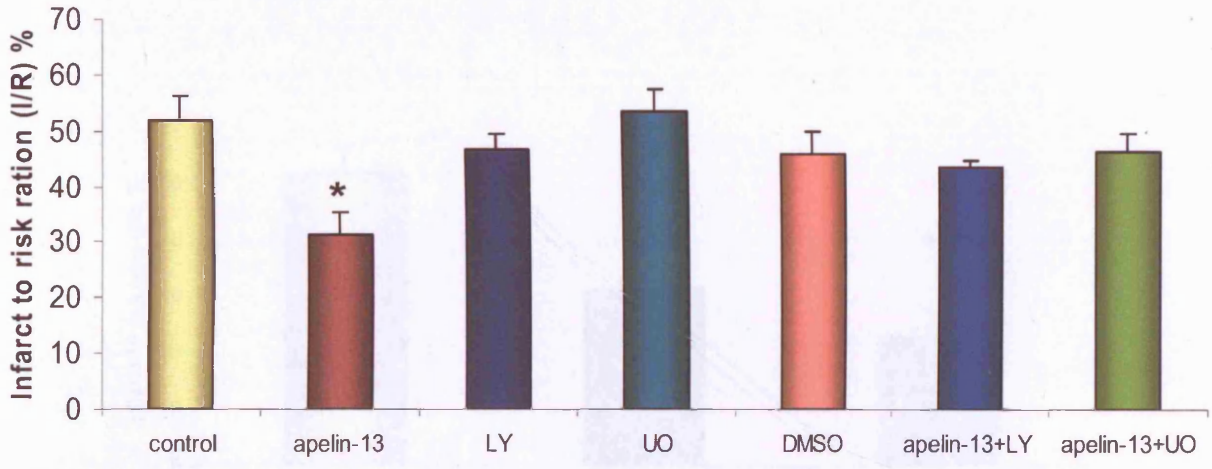

Figure 5-9 The influence of inhibitors of the RISK pathway on apelin-induced infarct size reduction
The graph shows the effects of the inhibitors UO126 (10μM) and LY249002 (15μM) administered with or without apelin-13 (1μM) on infarct size. (*$p<0.05$ n=6-9).

The kinase inhibitors alone did not influence infarct size, values for LY294002, UO126 and control of 46.5% ± 2.84, 53.7% ± 3.85 and 52.93% ± 4.79 respectively being obtained. Similarly, the vehicle for both inhibitors, DMSO, had no effect on infarct size (45.9% ± 3.80, DMSO vs 52.93% ± 4.79, control). When, however, LY294002 was

administered together with apelin-13 at reperfusion the protective effect of apelin-13 was lost (43.4% ± 1.41, LY294002 + apelin-13 vs. 31.40% ± 4.03, apelin-13). UO126 when administered together with apelin-13 also blocked the cardioprotective effect of apelin-13 (46.3% ± 3.08, UO126 + apelin-13 vs. 31.4 ± 4.03%, apelin-13)

5.3.1.4 The effect of apelin-F13 on apelin-13 induced cardioprotection

The effect of the modified peptide, apelin-F13, on infarct size when administered prior to apelin-13 was also examined (see Figure 5-10). Interestingly, an effect opposite to that which might have been expected was obtained i.e. apelin-F13 pre-treatment of hearts did not prevent the cardioprotective action of apelin-13 compared to controls (22.27% ± 3.97 vs. 52.93% ± 4.79 $p<0.001$). In fact, mean infarct size was less in apelin-F13 treated hearts than that seen with hearts treated with apelin-13 alone, although the difference was not statistically significant (22.27% ± 3.97 vs. 31.40% ± 4.03 $p=0.162$).

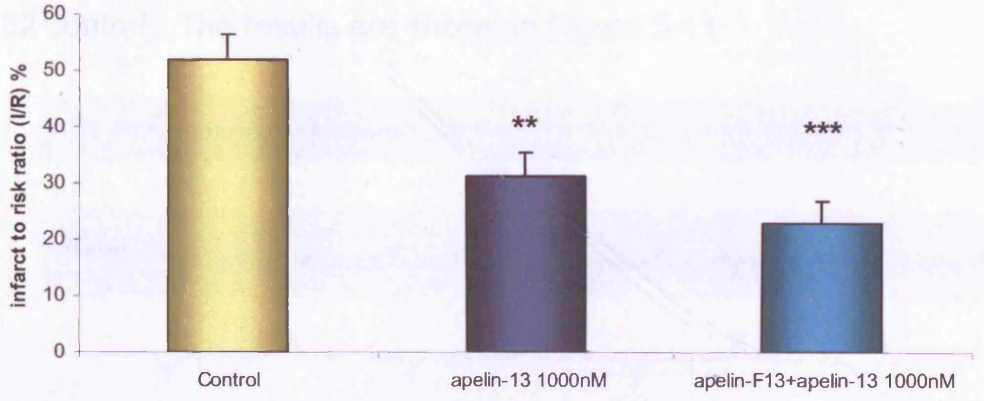

Figure 5-10 The effect of apelin-F13 pre-treatment on apelin-13 induced protection.
The graph shows the effects of apelin-13 (1μM) in the absence and presence of apelin-F13 (1μM) on infarct size compared to control (**$p<0.01$ and ***$p<0.001$ n=7-9).

5.3.2 Western blot analysis

The results of experiments in which the influence of apelin-13 on the phosphorylation of kinases implicated in cardioprotection are described in this section. The data are presented as relative densities which have been normalised against β-actin. The general equation used when calculating data was: (relative density [antibody bound kinase]) / (relative density β-Actin). Cardiac samples were taken at two points during reperfusion, i.e. 5 minutes and 10 minutes, to establish when maximal phosphorylation occurred.

5.3.2.1 PI3K/Akt phosphorylation

5.3.2.1.1 5 minute reperfusion

The administration of apelin-13 increased Akt phosphorylation significantly (apelin-13 3.95 ± 0.49 vs. control 2.29 ± 0.32 relative density (RD), $p<0.05$ n=4). This increase was lost when the PI3K inhibitor LY294002 was present, pAkt relative densities being significantly reduced in both the LY294002 and LY294002+apelin-13 groups when compared with control (0.255 ± 0.085, $p<0.001$ and 0.573 ± 0.132, $p<0.001$ respectively vs. 2.29 ± 0.32 control). The results are shown in Figure 5-11

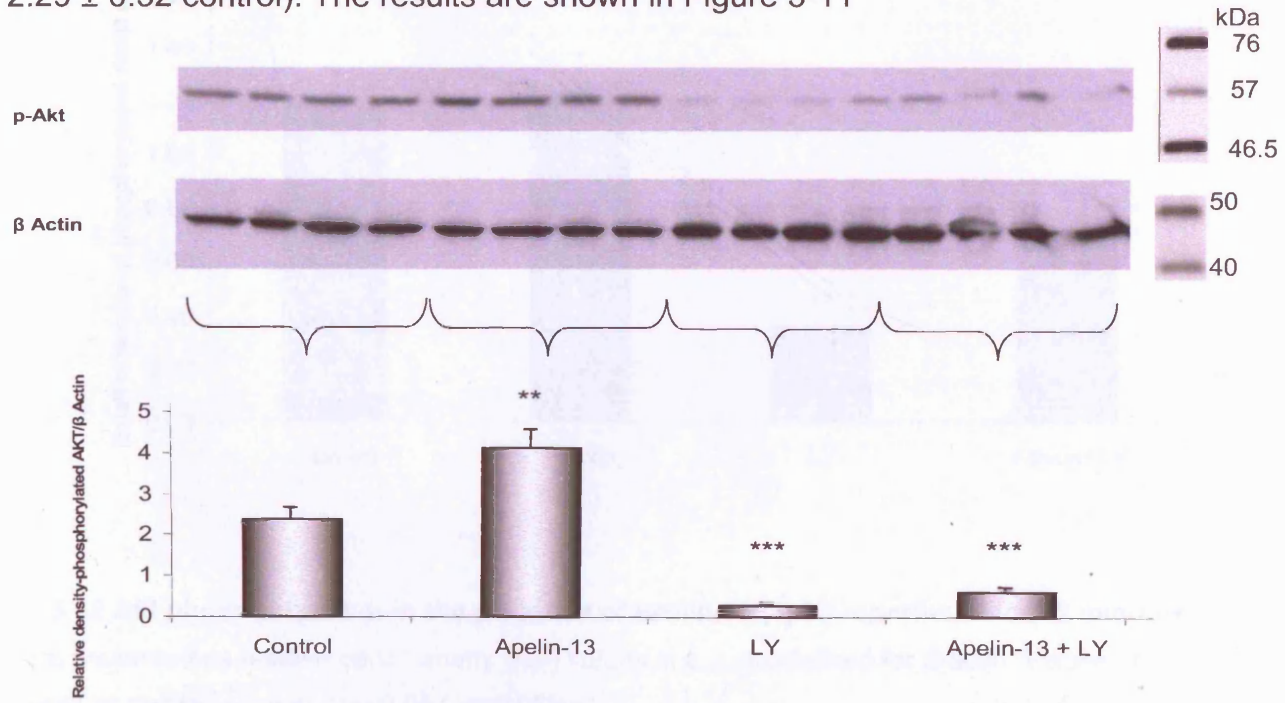

Figure 5-11 Effect of apelin-13 (1μM) on Akt phosphorylation at 5 minutes reperfusion.
Data are presented as relative densitometry (RD) values in a.u. normalised for *β*-actin. Values are expressed as means ± s.e.m. (**p<0.01 ***p<0.001; n=4)

5.3.2.1.2 10 minute reperfusion

The relative density data obtained for Akt phosphorylation at 10 minutes reperfusion in the presence and absence of apelin-13 revealed no significant difference from control (1.26 ±0.104 vs. 1.17 ± 0.141, apelin-13 vs. control, p=0.66). LY294002 treatment, however, resulted in a significant decrease in Akt phosphorylation for both the LY294002 and LY294002+apelin-13 groups compared with control (0.35 ± 0.050, $p<0.01$ and 0.67 ± 0.099, $p<0.05$ vs. control 1.26 ± 0.141). The results are shown in Figure 5-12

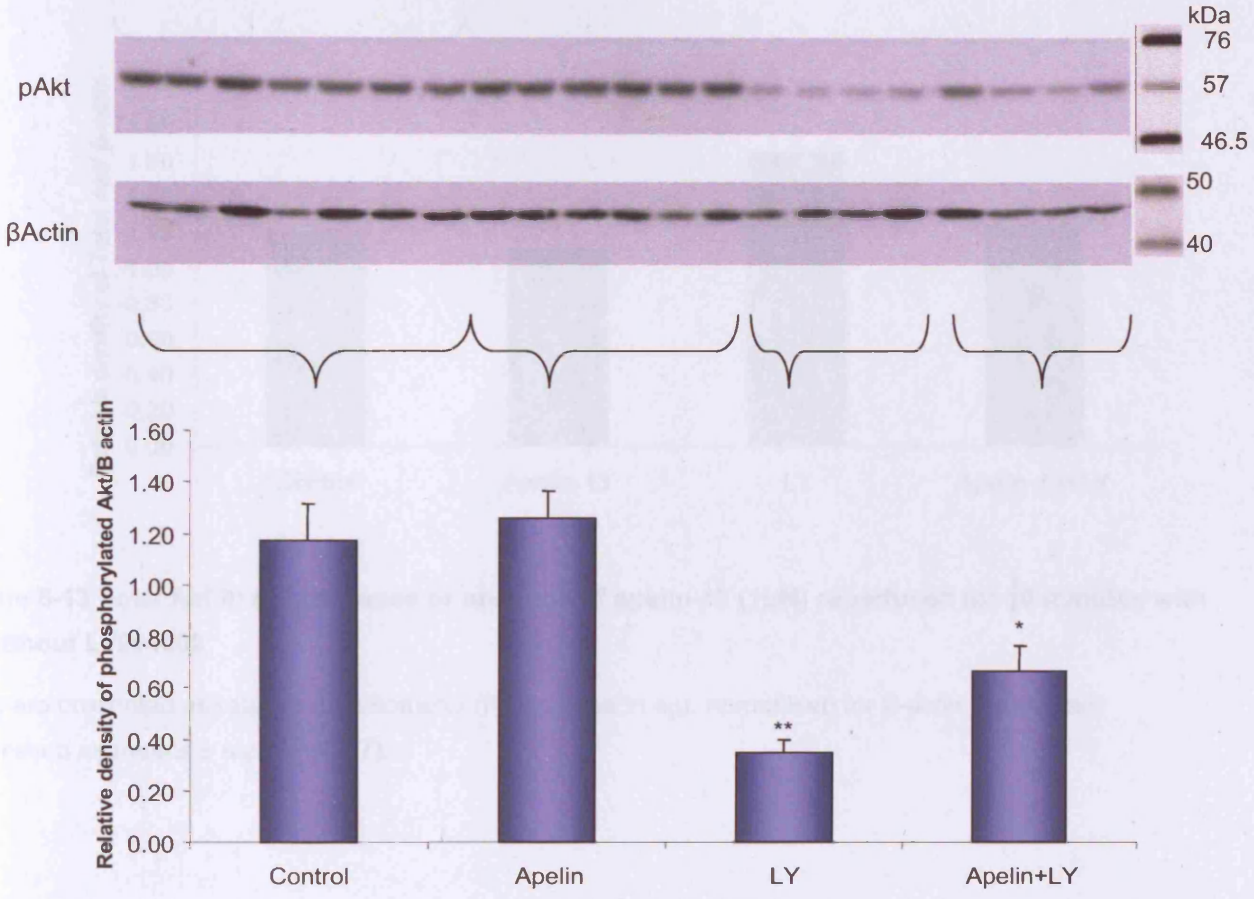

Figure 5-12 Akt phosphorylation in the presence of apelin-13 (1μM) reperfused for 10 minutes. Data are presented as relative densitometry (RD) values in a.u. normalised for β-actin. Values are expressed as means ± s.e.m. (*$p<0.05$ **$p<0.01$;n=4-7)

The data obtained for total Akt are shown below (Figure 5-13). Total Akt levels were unaffected by any the treatments examined.

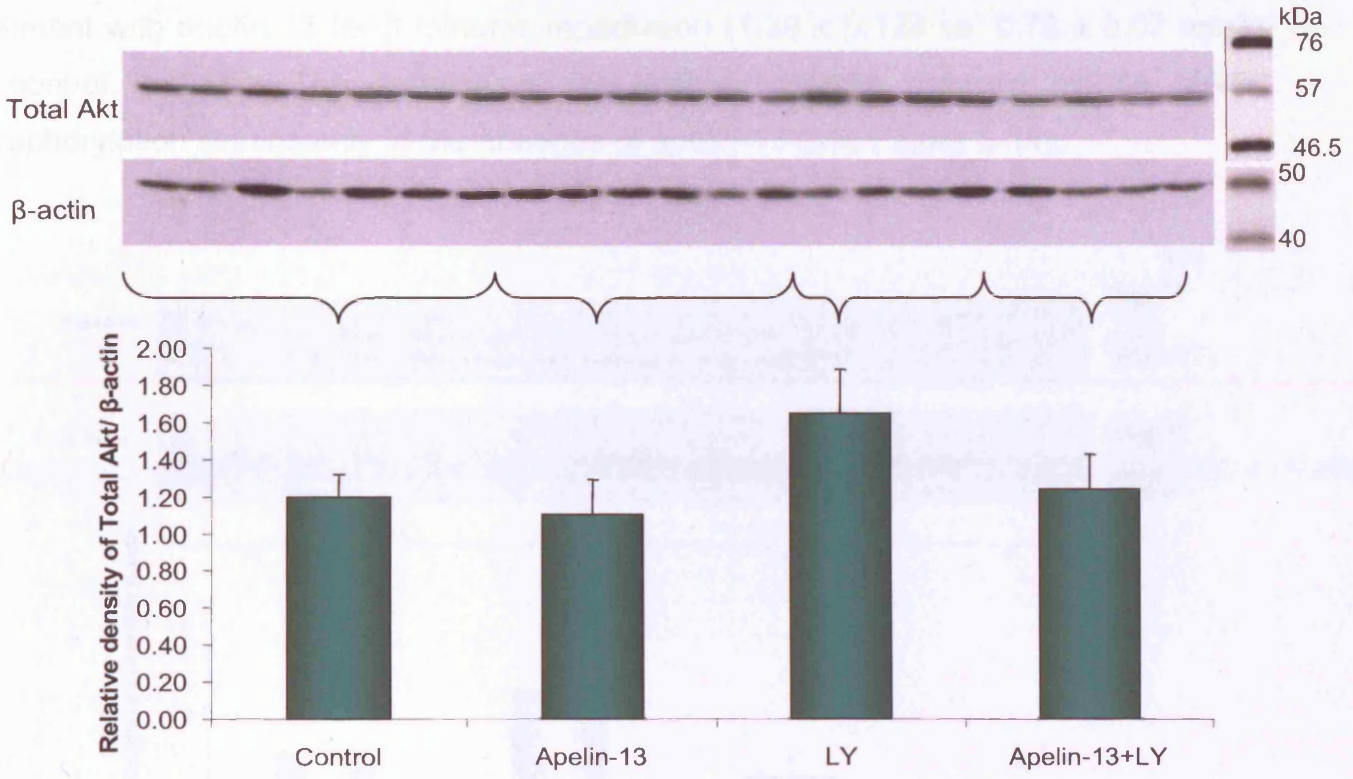

Figure 5-13 Total Akt in the presence or absence of apelin-13 (1μM) reperfused for 10 minutes with or without LY294002

Data are presented as relative densitometry (RD) values in a.u. normalised for *β*-actin. Values are expressed as means ± s.e.m (n=4-7).

5.3.2.2 P42/44 MAPK phosphorylation

5.3.2.2.1 5 minutes reperfusion

As for Akt, a significant increase in p42/44 MAPK phosphorylation was observed on treatment with apelin-13 for 5 minutes reperfusion (1.29 ± 0.124 vs. 0.78 ± 0.07 apelin vs control, $p<0.05$). The addition of the inhibitor UO126 reduced p42/44 MAPK phosphorylation significantly in the absence of apelin-13 (see Figure 5-14).

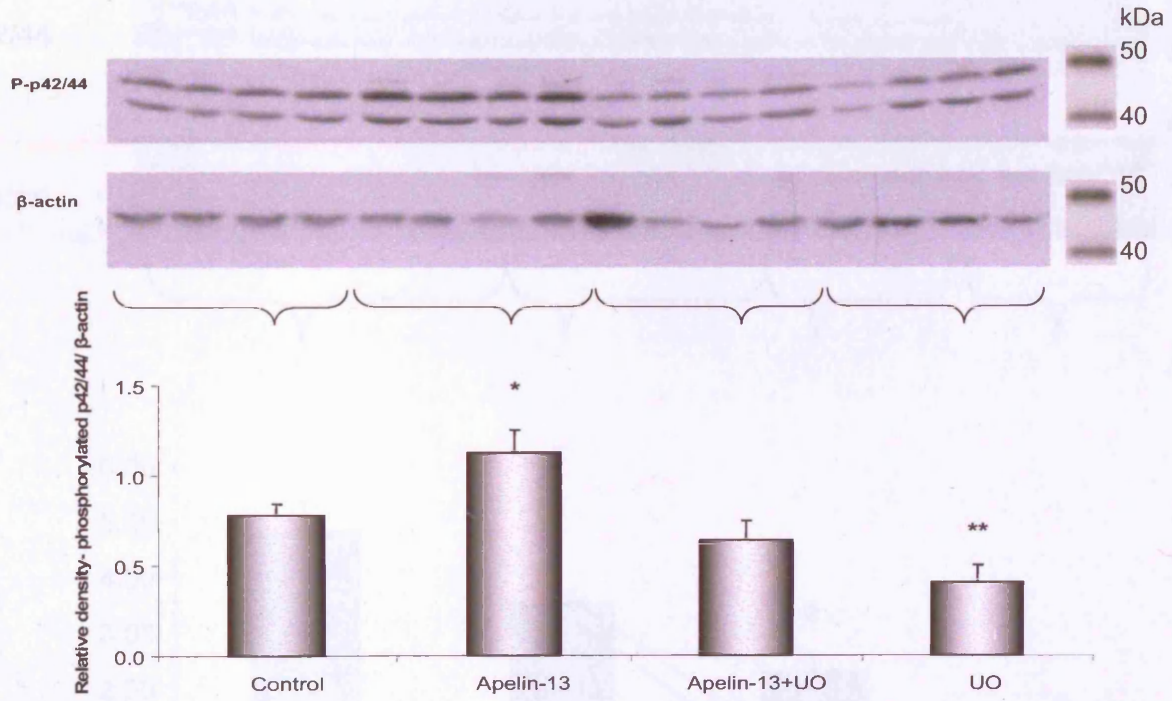

Figure 5-14 The effect of apelin-13 (1μM) on p42/44 phosphorylation at 5 minutes of reperfusion with or without UO126

A smaller relative density was seen with apelin-13 (1μM) + UO126 but this was not significantly different from control (Apelin-13 + UO126, 0.637 ± 0.11 vs. control, 0.78 ± 0.07 $p=0.32$) (*$p<0.05$ **$p<0.01$, $n=4$).

5.3.2.2.2 10 minutes reperfusion

No significant difference occurred between control and apelin-13 treated hearts with regard to p42/44 phosphorylation at 10 minutes reperfusion (4.77± 1.23 vs. 3.48± 0.54). UO126 blocked p42/44 phosphorylation both in the absence and presence of apelin-13 (1μM): 2.27± 0.52 vs. 4.77 ±1.23, UO126 vs. control, $p<0.05$; 0.64 ± 0.23 vs. 4.77 ±1.23 UO126+apelin-13 vs. control, $p<0.05$. The results are shown in Figure 5-15.

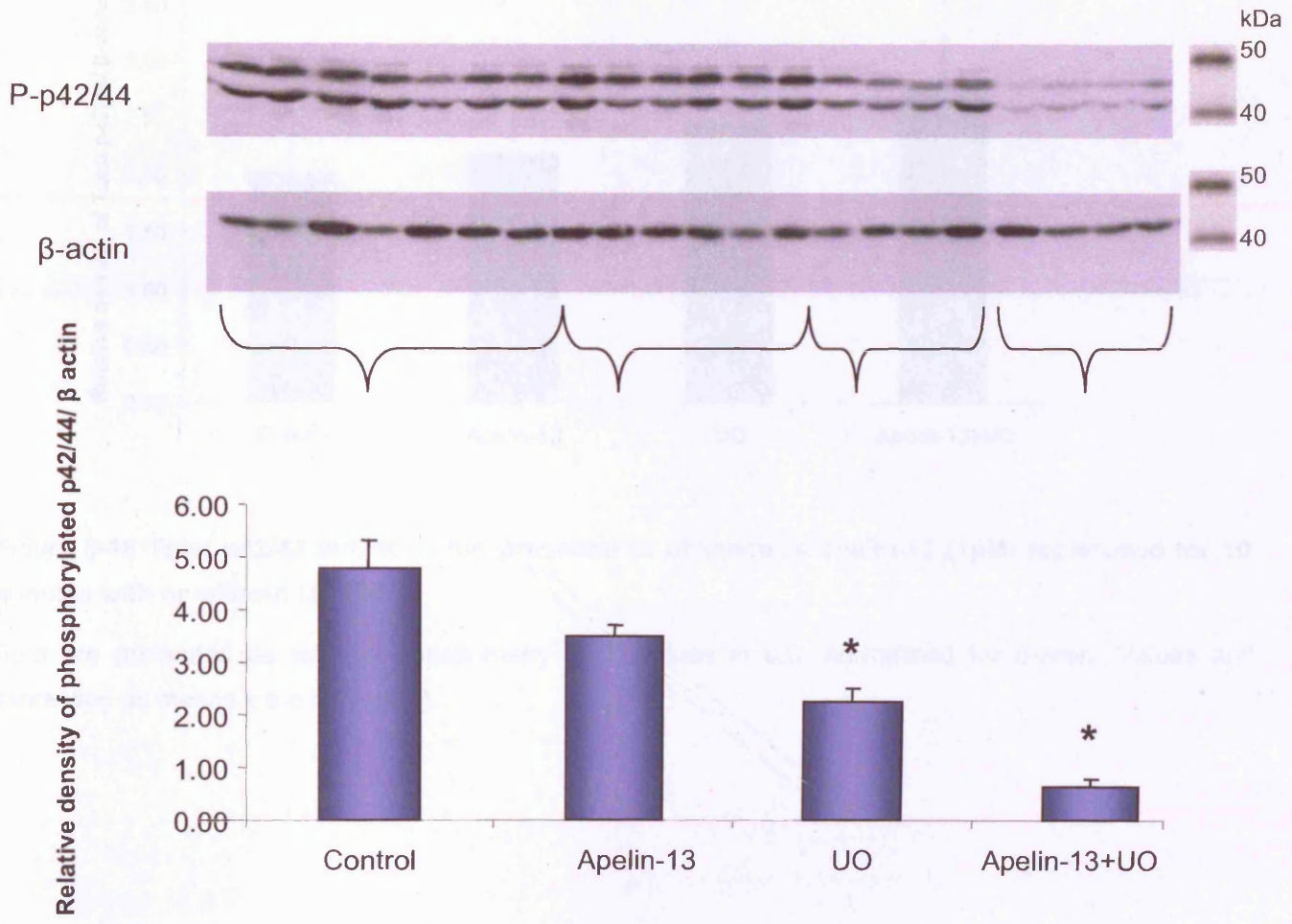

Figure 5-15 Effect of apelin-13 (1μM) on p42/44 phosphorylation at 10 minutes reperfusion.
Data are shown as relative density values (a.u.) normalised for *β*-actin loading and indicate that UO126 blocked p42/44 phosphorylation both in the absence and presence of apelin-13 (*$p<0.05$, n=4-7).

Total p42/44 MAPK levels were unaffected by any of the treatments applied (see Figure 5-16).

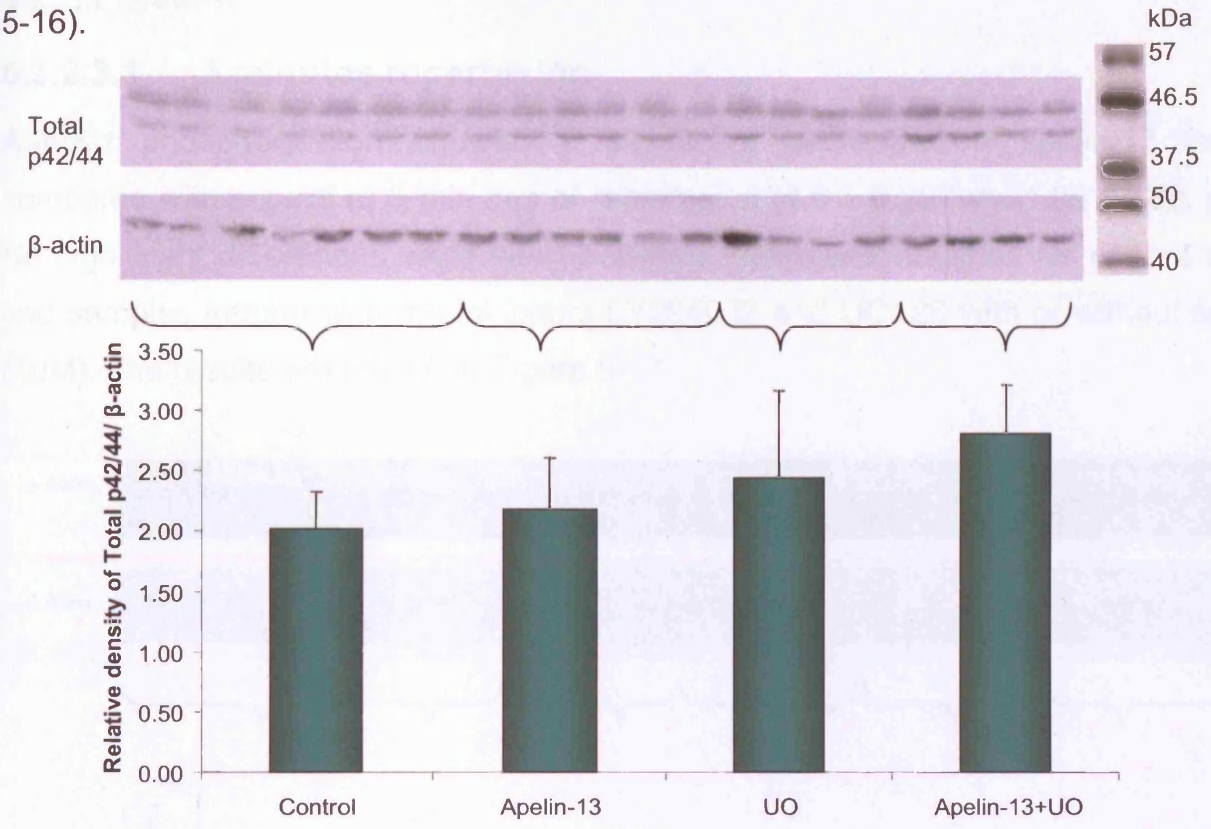

Figure 5-16 Total p42/44 MAPK in the presence or absence of apelin-13 (1μM) reperfused for 10 minutes with or without UO126

Data are presented as relative densitometry (RD) values in a.u. normalised for *β*-actin. Values are expressed as means ± s.e.m (n=4-7).

5.3.2.3 pAMPK

5.3.2.3.1 5 minutes reperfusion

AMPKα phosphorylation showed a significant decrease on apelin-13 treatment compared with control at 5 minutes of reperfusion (4.0 ± 0.227 vs. 1.9± 0.389, $p<0.01$). No significant differences were seen between the values obtained for control samples and samples treated with the inhibitors LY294002 and UO126 with or without apelin-13 (1μM). The results are shown in Figure 5-17.

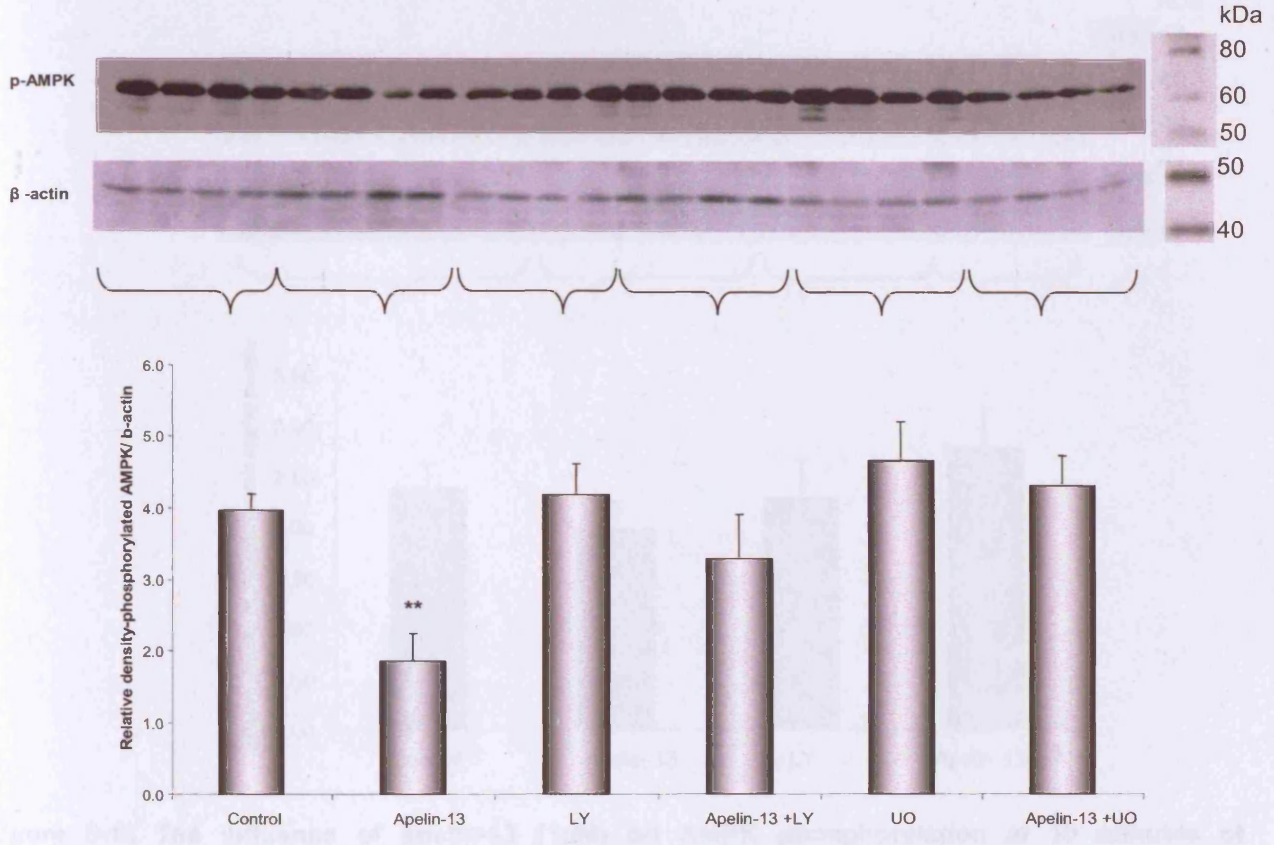

Figure 5-17 The influence of apelin-13 (1μM) on AMPK phosphorylation at 5 minutes reperfusion. Data are shown as RD values (a.u.) normalised for β-actin loading and indicate that apelin induces downregulation of AMPK phosphorylation. Values are presented as mean ± s.e.m. (**$p<0.01$, n=4).

5.3.2.3.2 10 minutes reperfusion

The results obtained for the phosphorylation of AMPK in the absence and presence of apelin-13 reperfused for 10 minutes are shown in Figure 5-18 and Figure 5-19. Even though 17% and 39% reductions in AMPKα phosphorylation were seen with apelin-13 (1μM) this did not reach statistical significance. There were also no significant differences between control values and the values obtained with the inhibitors LY294002 and UO126 with or without apelin-13 (1μM).

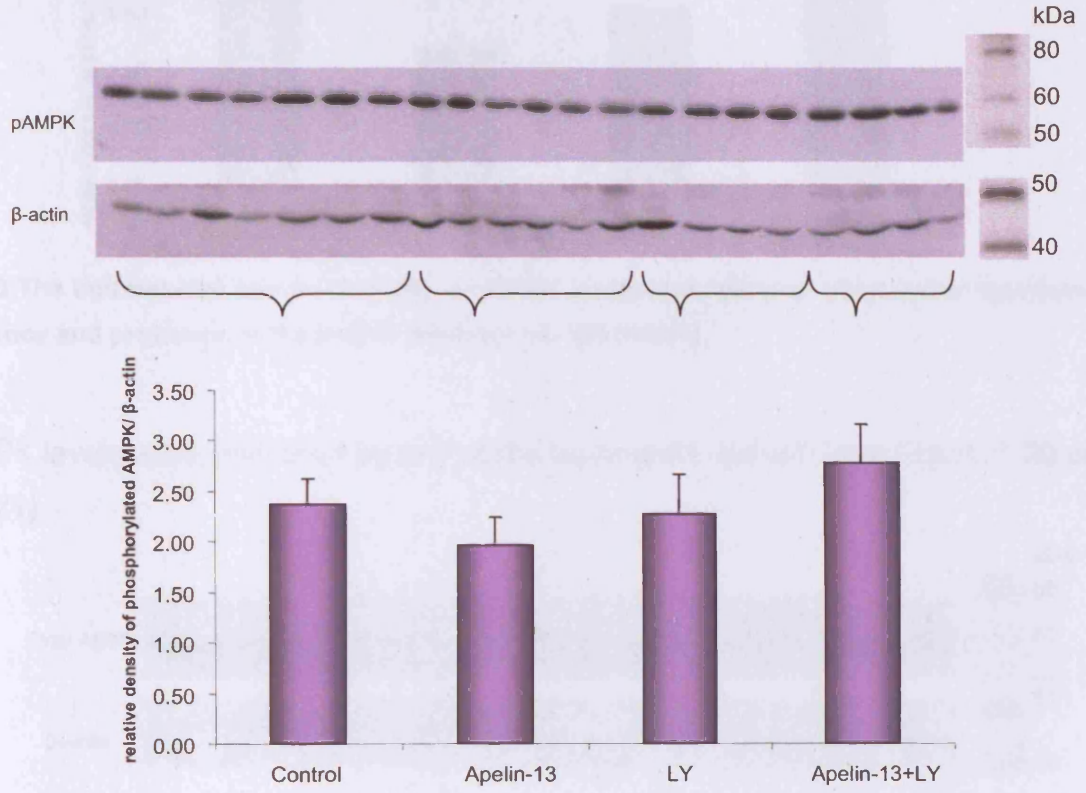

Figure 5-18 The influence of apelin-13 (1μM) on AMPK phosphorylation at 10 minutes of reperfusion in the absence and presence of the PI3K-Akt inhibitor LY294002 (n=4-7).

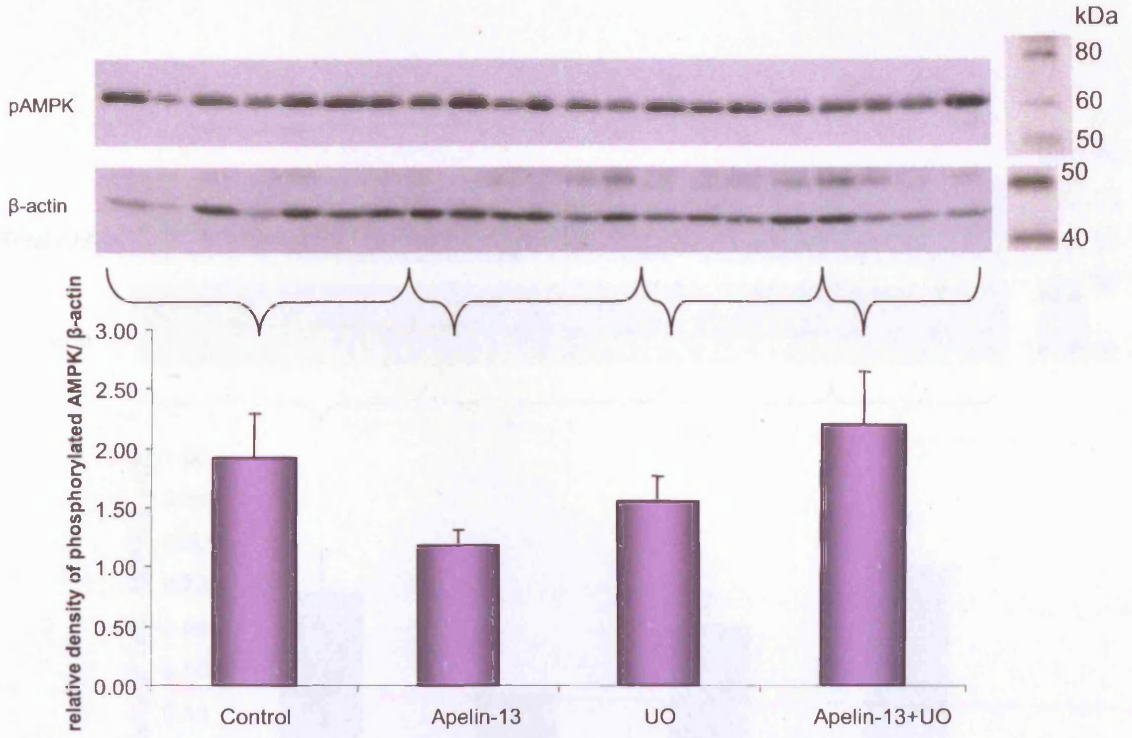

Figure 5-19 The influence of apelin-13 (1μM) on AMPK phosphorylation at 10 minutes reperfusion in the absence and presence of the p42/44 inhibitor UO126 (n=4-7).

Total AMPK levels were unaltered by any of the treatments applied (see Figure 5-20 and Figure 5-21)

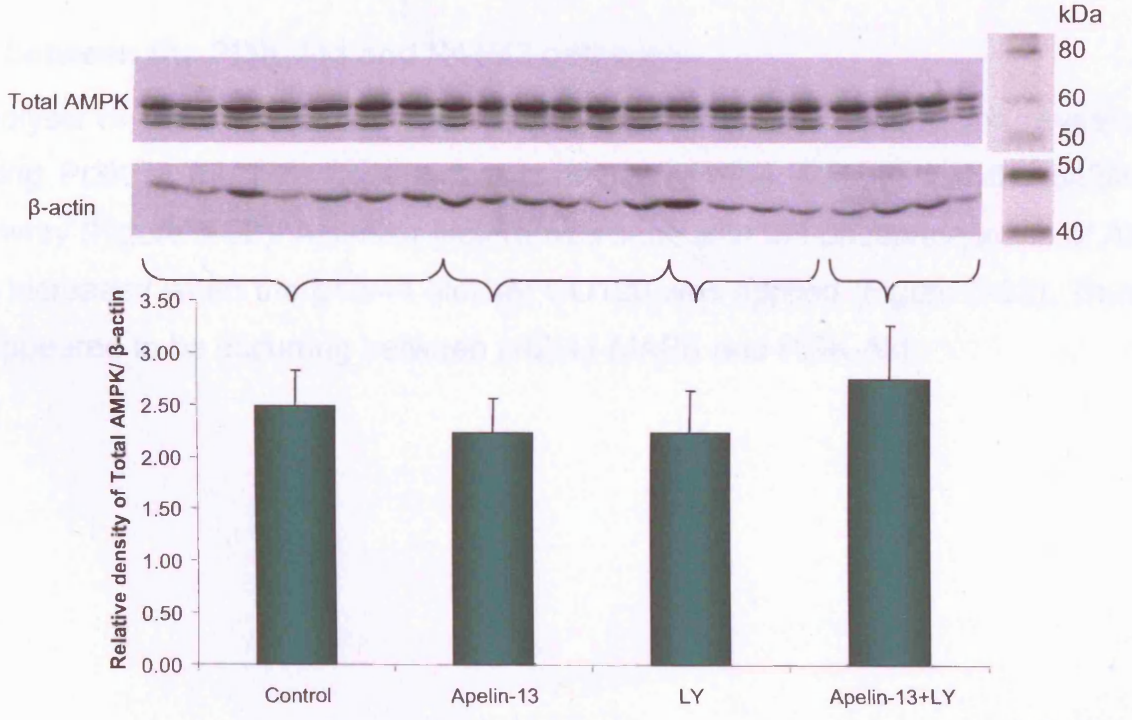

Figure 5-20 Total AMPK levels following 10 minutes reperfusion with apelin-13 in the absence and presence of the PI3K-Akt inhibitor LY294002 (n=4-7).

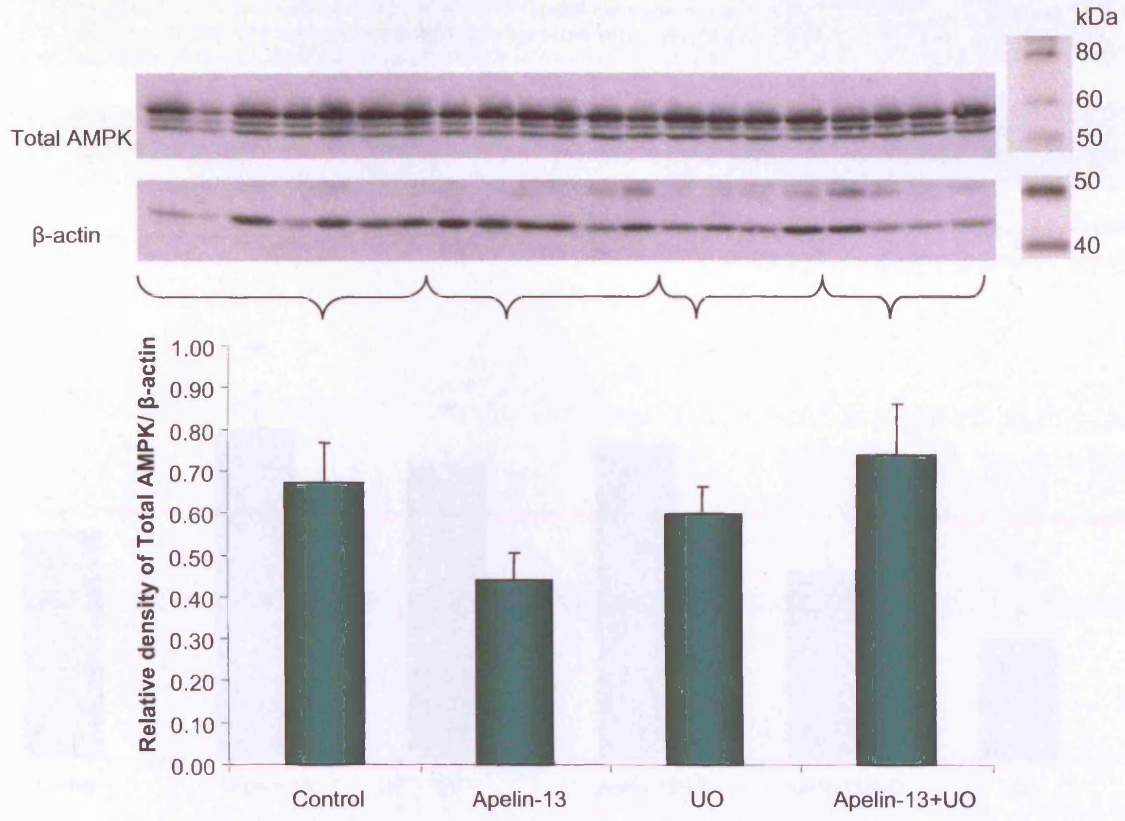

Figure 5-21 Total AMPK levels following 10 minutes reperfusion with apelin-13 with or without the p42/44 inhibitor UO126 (n=4-7).

Crosstalk between the PI3K-Akt and P44/42 pathways

Further analysis of the Western blot data obtained at 5 minutes reperfusion revealed that inhibiting PI3K at the time of reperfusion resulted in the activation of the p42/44 MAPK pathway (Figure 5-22). A similar picture was seen with the phosphorylation of Akt which was increased when the p42/44 blocker UO126 was applied (Figure 5-23). Thus, crosstalk appeared to be occurring between p42/44 MAPK and PI3K-Akt.

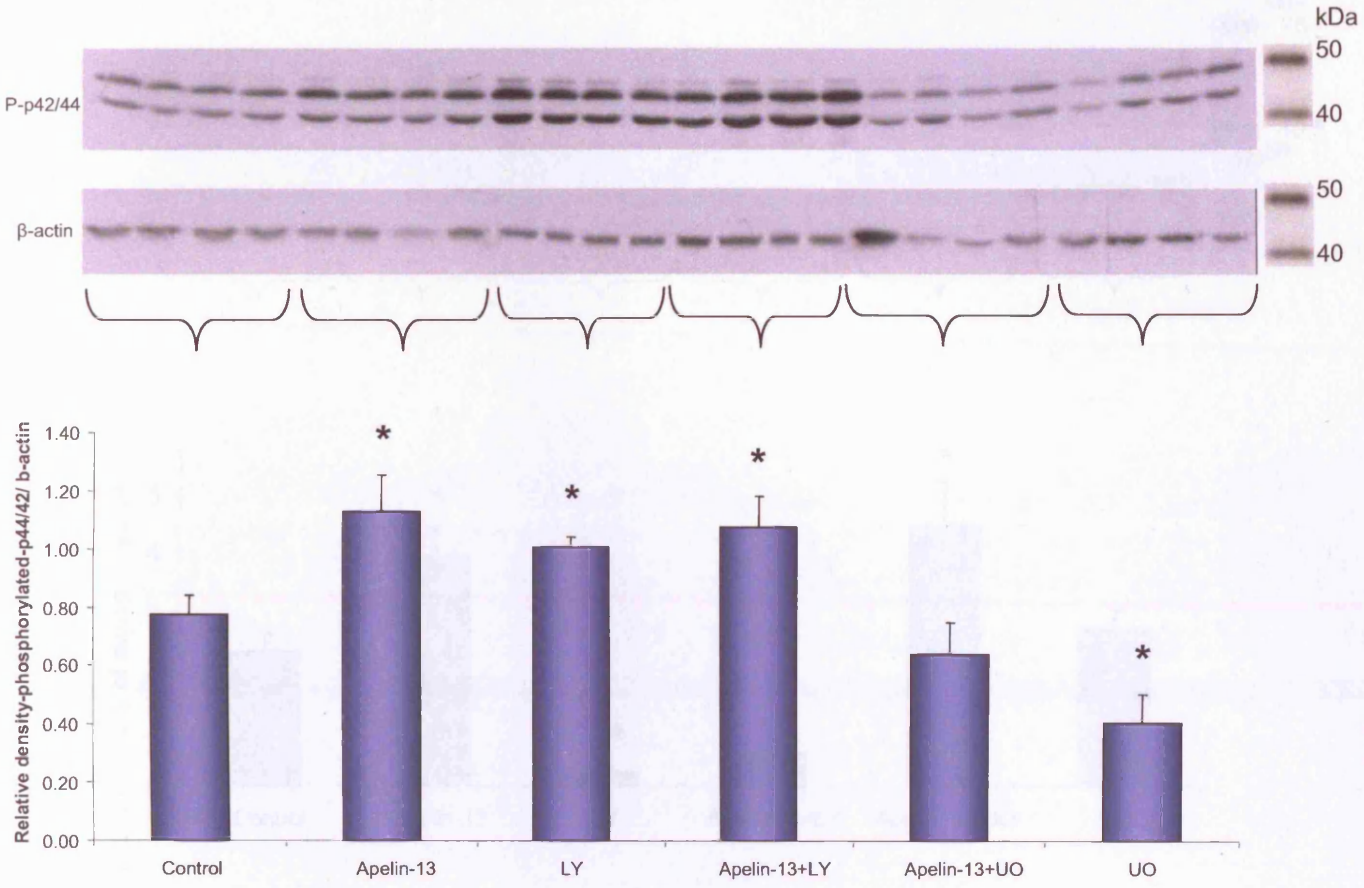

Figure 5-22 The effect of apelin-13 (1μM) on p42/44 MAPK phosphorylation in the presence of LY294002 or UO126.

Data are shown as relative density values (a.u.) normalised for *β*-actin loading and indicate that not only is there a significant increase in phosphorylated p42/44 with apelin-13 (1μM) when compared to control, but there is also a significant increase when the PI3K-Akt inhibitor, LY294002 is administered in the presence or absence of apelin (*$p<0.05$, n=4)

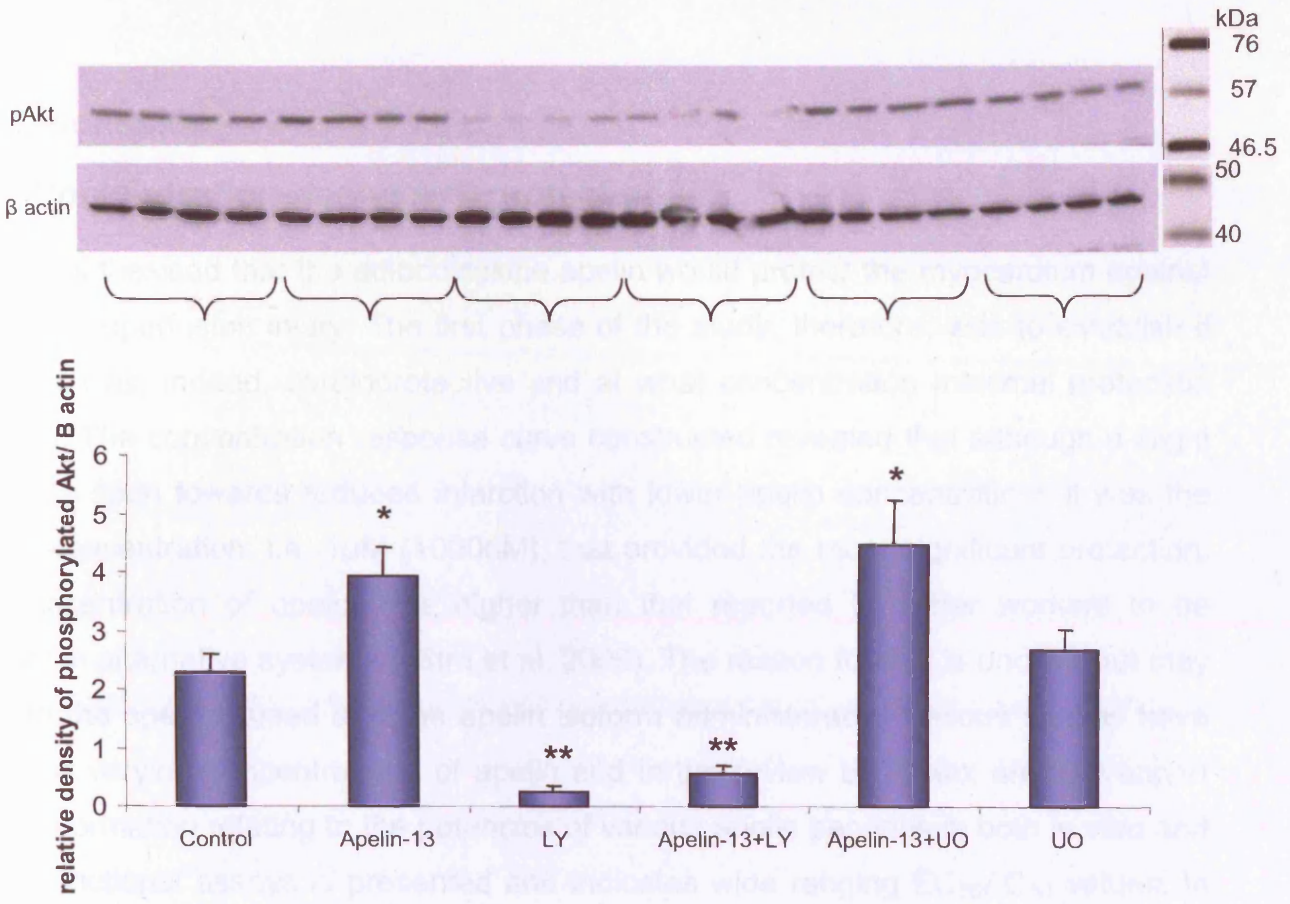

Figure 5-23 The effect of apelin-13 (1μM) on Akt phosphorylation in the presence and absence of LY294002 or UO126

Data are shown as RD values (a.u.) normalised for β-actin loading and demonstrate that apelin-13 (1μM) +UO126 significantly increases the phosphorylation of PI3K-Akt (**p<0.01 *p<0.05, n=4).

5.4 Discussion

5.4.1 Concentration-dependent protection

It was hypothesised that the adipocytokine apelin would protect the myocardium against ischaemia-reperfusion injury. The first phase of the study, therefore, was to establish if apelin-13 was, indeed, cardioprotective and at what concentration maximal protection occurred. The concentration response curve constructed revealed that although a slight trend was seen towards reduced infarction with lower apelin concentrations it was the highest concentration, i.e. 1μM (1000nM), that provided the most significant protection. This concentration of apelin was higher than that reported by other workers to be effective in alternative systems (Mitra et al. 2006). The reason for this is unclear but may relate to the species used and the apelin isoform administered. Previous studies have employed varying concentrations of apelin and in the review by Kleinz and Davenport (2005) information relating to the potencies of various apelin peptides in both in vitro and in vivo functional assays is presented and indicates wide ranging EC_{50}/IC_{50} values. In the present study it is possible that effects were missed, i.e. peptide concentrations between 100nM and 1000nM were not tested and apelin-induced infarct size reductions might have occurred over this range. Alternatively, it is possible that higher concentrations (>1000nM) of peptide might have produced more marked effects on infarct size. Whatever the reasons for the observations made the fact remains that effects were seen, albeit with an apelin concentration orders of magnitude greater than physiological/pathophysiological levels (Foldes et al. 2003), and equivalent to a pharmacological dose.

5.4.2 Isoform-dependent protection

The Langendorff perfused mouse heart model demonstrated that both apelin-13 and apelin-36 protected the heart when given at reperfusion. Apelin-13 treatment appeared to result in smaller infarct sizes when compared to apelin-36, although the values obtained were not statistically different. These data may, however, be consistent with previous reports that the biological potency of apelin-13 exceeds that of apelin-36 (Masri et al. 2004). It has been shown that apelin is produced as a large prepropeptide (Lee et al. 2000) which is processed to yield shorter more biologically active forms. Enzymatic

cleavage of large precursor molecules leading to the generation of a shorter more active peptide is commonly seen in endocrine systems (Rehfeld, 1998; Garden et al. 1999). Hence, the data obtained in the current study are in keeping with the consensus that the potency of apelin is inversely proportional to its peptide length (Tatemoto et al. 1998, Kawamata et al., 2001, Medhurst et al. 2003, Reaux et al., 2001, Beltowski 2006).

5.4.3 The involvement of the pro-survival (RISK) pathways

The study clearly demonstrated that the reductions in infarct size seen with apelin-13 were associated with PI3K-Akt and p44/42 activation. The inhibition, with appropriate chemical inhibitors, of both PI3K-Akt and P42/44 MAPK during Langendorff perfusion abrogated the protection afforded by apelin-13. These findings were substantiated by Western blot analysis, although, interestingly, the time of sample collection during reperfusion influenced the magnitude and statistical significance of the effects observed. Samples taken at 5 minute reperfusion revealed that significant phosphorylation of the pro-survival kinases had occurred, whilst, by 10 minutes of reperfusion statistical significance had been lost. These data suggest that a time window exists during which phosphorylation of the pro-survival kinases occurs. When phosphorylation/activation of these kinases is maximal during the reperfusion phase is, however, open to conjecture. Previous studies have examined the phosphorylation of pro-survival kinases at various time points (Masri et al., 2002, Xie et al., 2006, Hashimoto et al., 2006). Apelin treatment in osteoblasts has been shown to increase the phosphorylation of Akt after 5 minutes of incubation, with maximal activation occurring at 15 minutes (Xie et al 2006). Apelin promoted a transient and concentration-dependent phosphorylation of ERK in Chinese hamster ovary cells expressing the APJ receptor which peaked at 5 minutes, the signal being lost by 60 minutes. Apelin also stimulated myosin light chain kinase phosphorylation in vascular smooth muscle cells; peak phosphorylation was seen at 2 minutes and lost by 10 minutes (Hashimoto et al., 2006).

In the current study it is possible that peak phosphorylation/activation of the pro-survival kinases occurred before 5 minutes, and that the window of maximal phosphorylation was missed. It is also conceivable that increased kinase phosphorylation is not necessarily accompanied by increased kinase activity. In a recent paper from our laboratory (Smith et al 2007) the temporal nature of the activation of the RISK pathway

components was examined. An Enzyme Linked ImmunoSorbent Assay (ELISA) was employed to investigate if Akt activity reflected the Akt phosphorylation state. The study concluded that under basal conditions of I/R (i.e. in the absence of the cardioprotective agent apelin) phosphorylation of the RISK pathway components, Akt and p44/42, increased in a time-dependent fashion in the early minutes of reperfusion. These increases in phosphorylation were, however not accompanied by increased kinase (i.e. Akt) activity. Contrasting with the results obtained under basal conditions, the application of apelin not only increased Akt phosphorylation at 5 and 10 min reperfusion but also increased Akt activity. It was concluded that maximal phosphorylation of the RISK pathway components, and specifically Akt, does not necessarily coincide with maximal kinase activity (i.e. Akt) activity. In addition, it was concluded that under basal conditions, i.e. in the absence of a cardioprotective agent such as apelin, kinase phosphorylation may occur as a result of the heart attempting to protect itself during ischaemia, (Smith et al., 2007). Thus, it is only with the addition of cardioprotective agents, such as apelin, which stimulate components of the RISK pathway to a sufficient extent that protection is observed. An extension of this study would be to carry out measurements of p44/42 kinase activity, as well as Akt activity. The recent availability of p44/42 ELISA assays will enable these investigations to be conducted and will resolve the situation with respect to the relationship between phosphorylation and activation of RISK pathway components and cardioprotection.

The influence of the PI3K-Akt and p42/44 kinase inhibitors LY294002 and UO126 on apelin-36 induced reductions in infarct size and kinase expression were not investigated. The main reason for this was that apelin-13 was more potent than the longer isoform with respect to cardioprotection, and therefore further investigation was deemed not necessary.

5.4.4 Apelin-F13

Previous work has suggested that manipulation of the N-terminal end of apelin influences its binding affinity to the APJ receptor and therefore its biological potency (Lee et al 2005). Thus, modification of this group was found to blunt the hypotensive response to apelin-13, leading to the conclusion that apelin-F13 was acting as an antagonist to apelin-13 (Lee et al. 2005). In the light of this work, a set of experiments was carried out to investigate the hypothesis that apelin-F13 may antagonise the cardioprotective effects of apelin-13. Interestingly, however, rather than blocking protection, apelin-F13 when given during stabilisation and prior to apelin-13, resulted in a greater reduction in mean infarct size compared to apelin-13 alone. This finding may have a number of explanations. The study by Lee et al. (2005), for example, focused on the actions of apelin-F13 on blood pressure with no data for binding affinities and receptor activation being presented. Thus, it is possible that the antagonistic action of apelin-F13 on apelin-13 induced hypotension does not involve the same signalling pathways that mediate apelin-13's mitogenic and anti-apoptotic effects. It may also be the case that apelin-F13 acts through partial inhibition of the APJ receptor whilst allowing stimulation of the pro-survival RISK pathways to occur. Due to limitations of time the actions of apelin-F13 were not fully characterised. Such experiments would have established whether or not apelin-F13 was itself cardio protective and if co-administration with apelin-13 influenced protection.

5.4.5 Nitric Oxide

Apelin exerts its haemodynamic actions via NO generation. Thus, the hypotensive effects of apelin were found to involve endothelial NO release and eNOS phosphorylation, and were blocked by L-NAME (Tatomoto et al. 2001, Ishida et al 2004). The data obtained in this study, were, however, not consistent with apelin having produced its cardioprotective actions through the modulation eNOS activity, i.e. Western blot analysis did not indicate that eNOS phosphorylation was increased following apelin treatment. Further studies using the inhibitor L-NAME would have clarified the situation as to whether NO played a role in the cardioprotective actions of apelin during I/R injury. Interestingly, studies carried out in this laboratory on the effects of the adipocytokine

leptin in I/R injury showed that L-NAME blocked protection (Smith et al. 2006). Leptin, like apelin, has been reported to cause activation of NOS in a number of tissues, including the heart (Nickola et al., 2000). In the study by Smith et al. (2006), however, a similar problem to that encountered with apelin occurred as regards the detection of significant leptin-stimulated changes in eNOS phosphorylation. It is possible that in this study and the present investigation that the eNOS signal had been dissipated by the time the hearts had been removed from the Langendorff apparatus. Further experimentation with apelin and L-NAME, however, will establish whether apelin's influence on NO generation has purely haemodynamic consequences or is involved in cardioprotection.

5.4.6 AMPK

The metabolic actions of the adipocytokines involve upregulation of AMPK activity (Kershaw et al. 2004, Fruhbeck et al 2006). Shibata et al. (2005) have reported that the cardioprotective actions of adiponectin involve AMPK activation. The importance of AMPK with respect to the cellular actions of apelin has yet to be established. However, it is intriguing that in the present study apelin treatment, rather than resulting in increased AMPK activity, was found to cause significant reductions in phosphorylated AMPK levels. Total AMPK levels were also reduced but not to a significant extent. These observations are reminiscent of previous findings from this laboratory regarding leptin; it was found that leptin-induced myocardial protection was associated with decreased total AMPK tissue contents (Smith et al., 2006). One explanation put forward for these reductions was that downregulation of total AMPK had occurred as a consequence of cross-talk between AMPK and p44/42, based on the observation that the leptin-induced decrease in total AMPK was abrogated in the presence of the p44/42 inhibitor UO126. A substantial body of evidence now exists for cross-talk between AMPK and various cell-signalling pathways. Kovacic et al. (2003) obtained evidence for cross-talk between the AMPK and Akt pathways and demonstrated that Akt activation led to decreased AMPK activity (Kovacic et al 2003). Meanwhile, in vascular smooth muscle evidence for cross-talk between p44/42 MAPK and AMPK was obtained (Rubin et al 2005). The results of the present study are in agreement with this finding i.e. decreased AMPK phosphorylation being coupled with Akt activation and probably occurring as a result of

cross-talk between Akt and AMPK pathways a phenomenon previously reported for Akt and p44/42 (see section below).

5.4.7 Crosstalk between PI3K and P42/44

The phenomenon of cross-talk between the RISK pathway components in which inhibition of one kinase results in the activation of another and vice versa, has been observed in tissues other than the heart, including the lens (Harada et al 2001) and neuronal cells (Van Der Heide et al 2003). The results of the present study confirmed that inhibiting PI3K, using LY 294002, at the time of reperfusion resulted in the activation of the p42/44 MAPK pathway. Interestingly, this cross-talk between the kinase cascades was not associated with cardioprotection, which suggests that the phosphorylation of both kinase cascades may be required at the time of reperfusion to mediate cardioprotection. In addition, it did not appear to be equal in that inhibition of MAPK p42/44 with UO126 did not significantly increase the phosphorylation of PI3K-Akt whilst, by contrast, inhibition of PI3-Akt with LY294002 at the time of reperfusion resulted in significant p42/44 phosphorylation. These findings suggest that crosstalk between kinase cascades are not balanced and that in the scenario of cellular survival, the PI3K/-Akt pathway is the more dominant cascade. Similar findings were reported by Hausenloy et al. (2004) and it was suggested that the MAPK p42/44 cascade may play a greater role in mediating growth and hypertrophy, whereas PI3K-Akt is more involved in cell survival (Hausenloy et al 2004).

5.4.8 Study limitations

The present study has a number of limitations which should be considered when interpreting the data obtained. First, whilst the Langendorff model allows study of apelin to the exclusion of other humoural influences, by the same token influences/factors which may be important in modulating the actions of apelin, e.g. its paracrine effects, are absent. Second, limitations exist as regards the technique of Western blotting, particularly in relation to the phosphorylation and activation of the RISK pathway kinases. Each blot represents one heart and in some cases large variations in the relative density values obtained contributed to non-significant differences between groups. Increasing the numbers of heart samples per group might, therefore, have

reduced this variability and altered the levels of significance obtained. Finally, the study relied heavily on the supposed specificity of the pharmacological inhibitors used. Thus, the concentrations employed being were the same as previously used in this laboratory (Efthymiou et al. 2006) and were well within the concentration ranges reported to produce specific kinase inhibition (Vlahos et al. 1994). It cannot, however, be categorically stated that these agents do not influence kinase pathways other than RISK pathway components.

5.5 Conclusion

In this study it has been demonstrated that apelin-13 and apelin-36, when given during reperfusion, protects the heart from I/R injury. These findings are in agreement with data obtained previously which indicated that other adipocytokines protected against I/R induced injury (Smith et al., 2006, Shibata et al., 2005). In the case of the more biologically potent isoform, apelin-13, protection was, importantly, associated with the activation of the RISK pathway.

In addition to demonstrating that apelin was cardioprotective, data were obtained which provide further evidence for cross-talk between p44/42 and AMPK in the myocardium, as well as between Akt and AMPK. It can, therefore, be concluded that the effects produced by apelin (and leptin) could indicate that some of the adipocytokines share common mechanisms of action, for example, in relation to the modulation of AMPK activity.

6 EFFECT OF APELIN ON MPTP OPENING IN ISOLATED ADULT RAT CARDIOMYOCYTES

6.1 Aims

The aim of the studies described in this chapter was to establish whether apelin influences the opening of the mPTP and the time to cardiomyocyte contracture. The mPTP may constitute an integral part of the machinery of the cell for reducing damage and promoting protection (Hausenloy et al., 2004). Recently the link between activation of the RISK pathway and prevention of mPTP opening was established with respect to cardioprotection (Davidson et al., 2006). Hence, it was hypothesised that apelin has direct effects on the mPTP delaying its opening and the time to myocyte hypercontracture. Evidence for possible mechanisms of protection was sought using inhibitors of the RISK pathway.

6.2 Methods

Rat ventricular cardiomyocytes were used to examine the effects of apelin on mPTP opening induced by oxidative stress. The model employed has been used extensively in this and other laboratories (Davidson et al. 2006, Jacobson & Duchen 2002) and is described in the review by Duchen (2000). Myocytes were isolated as described in chapter 3.7. and subjected to laser stimulation leading to mitochondrial reactive oxygen species (ROS) production, simulating the ROS production which occurs during reperfusion. The fluorescent dye, TMRM, was used to assess mitochondrial depolarisation and calculation of the times taken to depolarisation and subsequent rigor allowed for comparison between different treatments.

6.3 Protocol

The effect of apelin-13 (1μM) on the times to depolarisation and rigor in the absence and presence of the kinase inhibitors MEK 1 and LY294002 was examined. Experiments with UO126 were not carried out as it is highly light sensitive and prone to degradation as a consequence of laser stimulation. Hence, a selective inhibitor of MEK (MEK inhibitor 1), which is upstream of p42/44 MAPK and chemically related to UO126 but

more stable, was used (Wityak et al., 2004). Pre-incubation with apelin (1μM), with or without LY294002 (15μM) or MEK1 (1μM) inhibitor was followed by laser stimulation. Pre-incubation with LY294002 or MEK1 alone was carried out to ensure that the inhibitors did not influence mPTP opening themselves, and with cyclosporin A (CSA) (200nM), a recognised inhibitor of mPTP opening, which was used as a positive control (see Figure 6-1). Experiments were conducted with a total of 100 rat myocytes isolated from at least three hearts.

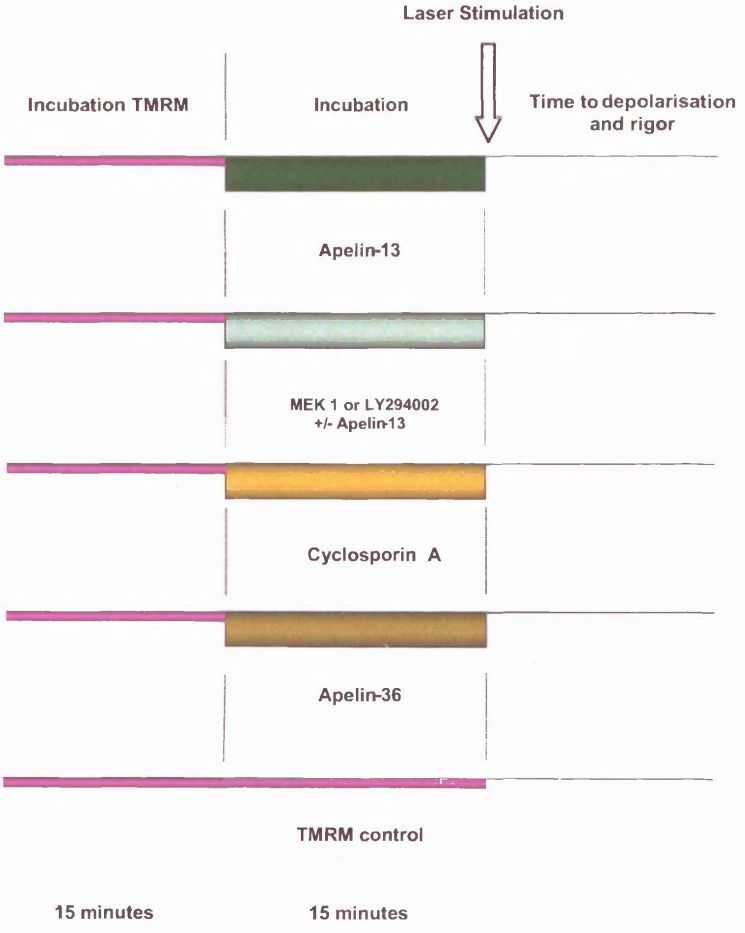

Figure 6-1 Protocols used to study the effects of apelin on mPTP pore opening induced by oxidative stress

The timeline of the experimental protocols used to examine mPTP opening under various conditions is shown above. All samples were pre-incubated with TMRM for 15 min, followed by 15 minutes incubation with the test reagent.

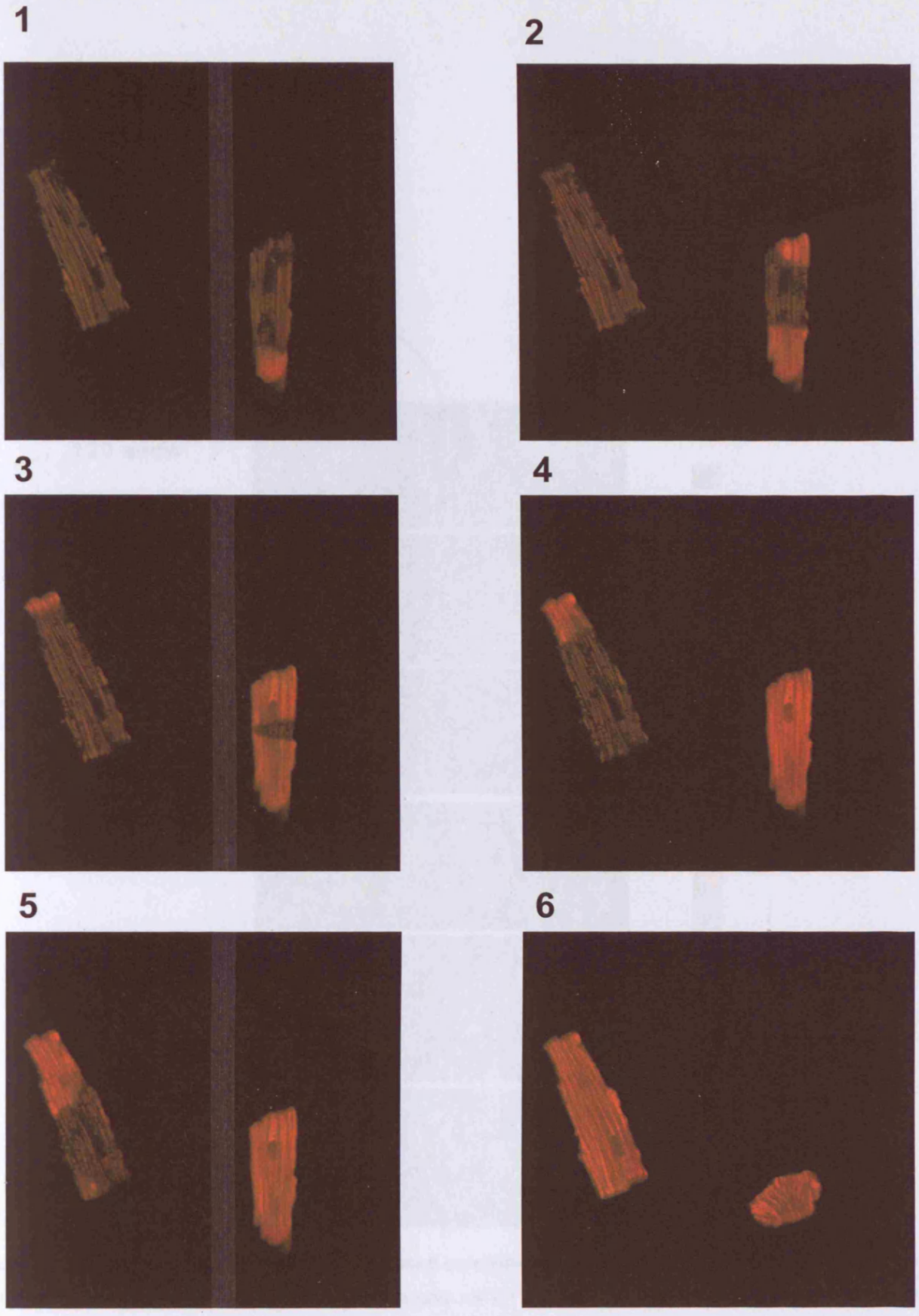

Figure 6-2 Cardiac myocyte depolarisation signifying mPTP opening and subsequent rigor

This figure shows the wave of depolarisation (1-6) occurring along the myocyte on laser stimulation and subsequent hypercontracture.

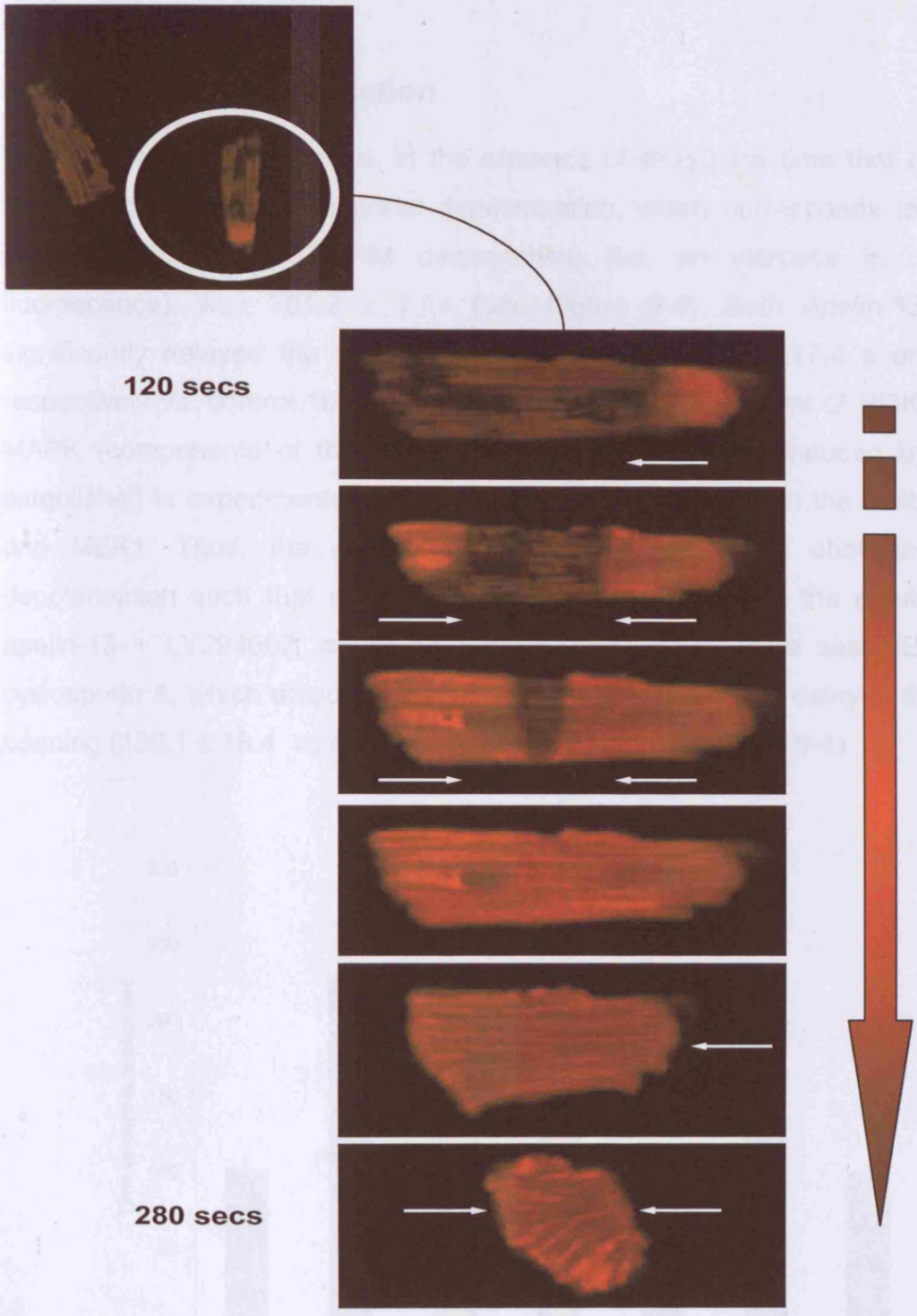

Figure 6-3 Depolarisation and rigor in an isolated cardiomyocyte.

This figure shows the process of depolarisation and mPTP opening in a single myocyte. The wave of depolarisation as it progresses along the myocyte can be clearly seen, along with the shortening of the myocyte as ATP becomes depleted and hypercontracture ensues. The times until depolarisation and rigor were recorded to allow comparison between treatment groups.

6.4 Results

6.4.1 Time to Depolarisation

Under control conditions (i.e. in the absence of drug.) the time that elapsed until the onset of complete mitochondrial depolarisation, which corresponds to mPTP opening and is indicated by TMRM dequenching (i.e. an increase in cytosolic TMRM fluorescence), was 101.2 ± 7.1s (see Figure 6-4). Both Apelin-13 and apelin-36 significantly delayed the time to mPTP opening (229.9 ± 17.4 s and 195.4 ± 9.6s respectively vs. control 101.2 ± 7.1 $p<0.001$). The involvement of PI3K-Akt and p42/44 MAPK (components of the RISK pathway) in the effects induced by apelin-13 was established in experiments in which cells were incubated with the inhibitors LY-294002 and MEK1. Thus, the addition of LY249002 and MEK abolished the delay in depolarisation such that no significant differences between the results obtained with apelin-13 + LY294002, apelin-13 + MEK and control were seen. Experiments with cyclosporin A, which acted as a positive control, significantly delayed the time to mPTP opening (188.1 ± 18.4 vs control 101.2 ± 7.1, $p<0.01$)(Figure 6-4)

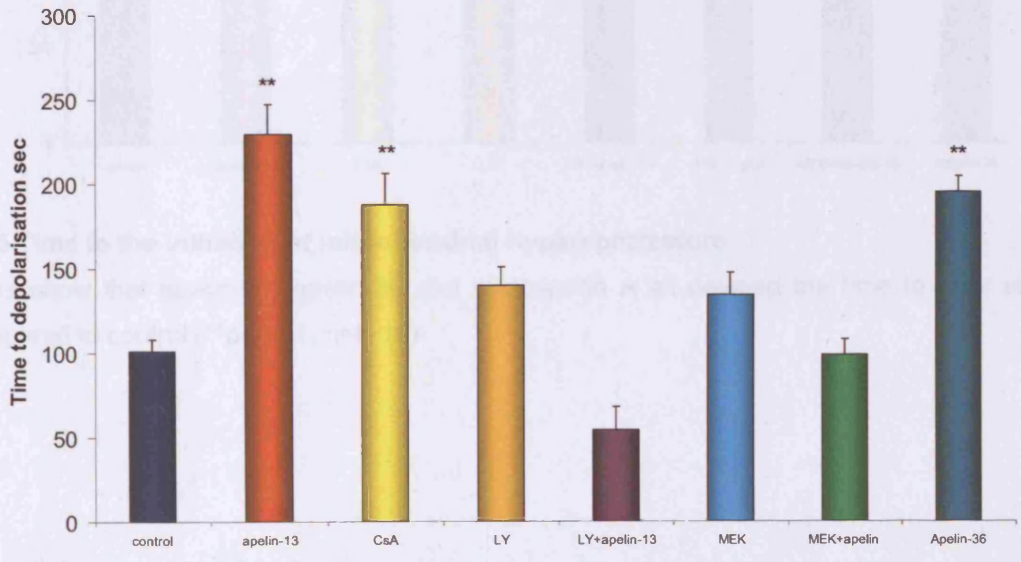

Figure 6-4 Time to the initiation of mitochondrial depolarization and mPTP opening

Apelin-13, Apelin-36 and cyclosporin all delayed mPTP opening significantly when compared to control (**$p<0.01$, n=6-16)

6.4.2 Time to Rigor

The time taken to rigor was significantly extended by apelin-13, apelin-36 and CsA compared to control (519.7 ± 38.7s, 502 ± 61.3s and 456.2 ± 33.8s, respectively, vs. control 285.8 ± 23.3s $p<0.01$) (Figure 6-5). Both LY294002 and MEK 1 blocked the delaying effects of apelin on mPTP opening, providing further evidence that the actions of apelin involve the PI3-K/AKT and p42/44 MAPK signalling pathways (see Figure 6-5). None of the inhibitors by themselves influenced the times taken to induce cardiomyocyte contracture or mPTP opening as compared to controls (Figure 6-4 and Figure 6-5).

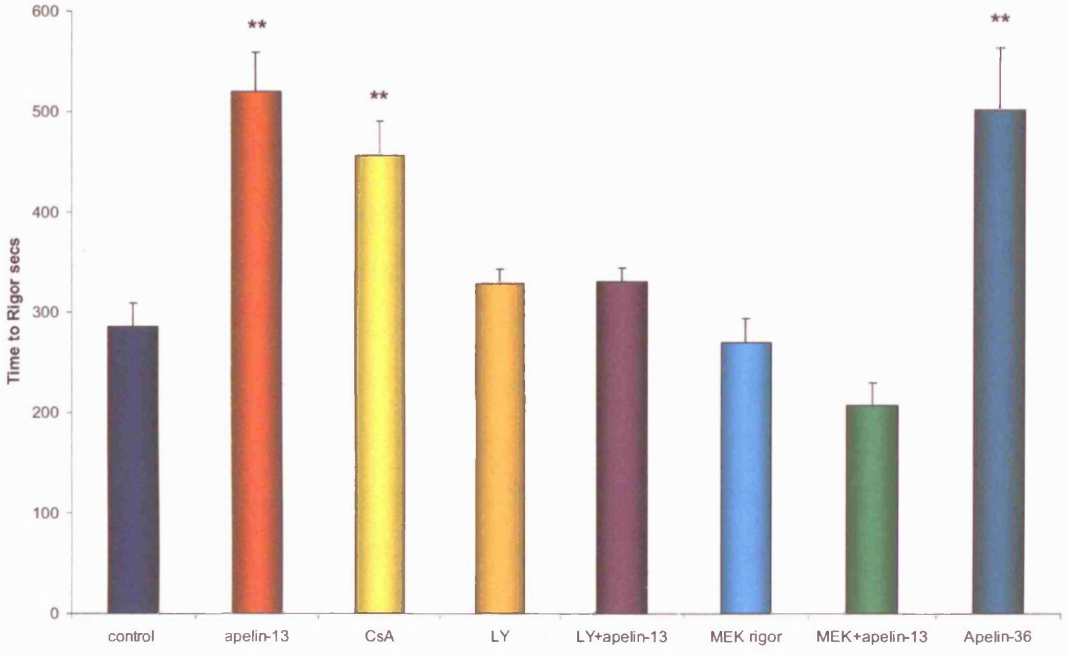

Figure 6-5 Time to the initiation of mitochondrial hypercontracture

The results show that apelin-13, apelin-36 and cyclosporin A all delayed the time to rigor significantly when compared to control (**$p<0.01$, n=6-16)

6.5 Discussion

Cardioprotection whether induced by ischaemic preconditioning or pharmacological agents involves activation of the RISK pathway and appears, ultimately, to be mediated through inhibition of mPTP opening (Hausenloy et al. 2003, Schinzel et al. 2005). The data presented in this chapter confirm the hypothesis that apelin-induced cardioprotection through activation of the RISK pathway occurs via inhibition of mPTP opening. This is in concordance with previous studies from this laboratory in which other cardioprotective strategies were used (Davidson et al 2006; Smith et al 2006). As seen with Langendorff experiments apelin-13 and apelin-36 produced effects in cardiomyocytes, both isoforms delaying pore opening. The delay with apelin-36, however, was not as prolonged as that seen with apelin-13, therefore lending further support for the theory that the shorter isoforms are more biologically active. The mechanism, by which RISK pathway activation mediates the inhibition of mPTP opening, is not fully understood. Akt may reduce pore opening via the phosphorylation of eNOS, which is known to inhibit mPTP opening (Kim et al. 2004). The anti-apoptotic action of Akt on the Bcl-2-associated death promoter (BAD) has previously been described (Chapter 1.4.2.2), and offers an alternative mechanism by which the mPTP may be inhibited (Jonassen et al. 2001). Whatever the pathways operating in cell death, pharmacological interventions that can inhibit mPTP opening, such as apelin, provide protection from reperfusion injury.

Previous findings from this laboratory have indicated that mice lacking the cyclophilin D component of the mPTP exhibit increased resistance to cardiomyocyte mPTP opening and reduced myocardial infarcts (Lim et al. 2007). From this we can postulate that the suppression induced by apelin and IPC of pathological mPTP opening occurring on reperfusion of the ischaemic myocardium is a likely target for cardioprotection.

In this study, apelin-13 and apelin 36, as well as cyclosporin-A, were given at the time of reoxygenation, immediately following hypoxia, to target the opening of the mPTP which occurs during the first few minutes of reoxygenation/reperfusion. These findings have implications for myocardial protection in the clinical setting, as a cardioprotective

strategy that can be applied during the reperfusion phase is clearly easier to implement given the unpredictable nature of acute myocardial infarction. Therefore, in the clinical setting of ischemia-reperfusion injury, such as after an acute myocardial infarction or at the time of cardiac surgery, intervening at the time of reperfusion offers a cardioprotective strategy can be more easily controlled by the clinician (Shanmuganathan et al. 2005).

6.5.1 Limitations

It has already been mentioned that a potential mechanism whereby Akt may delay mPTP opening is via the modulation of eNOS production. This mechanism could have been explored further in the present study using the eNOS inhibitor, L-NAME. Employing two additional treatment groups, i.e. L-NAME, and apelin-13+L-NAME, the potential role played by eNOS in apelin's actions on pore opening could have been investigated.

As described earlier cardiomyocytes from Sprague-Dawley rat hearts were used for these studies. Ideally, cells from C57/Bl6J mice should have been used, given that Langendorff and in vivo studies with apelin employed this species. In this laboratory the development of methods for the efficient preparation of cardiomyocytes from the murine heart is currently underway and hopefully in the near future studies with these cells will be possible, thus allowing species continuity. The preparation of murine cardiomyocytes is, however, technically difficult as these cells are less robust than those from rat heart and yields can be poor.

6.6 Conclusion

The mPTP represents a vital element of the intracellular machinery for limiting tissue damage (Hausenloy et al., 2004, Argaud et al.2005). Both apelin-13 and apelin-36 were shown to delay mPTP opening and cardiomyocyte contracture, the PI3K-Akt and p42/44 pathways appearing to be involved. In this respect apelin has similarities with leptin (Smith et al., 2006) which was shown to produce comparable actions on the mPTP, as well as reducing infarct size. The results of the present study, therefore, lend further credence to the theory that RISK pathway mobilisation and suppression of mPTP

opening are key mediators of cardioprotection (Hausenloy et al., 2004, Javadov et al., 2003, Argaud et al., 2005, Bopassa et al., 2006).

In the future the administration of pharmacological agents which activate these kinases and inhibit mPTP opening may prove valuable as adjuvant therapy to current myocardial reperfusion strategies such as thrombolysis and primary percutaneous coronary intervention, thereby offering further cardioprotection over and above that provided by reperfusion itself.

7 THE INFLUENCE OF APELIN ON INFARCT SIZE IN VIVO

7.1 Aims

The initial part of this study entailed examining if apelin, when administered intravenously, produced any haemodynamic effects. Thus, the aim of these preliminary experiments was to establish if apelin could be safely employed in a protocol designed to investigate if apelin protects against ischaemia/reperfusion injury in vivo. This work was intended to compliment the studies undertaken in the Langendorff heart but also to examine the effects of apelin in a more physiological model. The experiments were carried out in this laboratory in collaboration with Dr S. Lim, an expert in murine in vivo ischaemia/reperfusion modelling.

The in vivo actions of apelin in the mouse have, until now, not been described, although the results of infarct studies in other rodent models and, more recently, a sheep model have been reported (Lee et al, Tatemoto et al, Charles et al. 2006). Thus, a series of experiments were carried out in which the effects of increasing concentrations of apelin-13 on mean arterial blood pressure (MABP) and heart rate (HR) were investigated. The concentrations of apelin used in previous studies have varied widely, with doses ranging between 3 and 15 µg/kg (Lee et al. 2000, 2005, Tatemoto et al 2001, El Massari et al 2004) producing a fall in MABP. Higher doses of apelin (100-250 µg/kg) administered intravenously have been reported to increase MABP, although information on the time-course of the effects observed were not presented. Thus, it is unclear whether the hypertensive response to apelin preceded a decrease in MABP.

7.2 Methods

C57 Bl/6J were anaesthetised by intraperitoneal injection with a combination of ketamine, xylazine and atropine (final concentrations of ketamine, xylazine and atropine were 10 mg/ml, 2 mg/ml and 0.06 mg/ml, respectively) and body temperature maintained at 37^0C. The external jugular vein and carotid artery were then isolated and cannulated with polyethylene tubing containing heparinised saline (15 U heparin/ml 0.9% saline) these vessels being used for drug administration and mean arterial blood pressure (MABP) measurement, respectively. A tracheotomy was performed for artificial respiration which was maintained at 120 strokes/min and a 220 µl stroke volume using a

rodent Minivent (type 845, Harvard Apparatus, Kent, UK), and supplemental oxygen was supplied. A limb lead I electrocardiogram (ECG) was recorded. A left anterior thoracotomy and a chest retractor were used to expose the heart. Ligation of the left anterior descending (LAD) coronary artery was performed ~2 mm below the tip of the left auricle using 8/0 prolene monofilament polypropylene suture. Successful LAD coronary artery occlusion was confirmed by the presence of ST elevation and a decrease in arterial blood pressure. At the end of reperfusion the heart was isolated and the aortic root cannulated and used to inject 2,3,5-triphenyltetrazolium chloride (TTC, 5 ml of 1%) in order to demarcate the infarcted tissue. The LAD coronary artery was then re-ligated and Evans blue dye (2 ml of 0.5%) was perfused to delineate the area at risk (AAR). The heart was frozen and sectioned perpendicular to the long axis (1-2 mm thick). The slices were then transferred to 10% neutral-buffered formalin for 2 hours at room temperature to stabilize the staining. The area at risk (AAR) and infarct size were determined by planimetry as described in Chapter 3.6 with the AAR being expressed as a percentage of the left ventricle and infarct size as a percentage of the AAR.

7.3 Protocol

In order to assess apelin potency in vivo with regard to haemodynamic parameters the peptide was administered at varying concentrations according to the protocol illustrated in Figure 7-1

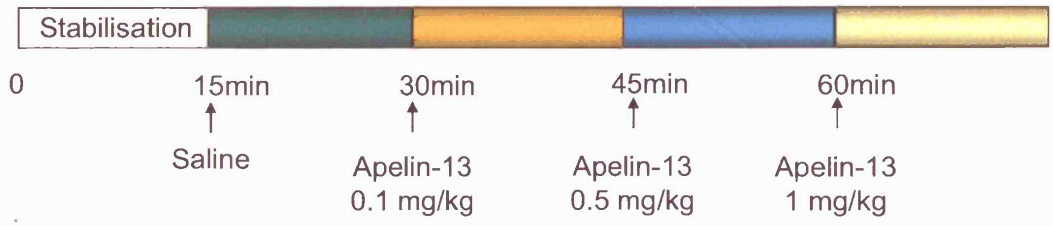

Figure 7-1 Experimental protocol for examination of the effects of apelin on haemodynamic parameters.

C57Bl/6J mice 8-10 weeks old, were administered apelin or saline in an injection volume of 0.05 ml/25 g via the jugular vein.

To investigate the effects of apelin on infarct size in vivo a series of experiments was conducted in which the heart was allowed to stabilise for 5-10 min prior to ligation. The suture was then tightened for 30 min to induce ischemia. After the ischemic period the ligature was released and a 120 min period of reperfusion followed. Apelin was administered via the external jugular line at the onset of reperfusion: in control experiments 0.9% saline was substituted for peptide (see Figure 7-2 for protocol).

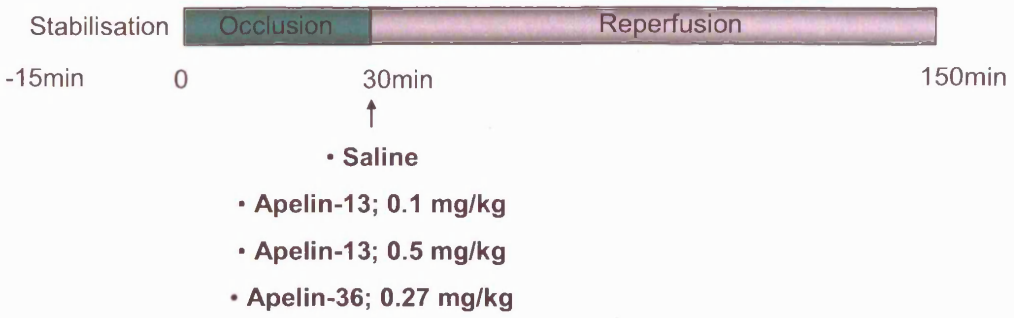

Figure 7-2 Experimental protocol for the examination of the effects of apelin on infarct size.

C57 Bl/6J mice, 8-10 weeks old, were given saline or apelin in an injection volume of 0.05 ml/25g via the jugular vein.

7.4 Results

7.4.1 Apelin haemodynamic dose response

The haemodynamic effects of apelin as reflected by changes in mean arterial blood pressure are shown in Figure 7-3 A typical response, with respect to mean arterial blood pressure (MABP), elicited by the administration of apelin Thus, after the injection of apelin a transient fall in MABP was observed followed by a rise. To quantify treatment-induced changes readings were taken at the trough and peak points indicated by 1 and 2 (Figure 7-3).

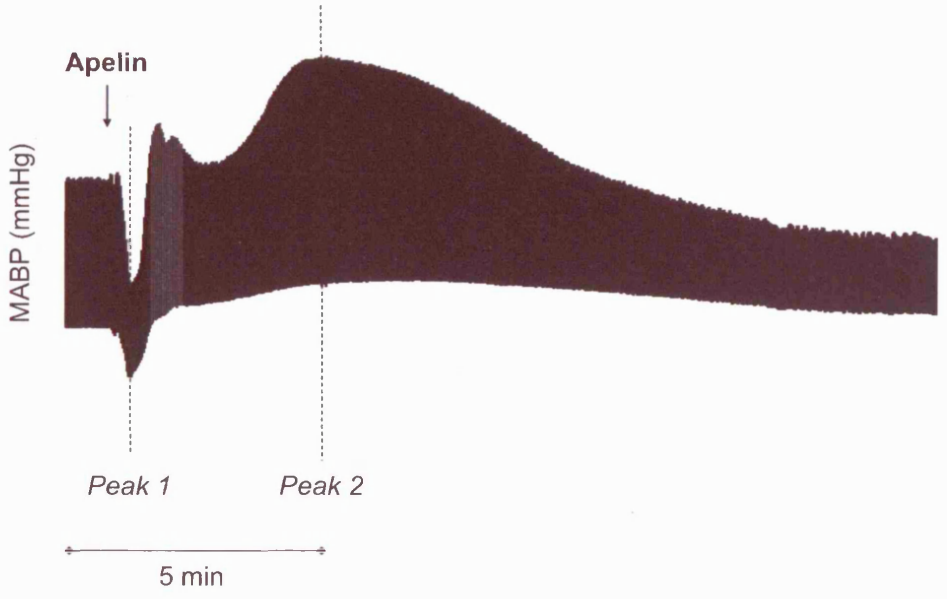

Figure 7-3 A typical response, with respect to mean arterial blood pressure (MABP), elicited by the administration of apelin

The effects seen were short-lived with pressures returning to control levels by 15 min post-bolus. The mean changes in MABP (i.e. the differences between peak 1 and peak 2 values) following control and apelin-13 (0.1-1mg/kg) treatments are shown in Figure 7-4. A transient drop in MABP is seen under control conditions i.e. after saline administration (peak 1), which returns to baseline values (peak 2). Apelin-13 (0.1-1mg/kg) caused a more pronounced drop in MABP but this was not statistically different from control. At higher doses apelin-13 (0.5mg/kg-1mg/kg) induced a biphasic response with an elevation in MABP (peak 2).

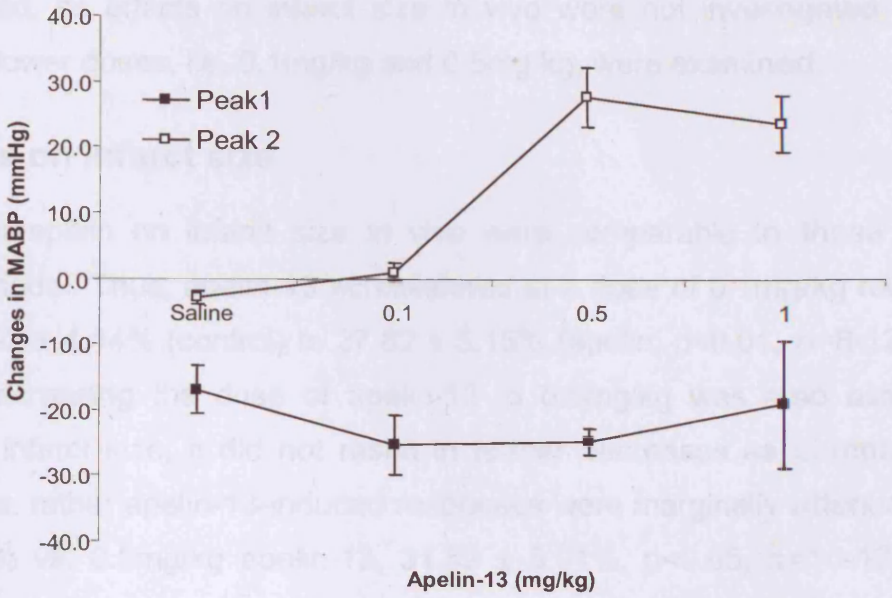

Figure 7-4 The influence of apelin on MABP.

The changes occurring in MABP following the administration of saline or apelin 0.1-1mg/kg (n=3).

It was noticed that during the course of experiments that higher doses of apelin (i.e. 0.5mg/kg and 1mg/kg) that the underlying heart rhythm was affected. In particular, higher peptide doses were found to increase the number of ventricular premature beats (VPB). By contrast, lower doses of apelin-13 (0.1mg/kg) did not influence VPB frequency during the reperfusion period.

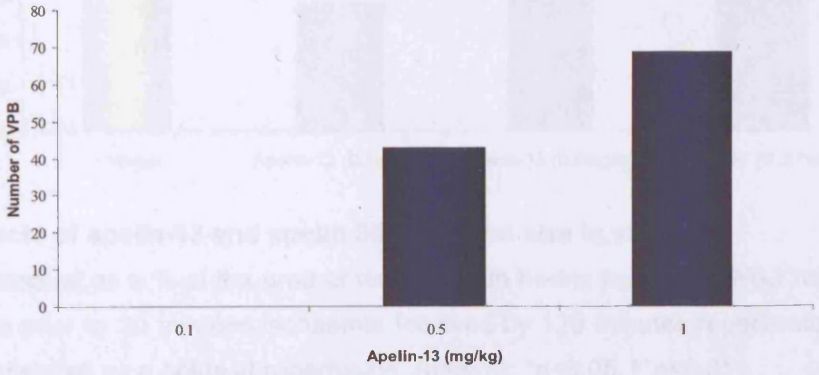

Figure 7-5 Frequency of ventricular premature beats with apelin-13.

In the light of the arrhythmogenic actions seen with the highest dose of apelin-13 (1mg/kg) tested, its effects on infarct size in vivo were not investigated. Rather, the effects of the lower doses, i.e. 0.1mg/kg and 0.5mg/kg, were examined.

7.4.2 Effects on infarct size

The effects of apelin on infarct size in vivo were comparable to those seen in the Langendorff model. Thus, apelin-13 administered at a dose of 0.1mg/kg reduced infarct size from 48.92 ± 4.44% (control) to 27.82 ± 5.15% (apelin, $p<0.01$, n=8-12; see Figure 7-6). Whilst increasing the dose of apelin-13 to 0.5mg/kg was also associated with reductions in infarct size, it did not result in further decreases as compared with the 0.1mg/kg dose, rather apelin-13-induced responses were marginally attenuated (control, 48.92 ± 4.44% vs. 0.5mg/kg apelin-13, 31.59 ± 5.01%, $p<0.05$, n=10-12; see Figure 7-6). This observation perhaps indicates that the infarct reducing effects of apelin-13 had reached their apogee and that with the higher concentration (0.5mg/kg) the dose-response curve had now entered the lag phase. As observed with Langendorff perfused heart experiments, treatment with apelin-36 (0.27mg/kg) resulted in reduced infarcts in the in vivo model also (control, 48.92 ± 4.44% vs. apelin-36, 32.94 ± 4.36%, $p<0.05$, n=8-12).

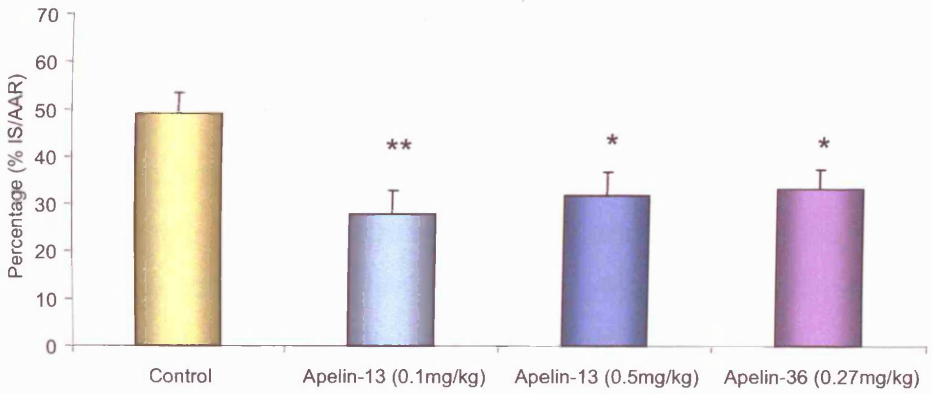

Figure 7-6 The effects of apelin-13 and apelin 36 on infarct size in vivo.

Infarct size (IS) expressed as a % of the area at risk (AAR) in hearts from C57 Bl/6J mice subjected to 15 minutes stabilisation prior to 30 minutes ischaemia followed by 120 minutes reperfusion. Apelin or saline (control) were administered as a bolus at reperfusion. (n=8-12; *$p<0.05$, **$p<0.01$)

The mortality rate for in vivo experiments was 20% and was evenly distributed between the groups.

7.5 Discussion

7.5.1 Haemodynamic effects

The first phase of the study focused on the haemodynamic responses elicited by increasing concentrations of apelin-13. The actions of apelin were found to be biphasic in nature, and were characterised by an initial fall in MABP followed by a compensatory increase. The effects were short-lived returning to baseline levels within 5-10 minutes. These data confirm the haemodynamic effects reported by Charles et al 2006 who reported a similar biphasic response to the bolus administration of apelin-13. The results are also in keeping with other studies in which similar biphasic haemodynamic responses (hypotension followed by hypertension) were seen with other vasoactive peptides, including endothelin (King et al. 1990, Rohmeiss et al 1990) and urotensin II (Gardiner et al. 2004). The mechanism(s) underlying this biphasic response may involve the generation of nitric oxide, as it is known to play a role in apelin-induced hypotension. As regards the subsequent hypertension, this may as suggested by Charles et al. (2006), be baroreceptor mediated. The initial investigation was carried out, primarily to identify any potential detrimental effects occurring as a result of the intravenous administration of apelin to mice. Interestingly, it revealed that at higher doses apelin induced VPB, a potential sign of myocardial irritability. This effect may indicate that a therapeutic window exists for apelin, with higher doses given i.v. producing negative effects on the conducting system of the heart.

The next logical step in these investigations would be to utilise animals that have been genetically modified, mice that are apelin deficient and apelin receptor deficient. This would allow for more detailed examinations of the mechanisms underlying the haemodynamic actions of apelin and its role as a modulator of vascular function. In this way characterisation of the short and long-term effects of the apelin-APJ system will be made possible. The evidence that apelin acts as a vasodilator, and that the sensitivity to apelin may be altered in disease states, makes apelin a promising target for the development of drugs for regulating blood pressure. The use of the novel APJ receptor antagonist apelin-13(F1A) could be employed in this model to assess its antagonistic effects on blood pressure.

It has already been mentioned that the APJ receptor homology with the transmembrane portion of the AT_1 angiotensin receptor (O'Dowd et al. 1993). Recent studies have suggested that apelin possibly induces a compensatory vasorelaxation to counter angiotensin II-mediated vasoconstriction (Ishida et al. 2004).Given that angiotensin II plays a central role in the development of atherosclerosis, further studies investigating the role played by the apelin-APJ pathway in this disease setting could prove important (Ashley et al 2006).

7.5.2 Effects on infarction

The primary goal of this study was to assess whether apelin was cardioprotective in vivo in the setting of ischaemia/reperfusion injury. Apelin-13 administered at a dose of 0.1mg/kg reduced infarct size significantly. This observation is in keeping with other studies which have investigated the in vitro therapeutic effects of apelin in myocardial injury (Jia et al. 2005). The present study, however, is the first to investigate the acute effects of apelin in vivo when given at reperfusion. Current evidence suggests that apelin and APJ receptor gene expression are down-regulated in the injured myocardium (Jia et al. 2005). Studies which have employed apelin as a therapeutic agent in the setting of heart failure and ISO-induced myocardial ischaemia (Ashley et al. 2004, Berry et al. 2004 and Jia et al. 2005) have all shown exogenously administered apelin to be beneficial. These findings suggest that the signalling capacity of cardiac APJ receptors is not exhausted even when endogenous apelin levels are increases or when APJ receptor expression is diminished (Japp et al. 2008)

7.5.3 Future Studies

Future studies involving the use of inhibitors of the RISK pathway and the APJ receptor antagonist, apelin-F13, will provide further information concerning the mechanisms underlying apelin's cardioprotective actions in vivo. The actions of apelin-F13 in I/R injury have not been fully investigated, including its potential role as a blocker of apelin induced cardioprotection. The use of genetically modified mouse models lacking apelin and the APJ receptor would allow for a thorough investigation of the role apelin-APJ

pathway plays in I/R injury. The use of inhibitors of the RISK pathway and NO would be important in determining whether the cardioprotective actions of the apelin-APJ system in vivo are mediated solely through the RISK pathway, or via direct actions on smooth muscle cells in the vascular wall.

The clinical use of apelin as adjunct therapy in minimising reperfusion injury is suggested by this study. The results of both Langendorff and in vivo studies indicate that apelin when given at reperfusion is cardioprotective. Extrapolation to the clinical situation can be envisaged when considered as a treatment for reperfusion injury during revascularisation following myocardial infarction.

8 CONCLUSIONS

8.1 Summary of findings: -

1. Apelin administered at reperfusion reduced infarct size both in vitro and in vivo
2. Apelin delayed the opening of the mPTP
3. The effects induced by apelin were associated with the activation of components of the RISK pathway i.e. PI3K-Akt and p44/42.

8.2 Conclusions

In this study the cardioprotective effects of apelin have been demonstrated. This leads one to suggest that the administration of apelin at the time of reperfusion during an acute coronary event may represent a novel form of adjunctive treatment that could lead to reduced ischaemic damage and improved myocardial function. These findings are in keeping with other studies which have reported that apelin protects from both ISO induced myocardial injury and ischaemic coronary occlusion (Jia et al. 2005, Kleinz and Baxter 2007). Reperfusion is essential in salvaging viable myocardium from infarction. Paradoxically, however, reperfusion itself can cause myocardial cell death via a lethal reperfusion-induced injury pathway that involves the opening of the mPTP and subsequent apoptosis (Kleinz and Baxter 2007). The results of the present study clearly show that reperfusion with apelin following a period of lethal ischaemia does, indeed, attenuate infarction via PI3-AKT and P44/42-dependent mechanisms, and delayed mPTP opening.

In recent years a number of adipocytokines, including apelin, have been discovered and many of their functions elucidated. More recently the discovery that some of these adipocytokines are upregulated in obesity and exert potentially beneficial effects has prompted the suggestion that these agents may play a role in tissue preservation. Indeed, the present study provides further support for the proposition that the adipocytokines may represent a class of endogenous substances that, apart from having potential in the treatment of metabolic disorders and, in the case of apelin, heart

failure could prove useful in limiting myocardial damage when administered following infarction.

Evidence for a protective role for the adipocytokines has come mainly from animal experiments and not studies conducted in patients (Shibata et al. 2005, Jia et al. 2005, Kleinz and Baxter 2007). Interestingly, however, certain circumstances obesity would appear to confer some protection in humans (Gruberg et al., 2002. It is known that obesity is an independent risk factor for the development of cardiovascular disease (Yusuf et al. 2004). However, data have been reported indicating that high BMI is associated with reduced short-term cardiovascular mortality; an observation termed the "obesity paradox" (Gruberg et al., 2002). In fact, in some cases obesity actually confers a mortality benefit (Fonarow et al., 2007). It is likely that these findings are dependent on many factors particularly that more aggressive management is likely to have been employed in this subgroup of patients (Steinberg et al., 2007). Another potential explanation might be that patients with coronary artery disease who are underweight or of normal weight may not have enough metabolic reserve to overcome the catabolic stress resulting from an acute coronary event and/or revascularisation procedure. The possibility that the pathophysiology of acute coronary syndrome may vary between patients with differing BMI cannot be excluded. Further research is therefore needed to investigate the true nature of the "obesity paradox" and the role played by the adipocytokines in cardiovascular pathogenesis.

The role of the apelin-APJ system in cardiovascular control is now considered to be an important area for further research and preliminary studies indicate that apelin may play an important role in blood pressure control. In keeping with previous studies the present investigation has yielded additional evidence for apelin's action on blood pressure control and cardioprotection. The development of non-peptide agonists of the apelin receptor may provide new therapeutic tools for treating cardiovascular diseases.

8.3 Clinical implications

The administration of apelin at the time of cardiac reperfusion in order to recruit innate cellular anti-apoptotic mechanisms and protect the heart from reperfusion-induced cell death offers an intriguing treatment option with regard to the management of acute coronary syndromes. Thus, it could be suggested that apelin could be given as an adjunct to thrombolysis, post-stenting and at the point of reperfusion during coronary artery bypass procedures. Further studies are required to quantify apelin levels during acute ischaemic events together with longitudinal studies to establish apelin profiles under pathological and non-pathological conditions. This will then open the way for clinical trials to establish whether modulation and enhancement of the apelin-APJ system has a therapeutic benefit in patients with ischaemic heart disease.

Previously, apelin has been associated with a positive haemodynamic profile in both normal and diseased hearts. Clinical medicine is lacking new pharmacological therapies for the treatment of heart failure. Many new interventions utilise mechanical or resynchronisation therapy as a modality for treating the disease. The vasodilatory, diuretic and cardioprotective effects of apelin raise the possibility that the apelin-APJ pathway might represent a novel endogenous "compensatory" system in heart failure. Further studies involving the manipulation of the apelin-APJ system may offer benefit with respect to heart failure ultimately leading to clinical applications in humans.

A role for apelin as a biomarker of heart failure and atrial fibrillation has recently been suggested. Depressed apelin levels in lone atrial fibrillation suggest that it may be possible to identify those individuals with a predisposition to arrhythmia even when they are not in atrial fibrillation (Ellinor et al. 2006). More recent studies, however, have failed to provide evidence that apelin represents a useful biomarker for diagnostic and prognostic purposes (Von Kimmenade et al. 2006).

8.4 Future directions

In future studies, hearts from animals that have been genetically modified, that is, mice that are APJ receptor deficient, should be examined. This would allow for a more detailed examination of the mechanisms by which apelin protects the myocardium against I/R injury.

The cardiovascular effects of acute apelin administration in rodents are now relatively well characterised but the impact of chronic administration requires further investigation. Studies are needed to confirm whether longer periods of treatment are cardioprotective and whether any apparent extra benefit, i.e. enhancement of cardiac performance, occurs without deleterious effects on cardiac remodelling e.g. left ventricular hypertrophy.

Greater understanding is also needed of the molecular mechanisms governing apelin and APJ gene expression and the effect disease states have on these processes. Alternative strategies that enhance endogenous apelin synthesis and secretion or preserve biological activity by inhibiting peptide breakdown need to be investigated (Japp et al. 2008). It is intriguing that treatment with the new anti-obesity drug Rimonabant has been found to be associated with increase plasma levels of the cardioprotective adipocytokine adiponectin. Studies to investigate if Rimonabant also modulates plasma apelin levels may yield a novel approach to a therapeutic intervention.

Although the cardiovascular profile of apelin in rodents suggests a potential therapeutic application, the relevance of the apelin-APJ pathway in human cardiovascular physiology and pathophysiology has yet to be established. In the first report of in vivo apelin administration to humans, a group from Edinburgh have confirmed that apelin has direct vasodilatory properties in man. This preliminary study will allow further characterisation of apelin's properties in humans and further clinical investigation to determine its role in cardiovascular homeostasis and disease (Japp et al. 2008).

In conclusion, it is now apparent that adipose tissue is not simply a storage reservoir of fat, but, in fact, constitutes the largest endocrine organ in the body, releasing factors that exert multiple effects on metabolism and cellular protection. Increased understanding of the adipocytokines and the intracellular pathways by which they may influence myocardial integrity could lead to the development of novel therapies.

9 PUBLICATIONS RESULTING FROM THIS PROJECT

PAPERS

Leptin, the obesity-associated hormone, exhibits direct cardioprotective effects.
Smith CC, Mocanu MM, Davidson SM, Wynne AM, Simpkin JC, Yellon DM

British Journal of Pharmacology. 2006 Sep;149(1):5-13. Epub 2006 Jul 17

Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemia reperfusion injury.
Simpkin JC, Yellon DM, Davidson SM, Lim SY, Wynne AM, Smith CC.

Basic Research in Cardiology. 2007 Nov;102(6):518-28. Epub 2007 Aug 13

Temporal changes in myocardial salvage kinases during reperfusion following ischemia: studies involving the cardioprotective adipocytokine apelin.
Smith CC, Mocanu MM, Bowen J, Wynne AM, Simpkin JC, Dixon RA, Cooper MB, Yellon DM

Cardiovascular Drugs and Therapy. 2007 Dec;21(6):409-14.

ABSTRACTS

Apelin a vasoactive adipocytokine, exhibits direct cardioprotective effects
JC Simpkin, CCT Smith, DM Yellon
UCL Cardiovascular Science and Medicine day 2 May 2006

Apelin a vasoactive adipocytokine, exhibits direct cardioprotective effects
JC Simpkin, CCT Smith, DM Yellon
International Society for Heart Research
European section meeting, Manchester, Wednesday 14th - Saturday 17th June 2006
Journal of Molecular and Cellular Cardiology, June 2006; (40)6: Page 956

Apelin a vasoactive adipocytokine, exhibits direct cardioprotective effects
JC Simpkin, CCT Smith, DM Yellon
Medical Research Society with the Academy of Medical Sciences in the Royal College of Physicians
Royal College of Physicians 28th February 2007 conference

Apelin exhibits cardioprotection against ischaemia reperfusion injury
James C. Simpkin, Derek M. Yellon, Sean M Davidson, Shiang Y Lim, Abigail M Wynne and Christopher C.T. Smith
British Cardiovascular Society Annual Scientific Conference, Glasgow, 4 – 7 June 2007
Heart, June 2007; 93(supplement 1): A212

Apelin, the potentially therapeutic adipocytokine, protects against myocardial ischemia-reperfusion injury.
James C Simpkin, Shiang-Y Lim, Sean M Davidson, Abigail Wynne, Christopher CT Smith, Derek M Yellon.
XIX World Congress of the ISHR
Bologna (Italy), 22-26 June 2007
Journal of Molecular and Cellular Cardiology, June 2007; 42,(6) (Supplement 1), S208

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