 Contribution of p38 mitogen-activated protein kinase isotypes to cardiac physiology

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Contribution of p38 mitogen-activated protein kinase isotypes to cardiac physiology

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ABSTRACT

The isoform-specific functions of the four isoforms of p38 mitogen-activated protein kinase (p38 α-, β-, γ- and δ- MAPK) in the adult heart are largely unknown, partly due to the lack of isotype-specific tools to manipulate or measure p38 MAPK isoform activity. Improved understanding of p38 MAPK isoform regulation will benefit development of selective pharmacological inhibitors and move towards eliminating the potential drawbacks of chronic systematic inhibition of this important kinase. This Thesis describes our investigation of endogenous expression of p38 MAPK in the murine heart; the functional contributions of endogenously expressed p38 MAPK in response to clinically-relevant stimuli such as such as pharmacological preconditioning, ischaemia and reperfusion, and pro-inflammatory cytokines (central in the progression of heart failure, such as TNF-α, IL-1) and osmotic stress; and the involvement of p38γ and δ MAPK isoforms in left ventricular (LV) remodelling in response to in vivo models of cardiac hypertrophy and following myocardial infarction (MI).

Using commercially available isoform-specific antibodies we have demonstrated that all p38 MAPK isoforms are expressed in the murine heart with p38α and γ being the most abundant. p38β and δ MAPK expressed at lower levels. The transcripts for all the p38 MAPK isoforms were detected. Immunocytochemistry of isolated cardiac myocytes demonstrated a diverse localization of p38 MAPK isoforms which suggest different functions.

In isolated perfused hearts, we showed that mice lacking the β isoform (p38β KO) are refractory to pharmacological preconditioning by the carbon monoxide-releasing molecule, CORM-3. Our data demonstrate that CORM-3 pre-treatment followed by a 5 min washout of hearts prior to an in vitro ischaemia-reperfusion results in decreased infarct size and preserved LV function. With respect to p38γ and δ isoforms, we observed a significant reduction in left ventricular developed pressure in response to sorbitol (osmotic stressor) in wild type (WT) hearts which was significantly ameliorated in p38γδ knockout (p38γδ KO) hearts. This was accompanied by a reduction in the level of p38 MAPK phosphorylation in transgenic mice compared with the WT. A comparable response was observed between WT and p38γδ KO mice in response to the other stimuli. The potential roles of p38γ and δ MAPK were examined in a model of isoproterenol (ISO)-induced cardiac hypertrophy. Our studies revealed no significant differences between the WT and the transgenic phenotypes in response to hypertrophic stimuli. Infusion of ISO resulted in comparable LV remodelling, as assessed by echocardiography. In addition, no differences were observed in the cardiac function (assessed by pressure volume analysis) between the two genotypes. These finding suggest that p38γ and δ MAPK are unlikely to be involved in geometric remodelling of hypertrophy. Investigating the possible contribution of p38γ and δ MAPK in post-MI remodelling in vivo (using a permanent left anterior descending artery ligation model) revealed no apparent difference between WT and p38γδ KO mice. Echocardiographic and pressure-volume analysis showed comparable LV dilatation in WT and p38γδ KO mice.

Our data confirmed that p38α MAPK is the dominant isoform in the murine myocardium and is activated in response to ischaemia, ischaemia reperfusion and a number of pro-inflammatory cytokines. We propose that p38β MAPK is implicated in pharmacological preconditioning whereas p38γ and δ isoforms appear to be important in the myocardial response to osmotic stress. p38γ and δ isoforms also seem to be implicated in LV remodelling and somehow contribute to functional changes during cardiac hypertrophy and following-MI.
LIST OF PUBLICATIONS

Full papers

Clark JE, Sarafraz N, Bodkin J, and Marber MS. p38β mitogen-activated protein kinase-mediated cardioprotection by a water soluble carbon monoxide releasing molecule in the isolated mouse heart. IN PREPARATION.


Abstracts

International Society for Heart Research, European Section, Athens, May 2008


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<table>
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<tbody>
<tr>
<td>AAR</td>
<td>Area at risk</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AMVM</td>
<td>Adult mouse ventricular myocytes</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>bpm</td>
<td>Beats per min</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CF</td>
<td>Cardiac Fibroblasts</td>
</tr>
<tr>
<td>CaM</td>
<td>Calcium calmodulin</td>
</tr>
<tr>
<td>CnA</td>
<td>Calcineurin A</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>CORM</td>
<td>Carbon monoxide releasing molecule</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNPT</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-Dithithretiol</td>
</tr>
<tr>
<td>DR</td>
<td>Drug resistant</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>G-protein</td>
<td>Heterotrimeric GTP-binding protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GSK</td>
<td>Glycogen synthase kinase</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human embryonic kidney, clone 293 (cell line)</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p</td>
<td>Intra-peritoneal</td>
</tr>
<tr>
<td>I-R</td>
<td>Ischaemia reperfusion</td>
</tr>
<tr>
<td>ISO</td>
<td>Isoproterenol</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun N terminal kinase</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LVAW</td>
<td>Left Ventricular anterior wall</td>
</tr>
<tr>
<td>LVDP</td>
<td>Left ventricular end diastolic pressure</td>
</tr>
<tr>
<td>LVID</td>
<td>Left ventricular interior dimension</td>
</tr>
<tr>
<td>LVPW</td>
<td>Left Ventricular posterior wall</td>
</tr>
<tr>
<td>LVS</td>
<td>Left ventricular septal wall</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAPKAK2</td>
<td>Mitogen activated protein kinase kinase kinase</td>
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</table>
MEF  Monocyte enhance factor
MI   Myocardial infarction
MMP  Matrix Metalloproteinase
MAPK Mitogen activated protein kinase
m-PTP Mitochondrial transition pore
μg   Microgram
μL   Microlitre
μm   Micromolar
mA   Milliohm
mg   Milligram
min  Minute
mL   Millilitre
mM   Milimolar
mmHg Millimetre Mercury
ms   Mili seconds
mTOR Mammalian target of rapamycin
MW   Molecular weight
NADPH Nicotinamide adenine dinucleotide
NaF  Sodium fluoride
NE   Norepinephrine
NGS  Normal goat serum
nM   Nanomolar
O₂   Molecular Oxygen
PAGE Polyacrylamide gel electrophoresis
PBS  Phosphate-buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKN</td>
<td>Protein kinase N</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PV</td>
<td>Pressure volume</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homology gene family</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SAP</td>
<td>Synapse associated protein</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress activated protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>TAK1 binding protein 1</td>
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<tr>
<td>TAK 1</td>
<td>TGF-β associate kinase 1</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline (with Tween)</td>
</tr>
<tr>
<td>TEMED</td>
<td>NNNN-tetraethylethalamine</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Tris</td>
<td>Hydroxymethyl methylamine</td>
</tr>
<tr>
<td>TTC</td>
<td>Triphenyl tetrazolium chloride</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
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</tbody>
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1 GENERAL INTRODUCTION

1.1 Ischaemic heart disease

Atherosclerotic vascular disease manifests predominantly as heart disease and stroke, which are the most frequent causes of death in the UK. Collectively, atherosclerotic vascular disease was responsible for 40% of total mortality in the UK in 2004. Despite recent reductions in this high mortality, morbidity is increasing as more patients survive myocardial infarction and stroke. For example, about 1.3M people in the UK have survived acute myocardial infarction, 2M have angina and 0.9M have heart failure. The extraordinarily high impact of atherosclerosis on the Nation’s health is reflected in its economic cost. Atherosclerotic disease of just the coronary arteries costs the UK healthcare system £3,500M with an estimated further £4,400M lost to the economy through premature death, illness and informal care.¹

Coronary atherosclerotic plaque rupture/erosion in the epicardial coronary arteries can result in myocardial infarction (MI) which is characterised by ischaemic injury leading to necrosis and apoptosis of cardiomyocytes. Ischaemia may manifest as angina discomfort, ST-segment (the end of electrical depolarisation and beginning of ventricular repolarisation) deviation on ECG and regional or global impairment of ventricular function. Infarction of the left ventricle (LV) results in a reduction in both blood pressure and cardiac output due to inability of the LV in accepting blood from the pulmonary vein, which in many patients causes symptoms such as breathlessness.

1.2 **Immediate effects of myocardial ischaemia**

1.2.1 **Biochemical changes**

Occlusion of the coronary arteries results in two immediate detrimental effects: pump failure and lethal arrhythmias (Janse and Wit, 1989). At the first instance, there is a halting in the delivery of oxygen to the myocardium and a reduction in oxidative metabolism of adenosine triphosphate (ATP) production. This is accompanied by accumulation of important metabolites (such as lactate) and a reduction in intra-myocardial pressure. Accumulation of such metabolites inhibits glucose uptake and glycolysis and leads to induction of hypoxia in the myocardium. There is an increase in the level of nicotinamide adenine dinucleotide phosphate (NADPH) in the cytosol due to its reduced removal by mitochondria and is counterbalanced by NADPH conversion to NAD through formation of lactate from pyruvate. This leads to development of acidosis and a drop in intracellular pH.

Progression of ischaemic damage inhibits the mitochondrial respiratory chain and results in the accumulation of reactive oxygen species (ROS). The energy transport from mitochondria to cytosol is also impaired. Adenine nucleotide translocase and mitochondrial creatine kinase (enzymes that are required for transportation of ATP from the mitochondria to the cytosol) is reduced with subsequent impaired transportation into the sites of utilization of ATP. Energy depletion (in the form of ATP depletion) and acidosis result in inhibition of the sodium pump (Na⁺/K⁺-ATPase) and enhanced activation of the sodium-proton exchanger (NHE) in an attempt to correct acidosis. This results in a rise in intracellular sodium which enhances osmotic pressure and swelling (Carmeliet, 1999).

1.2.2 **Cell injury**

Lack of available oxygen together with accumulation of metabolites initiates cell injury. The extend of cell injury depends on severity, duration, the temporal sequence of ischaemia, changes in metabolite environment (e.g. hypothermia vs. normothermia, pre-ischaemic myocardial glycogen content) as well as the involvement of the inflammatory response. Cell death occurs by two major processes: apoptosis and necrosis. Necrotic cell death has been shown to peak after 24 h of reperfusion and apoptotic cell death is increased after 72 h of reperfusion, in a canine model of myocardial infarction (Zhao et al., 2001). Necrosis is characterised by membrane disruption, cell swelling and triggers the inflammatory response.
Apoptosis (programmed cell death) results in chromatin condensation, DNA fragmentation with preserved cell membrane integrity and does not involve the inflammatory response.

1.2.3 Inflammatory response

Cardiac muscle necrosis is associated with an inflammatory cascade that clears the infarct from dead cells and matrix debris and ultimately results in replacement of the damaged tissue with scar (Frangogiannis, 2006). Cells dying by necrosis release their intracellular contents and initiate an intense inflammatory response by activating innate immune mechanism. Cell surface receptors sense endogenous ligands released upon cell injury and activate cytokines and chemokines-mediated pathways. Activation of the complement cascade, generation of reactive oxygen species (ROS) and Toll-like receptor mediated signals play a significant role in triggering the post-infarction inflammatory response by activating the nuclear factor -κβ (NF-κB) system, resulting in up-regulation of chemokines and increased expression of adhesion molecules by endothelial cells (Frangogiannis, 2006). The interaction between the circulating leukocytes and adhesion molecules results in integrin-mediated adhesion, followed by diapedesis of leukocytes into the sub-endothelial space. Infiltrating leukocytes clear the infarct of dead cells, through the induction of cytokines and growth factors. Major cytokines released at the site of ischaemia include tumour necrosis factor -α (TNF-α) interleukin (IL-1). TNF-α activation leads to recruitment of macrophages in the ischaemic area and acts as an autocrine contributor to myocardial dysfunction (Bozkurt et al., 1998; Bryant et al., 1998). It also activates various signalling pathways during ischaemia which are implicated in cardiomyocyte death and transcription of genes required for the production of cytokine. Experimental studies have suggested that members of the IL-1 family are markedly and consistently upregulated in the infarcted heart. IL-1 is the prototypic multifunctional inflammatory cytokines and consists of two distinct ligands, IL-1α and IL-1β, with high sequence homology and indistinguishable biological activities (Allan et al., 2005). Both IL-1α and β are synthesised as large precursor proteins. Pro-IL-1α is biologically active and is cleaved by calpain (a family of calcium dependent non-lysosomal cysteine proteases) to generate the mature proteins, IL-1α remains intracellular unless released by a dying cell. In contrast, pro-IL-1β is biologically inactive until is enzymatically cleaved by the active IL-1β converting enzyme (caspase-1) to generate 17.5-kDa protein (p17) (Thornberry et al., 1992). Both IL-1α and β bind to two primary receptors. The IL-1 type I receptor (IL-1RI) associates with the IL-1 receptor accessory protein (IL1RAcP),
forming a complex that transduces a signal and is responsible for most IL-1 mediated action. In contrast, the type II IL-1 receptor (IL-1R II) lacks an intracellular signalling domain and does not initiate signalling when IL-1 binds. Thus IL-1RII serves as a decoy receptor acting as a molecular trap for its ligand (Colotta et al., 1993; Mantovani et al., 2001). Both IL-1α and β exert complex biological effects by modulating gene expression and behaviour in a wide variety of cell types. Activation of IL-1RI triggers multiple and sequential phosphorylation that results in nuclear translocation of transcription factors. Post-receptor amplification is responsible for the potent effects of IL-1 signalling despite the relatively low expression of IL-1RI on many cell types. IL-1β induction has been reported in rodent models of reperfused (Dewald et al., 2004) and non-perfused (Deten et al., 2002) infarction. Furthermore, a clinical investigation showed that IL-1β levels were elevated in patients with acute myocardial infarction within the first few hours after the onset of chest pain (Guillen et al., 1995). Although, IL-1α and IL-β exerts their functional responses via the same receptor, their synthesis and regulation are very different and they are product of different genes (Dinarello, 1996). The IL-1β gene promoter contains a number of transcription factor binding sites, including NF-κB, activator protein-1 (AP-1) (Dinarello, 1996) whereas only AP-1 and Sp1 (a transcription factor involved in early development) have so far been identified in IL-1α promoter gene (McDowell et al., 2005). Recently in an elegant study it was demonstrated that TNF-α stimulates IL-β m-RNA expression via important signalling cascades such as p38 mitogen activated kinase (p38 MAPK), phosphatidylinositol-3-kinase (PI3K/Akt) and NF-κB (Turner et al., 2007). Although inhibition of p38 MAPK and PI3K prevented TNF-α induced IL-α expression, inhibition of NF-κB increased IL-1α expression in human cardiac fibroblasts (CF) (Turner et al., 2007). In addition, post-translational regulation of IL-1α and IL-β is very different and vary amongst species. IL-1α is not secreted from human cells, but accumulates at the plasma membrane and intracellularly (Dinarello, 1996; Turner et al., 2007). Although, constitutive IL-β secretion has been observed in un-stimulated rat and mouse CF cultures, (Jaffre et al., 2004; Nagamatsu et al., 2006), this mechanism is absent in cultured human CF (Ancey et al., 2002; Turner et al., 2007).

In addition, IL-6 has shown to be elevated in patients with acute myocardial infarction (Yamauchi-Takahara et al., 1995). It is released from mononuclear cells in the ischaemic area and regulates contractile function by its acute effect on calcium transients (Yamauchi-
Takihara et al., 1995). In general, cytokines such as TNF-α and IL-1β and IL-6 have negative inotropic effects whereas chemokines exert their effects by activation of inhibitory mediators that suppress inflammation, but induce fibrosis (Stangl et al., 2002). IL-8 is also released at the ischaemic site and is thought to be involved in neutrophil recruitment (Ren et al., 2003). Neutrophils are rich in pro-oxidant species and proteolysis enzymes and further contribute to cell injury (Ren et al., 2003).

1.2.4 Contractile dysfunction

Under normal conditions, contraction of the ventricles results when the heads of thick myosin filaments interact with the thin filaments (strong cross bridge). This is initiated by triggered release of calcium from the sarcoplasmic reticulum (SR) (Solaro, 1999). During prolonged ischaemia strong interaction between the myosin heads and actin filaments are maintained due to ATP depletion and rise in adenosine diphosphate (ADP) levels (Stapleton and Allshire, 1998). Although this effect is moderate, it leads to cytoskeletal defects and cardiomyocytes become more fragile and susceptible to mechanical damage; particularly during ischaemia-and reperfusion. ATP depletion also results in opening of the sarcolemmal and mitochondrial ATP- dependent potassium channels (KATP) and as a result potassium leaks out of cells. Potassium leakage is further enhanced with negatively charged lactate, phosphate ions and inactive Na⁺/K⁺-ATPase. Potassium loss causes membrane action potential shortening and may prevent excessive calcium entry into the cell. Shortening of the action potential also occurs from injury currents generated from current differences between area of ischaemic and non-ischaemic regions (particularly at the border zone). Changes in action potential and its conductance constitute the basis of ischaemic and reperfusion arrhythmias (Carmeliet, 1999).
Eventually, the biochemical changes, cell injury and the inflammatory response together with the lack of oxygen and ATP following MI, will result in production of a vicious circle causing more damage and contractile dysfunction (Figure 1-1).

![Diagram of Myocardial Infarction](image)

**Figure 1-1 Consequences of myocardial oxygen supply-demand imbalance following ischaemia.**

Reduction in oxygen due to ischaemia results in depletion of ATP production and acidosis. This, along with increase in intracellular sodium, glucose oxidation and calcium overload leads to the negative inotropic effect and myocardial dysfunction.

### 1.2.5 Ischaemia-reperfusion injury

Reperfusion of the ischaemic myocardium is often used to achieve survival of the remaining tissue. Reperfusion is necessary in order to salvage any viable myocardium and if it is not performed soon (20-30 min) following the occlusion, injury will progress from reversible to irreversible, within the ischaemic risk zone (Fujiwara et al., 1989). However, this intervention may lead to further complications such as diminished myocardial function (stunning) and arrhythmia. Moreover, some cells subjected to ischaemia reperfusion display hallmarks of apoptosis. This phenomenon is termed “ischaemia-reperfusion injury”.

26
Although the mechanisms initiating apoptosis are still unknown, it seems likely that opening of mitochondrial permeability transition pore (mPTP) during reperfusion serves as a key mechanism of cell death, amplifying or accelerating cell death to produce necrosis (Gottlieb et al., 1994; Halestrap et al., 2004).

### 1.3 Chronic effect of myocardial ischaemia

Although the acute condition alone may result in death due to ventricular arrhythmias or pump failure, in patients with extensive infarction that survive, a chronic phase of ventricular remodelling occurs. Remodelling is a maladaptive process characterised by cardiomyocyte apoptosis, fibrosis, CF proliferation, thinning of the ventricular wall at the site of infarction, ventricular chamber enlargement and hypertrophy of surviving cardiomyocytes (Pfeffer and Braunwald, 1990; Swynghedauw, 1999; Udelson et al., 2003). These events may, eventually, lead to heart failure which is frequently lethal despite current best care. Therefore, interventions to minimise pathological cardiac remodelling is highly desirable to reduce the mortality and the incidence and severity of congestive heart failure after MI. Post infarction remodelling is divided into an early phase and a late phase. The early phase (<72 h) involves expansion of the infarct zone (Erlebacher et al., 1984; Pfeffer and Braunwald, 1990), which may result in early ventricular rupture or aneurysm formation. Late remodelling (> 72 h) involves the left ventricle globally and is associated with time-dependent dilatation and the distortion of ventricular shape by cardiac hypertrophy and remodelling.

#### 1.3.1 Role of cardiac fibroblasts

Fibroblast and endothelial cell proliferation marks the transition from the inflammatory to proliferative phase of scaring post infarction. CFs are the key element in the regulation and turnover of extracellular matrix (ECM). They are crucial to normal myocardial homeostasis as well as in the response to pathological stimulation (Porter and Turner, 2009).

Various hormones, growth factors and pro-inflammatory cytokines elevated in the remodelling heart result in proliferation of CF which then secrete cytokines such as IL-1β and TNF-α that can activate matrix metalloproteinases (MMPs) (Turner et al., 2007). As the scar matures, leukocytes undergo death and are cleared from the infarcted area. This is induced by the expression of inhibitory mediators such as transforming growth factor (TGF)-β and IL-10, that suppress inflammatory cytokines and chemokines synthesis.
(Frangogiannis, 2008; Zymek et al., 2006). During the remodelling process, CF undergo phenotypic modulation to myofibroblasts (myoFb) (Cohn et al., 2000; Weber, 2004). The trans-differentiation is activated in response to various cytokines and growth factors such as TGF-β and promotes extracellular matrix deposition in the infarcted area by myoFb (Weber, 2004). Highly proliferative and invasive myoFb increases secretion of ECM-degrading MMPs and collagen turnover which result in the active remodelling of the cardiac interstitium (Weber, 2004). MyoFb are mainly observed in the infarct site where synthesis and deposition of collagen promotes scar formation and fibrosis (Sun and Weber, 1996). In the healing skin, myoFb are particularly important in maintaining the structural integrity of scars (Sun and Weber, 2003). They anchor and contract which closes the wound and limit scarring (Gabbiani, 2003). They also provide tensile strength by deposition and synthesis of collagen. However unlike the healing skin, the presence of myoFb persists in the infarcted myocardium for months and possibly years (Jugdutt, 2003; Willems et al., 1994). Thus, they have been thought to facilitate hypertrophic scarring and the fibrosis that influence pathological remodelling, compromising cardiac function and ultimately leads to heart failure (Frangogiannis, 2006; Sun and Weber, 2000). Although it is not clear why their presence persist, it has been shown that a diverse array of environmental stimuli that occur during the remodelling process such as mechanical stretch and hypoxia activate this response (Clancy et al., 2007; Gupta and Grande-Allen, 2006). In addition, various neurohormonal stimuli such as catecholamines (via β2-adrenergic receptor (β2-AR)), Angiotensin II (Ang II) via AT1R, endothelin (via ETA and ETB) as well as pro-inflammatory cytokines (TNF-α, IL-1) and pro-fibrotic TGF-β can contribute to the persistent proliferation of myoFb at the infarct site (Bouzegrhane and Thibault, 2002; Modesti et al., 1999; Porter et al., 2004; Turner et al., 2007).

### 1.3.2 Cardiac Hypertrophy

Pathological cardiac hypertrophy is an adaptive response during post-infarction remodelling that offsets increased load, attenuates progressive dilatation and stabilises contractile function (Pfeffer and Braunwald, 1990). Cardiac hypertrophy is initiated by an interaction among a number of control mechanisms that translate an increase in the work of the heart into a signal that increases cell growth. The key elements include stretch receptors, extracellular growth factors (such as a fibroblast growth factor (FGF)), TGF-β, neurotransmitters and hormones (Parker et al., 1990). However, not all forms of cardiac
Hypertrophy are detrimental, as extensive aerobic conditioning through exercise induces a state of physiological growth that is thought to be adaptive, in the long term. Physiological growth is associated with exercise and pregnancy and is characterised by an increase in ventricle chamber size dimension and is referred to as eccentric (Levy et al., 1990). In this phenotype, cardiac hypertrophy occurs due to the addition of sarcomeres in series to lengthen the cell and in parallel on the periphery to increase the width of the cell (Figure 1-2 A).

Pathological cardiac hypertrophy, on the other hand, can produce concentric hypertrophy in which the ventricular wall and septum thicken with a net decrease in ventricular chamber dimensions to meet increased mechanical demand (Figure 1-2 B). This remodelling is associated with a greater increase in cardiac myocyte width than length. Hypertrophy reduces total energy expenditure and the tension that must be developed by each sarcomere in the wall of the failing heart (Boudina and Abel, 2007). Because energy utilization during systole is inversely proportional to wall tension, hypertrophy increases myocardial efficiency and so has an energy-sparing effect. This adaptation is best suited for the short-term because when the stimulus for hypertrophy is sustained for months and years, important deleterious effects begin to appear. These include abnormalities in architecture, blood supply and ultrastructure that exacerbate energy starvation in the overloaded heart.

Cardiac hypertrophy also results in expression of, so-called, early genes that encode the foetal isoforms of several myocardial proteins such as β-myosin heavy chain (β-MHC), skeletal α-actin (skACT) and arterial and brain natriuretic peptides (ANP and BNP) (Wakatsuki et al., 2004). In conjunction with these changes, a decrease in the adult cardiac non-specific genes, α-myosin heavy chain (α-MHC) and sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) also occurs (Izumo et al., 1988; Komuro et al., 1989; van den Bosch et al., 2006). ANP, β-MHC and skACT genes are normally expressed in late foetal and early neonatal heart tissues and are extinguished in adult ventricular myocardium. Although the significance of these changes has still not been fully understood, the re-expression of early genes could represent one facet of the complex adaptive system that serves to reduce energy demand under increased haemodynamic burden. For example β-MHC would facilitate cardiac muscle to work more efficiently when chronically overloaded because it contracts and relaxes more slowly than α-MHC (Izumo et al., 1988) while expression of ANP would contribute to limit cardiac energy consumption through its natriuretic and anti-hypertrophic properties (Holtwick et al., 2003).
**Figure 1-2 Physiological and pathological hypertrophic response to stimuli.**

A) Physiological remodelling is a reversible process which occurs during pregnancy and chronic exercise. It is characterised by eccentric remodelling in which a uniform growth of the LV and septum is matched with an increase in chamber dimension. This phenotype occurs due to addition of sarcomeres in series to lengthen the myocytes.

B) Pathological hypertrophy is an irreversible process, associated with concentric hypertrophy in which the ventricular wall and septum thicken with a decrease in left ventricular (LV) chamber dimensions. There is also an increased rate of myocyte death by apoptosis and necrosis. The dilatation of LV is due to a growth response in which sarcomeres are predominately added in series to individual myocyte. Adapted from (Heineke and Molkentin, 2006).

### 1.3.3 Cardiac Remodelling

Cardiac remodelling is commonly defined as a physiological or pathological state that occurs after conditions such as large MI, idiopathic dilated cardiomyopathy or volume overload (Figure 1-3). This process is characterised by replacement of necrotic myocardium with fibrotic tissue, increased size of myocytes and fibrosis in the remote area, as well as alteration in the LV structure (Warren et al., 1988). The process of cardiac remodelling is largely influenced by haemodynamic load, neurohormonal activation and additional factors such as endothelin, cytokines, nitric oxide production and oxidative stress shortly after
in infarction, the degradation of the inter myocytes collagen struts by serine proteases and the activation of MMPs released from neutrophils (Cleutjens et al., 1995).

**Figure 1-3 Cardiac Remodelling.**

Left ventricular remodelling (LV) is a dynamic process characterised by necrosis and thinning of the infarcted myocardium and LV dilatation. Fibrosis occurs at the site of the infarct and in non infarcted myocardium with hypertrophy of viable myocytes which results from addition of sarcomeres in series to individual myocyte. Adapted from (Heineke and Molkentin, 2006).

This results in expansion of the infarction which then leads to wall thinning and ventricular dilatation and elevation of diastolic and systolic wall stresses. Early ventricular dilatation due to infarct expansion has been unequivocally demonstrated in man. Increased wall stress is a powerful stimulus for hypertrophy mediated by mechanoreceptors and transduced to intracellular signalling partly via Ang II release, which initiates the increased synthesis of contractile assembly (Sadoshima et al., 1993). Wall stress is also a major determinant of ventricular function. Infarct expansion causes the deformation of the border zone and remote myocardium, which alters Frank/Starling relations and augments shortening (Lew et al., 1985). Consequently adaptive responses are invoked that preserve stroke volume by involving the non-infarcted remote myocardium (Lew et al., 1985). Perturbation in circulatory haemodynamics triggers the sympathetic adrenergic system, which stimulates catecholamine synthesis by the adrenal glands and activates the renin-angiotensin-aldosterone system (RAS), and stimulates the production of ANP and BNP (Gardner et al.,
2007; Richards, 2007). Augmented shortening and increased heart rate from sympathetic stimulation result in hyperkinesis of the non-infarcted myocardium and temporary circulatory compensation. In addition, the natriuretic peptides reduce intravascular volume and systemic vascular resistance, normalise ventricular filling, and improve pump function.

At the later phase of remodelling, changes in the architecture of the ventricles distributes the increased wall stress more evenly as the ECM forms a collagen scar to stabilise the distending forces and prevent further deformation.

Various intracellular signalling pathways are thought to play a critical role in the myocardial response to ischaemia, cardiac hypertrophy, and remodelling after myocardial infarction. Multiple mitogen-activated protein (MAP) kinase pathways are activated during ischaemia and may contribute to the structural and functional changes.

1.4 **Kinase signalling in stressed myocardium**

MAP kinases (MAPK) are highly conserved serine/threonine kinases that are activated, by a dual phosphorylation of a Thr-X-Tyr motif, in response to a wide variety of stimuli such as cytokines, osmotic and other environmental stresses. Consequently, these play a role in numerous cell functions including growth and proliferation (English et al., 1999; Pearson et al., 2001). Three of the five major MAPK cascades have been extensively studied in the heart: extracellular signal-regulated kinase (ERK1 and ERK2), c-Jun N-terminal kinases (JNK1 and JNK2) and p38 MAPK.

MAPK cascades are activated by various stimuli such as growth factors and environmental stresses. The pathway consists of a series of three protein kinases- a MAPK and two upstream components, MAPK kinase (MAPKK or MEK) and MAPKK kinase (MAPKKK) (Figure 1-4).
Mitogen activated protein kinases (MAPKs), which integrate and process various extracellular signals. The MAPK cascade consists of a series of three protein kinases - a MAPK and two upstream components, MAPK kinase (MAPKK or MEK) and MAPKK kinase (MAPKKK). ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase (Kumar et al., 2003).

A large number of studies have highlighted the activation of MAPK cascade signalling during myocardial stresses such as ischaemia, myocardial remodelling post MI and hypertrophy.

1.4.1 Kinase signalling in cardiac hypertrophy

Many of the signalling effectors that are implicated in the regulation of cardiac hypertrophy were first identified in cultured neonatal rat primary cardiac myocytes, which were initially used as the model system for the analysis of cardiac growth mechanisms. Recently, development of genetically modified mouse models has greatly advanced understanding of the complexities implicated in cardiac hypertrophy. The hypertrophic response is initiated in response to various neurohormonal and endocrine hormones such as catecholamine (such as noradrenaline (NA) and adrenaline, endothelin-1, Ang II and IGF-1) in cardiac myocytes.
Binding of the ligands to various membrane receptors activates a series of signalling pathways leading to hypertrophy (Figure 1-5). The hypertrophic response is also initiated by mechanical stimuli such as stretch at the cell membrane (Sugden and Clerk, 1998).

Ang II, endothelin-1 and catecholamine (via \(\alpha\)-adrenergic stimulation) bind to heterotrimeric G-protein of \(G_{aq/II}\) subclass membrane receptors which are coupled to phospholipase C\(\beta\) (PLC\(\beta\)). This induces generation of diacylglycerol (DAG), which functions as an intracellular ligand for protein kinase C (PKC). Activation of PKC is accompanied with the production of inositol-1,4,5-trisphosphate (Ins\((1,4,5)P3\)) (Rockman et al., 2002). Ins\((1,4,5)P3\) binds directly to the endoplasmic reticulum or the nuclear envelope. This leads to the mobilisation of internal Ca\(^{2+}\). Recently it was shown that this pathway mediates hypertrophic signalling through calcineurin – nuclear factor of activated T-cell (NFAT) activation or calmodulin-dependent kinase (CaMK) inactivation (Wilkins and Molkentin, 2004; Wu et al., 2006).

One of the key signalling pathways activated by hypertrophy is MAPK signalling. The MAPK signalling cascade is initiated by GPCRs receptors such as G-protein coupled receptors (GPCR), tyrosine kinases (IGF-1, and fibroblast growth receptor (FGR)), receptor serine/threonine kinases (TGF-\(\beta\)) and cardiotophin-1 (gp130 receptor) (Heineke and Molkentin, 2006). Activated p38 MAPK, JNK and ERK each phosphorylate multiple intracellular targets, including various transcription factors involved in the induction of cardiac gene expression reprogramming. In one study, JNK activation appeared to occur earliest and was fully activated within three hours, whereas ERK and p38 MAPK activation occurred within 7 days of surgery (Esposito et al., 2001). Amongst the three MAPKs, ERK has shown to play a central role in the development of hypertrophy. In contrast to ERK, both p38 MAPK and JNK have shown to be the negative regulators of hypertrophy (Figure 1-5). Overexpression studies involving the upstream activators of p38 MAPK and JNK suggest that activation of these kinases influence the hypertrophic response by reducing cardiac myocyte apoptosis (Nishida et al., 2004).

Studies using genetically modified mouse models have confirmed the induction of pathological hypertrophy through \(G_{aq/II}\) signalling. The overexpression of wild type \(G_{aq}\) (or an active form of \(G_{aq/II}\)) in the heart induced a uniform profile of cardiac hypertrophy that lead to heart failure (Adams et al., 1998; D'Angelo et al., 1997; Fan et al., 2005). The
combined distribution of gene encoding \(G_{\alpha q}\) and \(G_{\alpha II}\) abolished hypertrophy following pressure overload in cardiomyocytes (Wettschureck et al., 2001).

ERK1 and 2 are activated by MAPK kinases (MAPKK), MEK1 and MEK2. These directly phosphorylate a dual site in the activation loop of ERK kinases. ERK signalling has shown to be a central regulator of cardiac hypertrophy. This was demonstrated through the analysis of transgenic mice that express the activated MEK1 under the transcriptional control of specific promoter, which showed a specific activation of ERK1/2 in the heart (Bueno et al., 2000). The mice developed a uniform profile of an increased heart weight to body weight ratio (25-30%) and small degree of fibrosis, a phenotype consistent with a form of compensated hypertrophy (Bueno et al., 2000). This is thought to be associated with an enhanced transcriptional activity of NFAT (Sanna et al., 2005). On the other hand, overexpression of the upstream regulator of MEK-ERK1/2 signalling, Ras, promoted pathological hypertrophy (cardiomyopathy) and premature death (Hunter et al., 1995). This could be the result of other Ras effectors such as the JNK branch of the MAPK cascade, PI3K and other intracellular signalling pathways (Molkentin and Dorn, 2001).

In contrast to ERK, p38 MAPK and JNK are the negative regulator of cardiac hypertrophy. The expression of activated upstream activator of p38 MAPK (MKK3 and MMK6) resulted in rapid development of heart failure as juveniles which was characterised by reduced cardiac function, fibrosis, thinned ventricular wall, but no significant hypertrophy at the organ level (Liao et al., 2001). With respect to JNK pathway, transgenic mice that express activated MKK7 (an upstream activator of JNK), in the heart showed specific activation of JNK. This was associated with lethal cardiomyopathy in juveniles without hypertrophic enlargement, reminiscent of transgenic mice with enhanced p38 MAPK activity (Petrich et al., 2002; Petrich et al., 2003). It is not clear how p38 MAPK and JNK result in negative regulation of cardiac hypertrophy. However, it is thought that these kinases may be acting through a calcineurin-NFAT dependent pathway (Liang et al., 2003). It has been shown that the expression of dominant-negative mutants of upstream kinases such as MKK3/6 or p38\(\alpha\) MAPK promotes increased cardiac growth at baseline and after pressure-overload stimulation through a calcineurin-NFAT dependent pathway (Braz et al., 2003).

Indeed, the calcineurin-NFAT circuit is one of the important signalling pathways implicated in cardiac hypertrophy. The Ca\(2^{+}\)-dependent serine/threonine protein-phosphatase, calcineurin was first identified as a central pro-hypertrophic signalling in the myocardium.
(Molkentin et al., 1998). It consists of a catalytic (CnA) and a regulatory (CnB) subunit. When the protein becomes activated it dimerizes through binding to Ca2+ adaptor protein calmodulin (Wilkins and Molkentin, 2004). Activated calcineurin then binds to transcription factors of the NFAT family and dephosphorylate the conserved N-terminus. This results in the translocation of NFAT into the nucleus and the transcription of pro-hypertrophic gene expression (Wilkins and Molkentin, 2004b). In addition, kinases such as glycogen synthase kinase 3β (GSK3β), p38 MAPK and JNK can induce phosphorylation of NFAT in cardiac myocytes (Antos et al., 2002; Braz et al., 2003b; Liang et al., 2003).

Other hypertrophic regulators include the PI3K-AKT (also known as protein kinase B (PKB)) pathway. This pathway is activated by the IGF-1 binding to the surface receptor. PI3K activation results in the sarcolemmal recruitment of the kinases such as AKT and phosphoinositide-dependent kinase-1 (PKD 1) (Cantley, 2002). When PKD1 and AKT/PKB are brought into close proximity, PKD becomes phosphorylated and thereby activates AKT/PKB (Cantley, 2002). AKT-1 null mice have 20% reduction in body size, with a reduction in the heart size, whereas AKT-2 null mice do not show any reduction in body growth (Cho et al., 2001b; Cho et al., 2001a; DeBosch et al., 2002). Activated AKT/PKB can induce cardiac hypertrophy with preserved function. However, constitutive overexpression of AKT/PKB can also lead to cardiac dysfunction over time in some models (Condorelli et al., 2002; Shioi et al., 2002; Matsui et al., 2002). This paradox seems to be due to the nature or distribution of the stimuli. Using transgenic models, it appears that AKT/PKB is beneficial when is activated under physiological condition in an acute manner but, still lead to pathological remodelling in a chronic model (Shioi et al., 2002). One of the important downstream targets of AKT/PKB is GSK3β which is normally active but becomes inhibited by AKT/PKB mediated phosphorylation. Active GSK3β negatively regulate hypertrophic transcriptional effectors such as GATA4 (a zinc-finger containing transcriptional factor that can regulate hypertrophic gene expression), β-catenin (a cytoplasmic protein that translocate to the nucleus and induces gene expression through binding to transduction factors), cMyc and NFAT. It can also inhibit the translation initiation factor eIF2β (Cantley, 2002; Dorn and Force, 2005; Proud, 2004; Rota et al., 2005). AKT/PKB also enhances protein synthesis by activating mTOR (Cantley, 2002; Proud, 2004). The inhibition of mTOR by Rapamycin attenuates pathological remodelling and can reverse myocardial dysfunction (McMullen et al., 2004; Shioi et al., 2002; Shiojima et al., 2005).
Another important regulatory paradigm in hypertrophy involves alterations in gene expression which are mediated by chromatin remodelling regulated by a group of enzymes called Class II histone deacetylase (HADC). These enzymes are regulated by phosphorylation by kinases such as protein kinase D (PKD), PKC and CaMK (Vega et al., 2004). After phosphorylation, HDAC are transported in the nucleus where regulate genes that are involved in the regulation of muscle growth (Miska et al., 1999).

In addition to GSK3β and HDAC as negative regulator of hypertrophy (anti hypertrophic properties), cytoplasmic phospholipase A2 (cPLA2) and cyclic guanosine monophosphate (cGMP)-dependent protein kinse-1 (PKG1) have critical functions (Haq et al., 2003; Takimoto et al., 2005; Wollert et al., 2002). Ubiquitously expressed cPLA2 generates arachidonic acid. Transgenic mice with cPLA2 deletion have shown to have increased developmental and pathological hypertrophic myocardial growth via an arachidonic acid dependent mechanism which enhances IGF-1 dependent signalling (Haq et al., 2003). ANP, BNP, free radical and NO that are produced within the heart also activate different types of guanyl cyclases, which in turn generate cGMP leading to activation of PKG1 (Takimoto et al., 2005). Inhibition of cGMP catabolism activates PKG1 resulting in inhibition of pathological cardiac hypertrophy (Takimoto et al., 2005). The overexpression PKG1 in cultured cardiomyocytes similarly resulted in reduced hypertrophic growth (Wollert et al., 2002). The mechanisms of reduced pathological cardiac hypertrophy appear to be due to the inhibition of the calcineurin-NFAT, PI3K-AKT/PKB and ERK1/2 pathway (Fiedler et al., 2002; Takimoto et al., 2005).

These are two more classes of kinases which have also been implicated as regulators of hypertrophy include CaMK and PKC. Both, CaMK and PKC can be activated by Ca $^{2+}$. The genetic inhibition of CaMK II exhibited reduced hypertrophy and improved cardiac function during infusion of isoproterenol (Passier et al., 2000; Zhang et al., 2003b). This indicated that CaMK is necessary for the induction of hypertrophy (Zhang et al., 2003b). The overexpression of PKC isoforms ($\alpha$, $\beta$ and $\epsilon$) can induce cardiac hypotrophy in transgenic mice. However, the inhibition of PKC isoforms by gene targeting or blocking peptides has shown that they are not implicated in the regulation of cardiac hypertrophy (Passier et al., 2000; Zhang et al., 2003b). These findings indicate that PKC isoforms might not be the primary regulators of this process, or that gene redundancy is a significant issue in vivo (Dorn and Force, 2005).
From the discussion above, it is clear that hypertrophy involves a complex series of signalling transduction pathways with a great degree of cross talk. Some of these signalling pathways seem to integrate specific neuro-endocrine signals at cell membrane and transmit this information to intracellular target proteins that are involved in transcription, protein synthesis and protein stability.
In response to pressure overload, there is an intra-myocardial release of ligands such as endothelin 1 (ET1), Angiotensin II (Ang II) and noradrenaline (NE), growth factors (GFs), including FGF1 and cytokines. These will then activate transmembrane GPCRs, RTKs (receptor tyrosine kinase) and cytokine receptor (not shown). This will activate an intracellular signal transduction including G-proteins (Gq/11) and Grb2-SOS complex, which promote activation of the MAPK cascades and the CaM (Ca\(^{2+}/\)calmodulin)-CnA (calcineurin A)-NFAT 3/4 (nuclear activated T-cells) cascade. Activation of these signalling pathways modulates the growth of cardiomyocytes. Specifically, ERK1/2 phosphorylate a variety of targets that may contribute to cardiomyocyte growth, including the transcription factors ELK-1 and GATA4 and several proteins that regulate the translational machinery, including Mnk1 and p90 RSK (receptor tyrosine kinase). On the other hand, JNK1/2 and p38 MAPK phosphorylate NFAT family members, resulting in inhibition of the CnA-NFAT hypertrophic pathway.
1.4.2 Kinase signalling in cardiac remodelling

Two major MAPK cascades, p38 MAPK and JNK, are thought to be activated in pathological remodelling after MI (Figure 1-6). Shortly after ischaemia, (in vivo model of MI) ERK1/2, JNK1/2 and p38α MAPK are all activated in the myocardium (Ren et al., 2005). However, amongst all the MAPKs, the level of p38α MAPK has shown to remain elevated most consistently for several weeks in the left ventricle after the initial insult (Qin et al., 2005; Tenhunen et al., 2006). Dominant negative p38α MAPK transgenic mice subjected to a permanent ligation of the left anterior descending (LAD) coronary artery, demonstrated a reduced degree of scarring at the site of infarction with a markedly declined cardiomyocyte apoptosis at the border zone compared to the wild type counterpart (Kaiser et al., 2004). This was associated with an increased expression of anti apoptotic gene, Bcl-2, at baseline in the transgenic animals, which was increased after ischaemia reperfusion injury (Kaiser et al., 2004).

It has also been demonstrated that rats subjected to permanent ligation of the LAD treated with pharmacological inhibitor of p38 MAPK show a reduction in fibrosis, reduced TNF-α and collagen I levels and increased LV contractile function (Yin et al., 2008). Therefore, it has been suggested that activation of p38α MAPK promotes pathological remodelling by reducing the activity or expression of the anti-apoptotic family members Bcl-xL and Bcl-2 and the production of inflammatory cytokines (Figure 1-6). It has also been shown that p38α MAPK can block cardiomyocyte mitosis (Engel et al., 2005). In this study, microarray analysis of neonatal rat cardiomyocytes treated with pharmacological inhibitor of p38 MAPK, SB203580, revealed an up-regulation of many cell-cycle proteins, including cyclin A2 and cyclin B (Engel et al., 2005). Cyclins are a family of proteins which control the progression of cells though the cell cycle by activating cyclin-dependent kinase (Cdk). Although this evidence is based on animal data, it seems likely similar mechanisms operate in the human heart since p38 MAPK is identically activated by ischaemia (Cain et al., 1999; Cook et al., 1999; Lemke et al., 2001) and early clinical trials indicate a potential benefit (de Winter et al., 2005). Thus, superficially at least, inhibitors of p38 MAPK have therapeutic potential in ischemic heart disease (Force et al., 2004).

The second MAPK signalling pathway involves activation of JNK1/2 by its upstream activator ASK1 (Figure 1-6). ASK1 is stress inducible MKKK in the heart and it is regulated by ROS via thioredoxin in a reduction/oxidation-sensitive manner (Kinugawa et al., 2000).
ASK-1\textsuperscript{+/−} transgenic mice subjected to MI have reduced cardiac remodelling, reduced fibrosis at the border zone and remote myocardium, reduced diastolic LV dilation, improved fractional shortening and reduced cardiomyocyte apoptosis in the border zone. This was accompanied by a reduction in the level of JNK1/2 at the infarct border zone (Yamaguchi et al., 2003).

Of all the kinases, however, p38 MAPK is best characterised and perhaps the most physiologically relevant kinase involved in inflammatory responses and is the focus of this Thesis.

p38 MAPKs are activated by a wide range of extracellular influences, including radiation, ultraviolet light, heat shock, osmotic stress, pro-inflammatory cytokines such as IL-1β and TNF-α, and certain mitogens (Sugden and Clerk, 1998) in addition to myocardial ischaemia (Bogoyevitch et al., 1996; Luss et al., 2000; Ping and Murphy, 2000). Furthermore, activation of p38 MAPK is intimately involved in multiple cellular responses including growth, proliferation, differentiation and death (English et al., 1999; Ono and Han, 2000). Perhaps, not surprisingly, these cellular effects have clear consequence(s), translating into involvement in complex pathophysiologies such as wound healing (Lim et al., 1998) inflammatory arthritis (Badger et al., 1996), sepsis (Kotlyarov et al., 1999) and malignant hypertension (Behr et al., 2001).

Four p38 MAPK isoforms (α, β, δ and γ) exist which have preserved structure but variable sensitivity to pharmacological inhibition. All four isoforms have a Thr\textsuperscript{180}-Gly\textsuperscript{181}-Tyr\textsuperscript{182} (TGY) dual phosphorylation motif which is used by investigators to infer activation. p38α and β MAPK have high sequence homology and share sensitivity to pharmacological inhibition by pyridinyl imidazole molecules (such as SB203580) but have only 60% homology with p38γ and δ MAPK (Figure 1-7), which are resistant to SB203580 (SB) inhibition (Eyers et al., 1999). Of the SB-sensitive isoforms p38α MAPK is the predominant form in human and rodent myocardium (Lemke et al., 2001; Rakhit et al., 2001; Sanada et al., 2001). Studies with knockout mice and cells have shown that p38α MAPK is essential for embryonic development as knockout of the α isoform results in embryonic lethality, but mice lacking p38β, p38γ, and p38δ MAPK are viable (Adams et al., 2001; Allen et al., 2000; Beardmore et al., 2005; Tamura et al., 2000).
Figure 1-6 Model of the MAPK cascade in pathological remodelling.

After MI, there is a local release of ligands such as noradrenaline (NE), cytokines, growth factor (GFs), including ROS. This leads to the activation of trans-membrane receptors and intracellular MAPK cascade. ROS may modify the activity of the MKKK ASK 1 by blocking the ability of thioredoxin to inhibit ASK1. The activation of the MKK-MKK3/6-p38α MAPK and the ASK1-MKK4/7-JNK cascades promote cardiac remodelling which includes cardiomyocyte apoptosis, inhibition of cardiomyocyte mitosis, inflammation and fibrosis. Specifically p38α MAPK promotes cardiomyocyte mitosis by inhibiting the activity or expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL.
Figure 1-7 Sequence comparison of human p38 isoforms.

Human p38α MAPK (also called SAPK2a, p40, RK, CSBP2, Mxi2 and XmP1), human p38β MAPK (SAPK2b), human p38γ MAPK (also called ERK6 and SAPK3), P38δ MAPK (SAPK4) and HOG1 (a MAPK homologue in yeast). Amino acids were aligned and gaps were introduced to maximize the homology. Amino acid identities between at least three of the five sequences are indicated by black bars. Asterisks denote the dual phosphorylation sites in the TGY motif of the activation domain (Goedert et al., 1997a).
1.5 Structure of p38 MAPK

The catalytic site of p38 MAPK lies in a pocket between the N- and C-terminal domains. These domains are connected by a single hinge and the L16 loop of the C-terminal domain which wraps back around the N-terminal domain, and controls the relationship between the relatively rigid domains (Figure 1-8). In addition, in the inhibitor bound non-phosphorylated state there is a misalignment between the N- and C- lobes which prevents the cooperation between a lysine residue (Lys^{53}) in the N-terminal lobe, and aspartic acid residue (Asp^{168}) in the C-terminal lobe, imperative to binding and stabilisation of the alpha phosphate group and adjacent ribose of ATP, respectively (Figure 1-8) (Gum et al., 1998; Wilson et al., 1996). Therefore, it is widely thought that un-phosphorylated form of p38 MAPK is inactive as a result of steric obstruction of the peptide-binding channel and low ATP affinity.

To date, four splice variants of the p38 MAPK family have been identified; p38α, p38β, p38γ (ERK 6, SAPK3) and p38δ (SAPK4) (Zarubin and Han, 2005). Of these, p38α and β are ubiquitously expressed while p38γ and δ are differentially expressed depending on tissue type. The expressions of these genes are differentially regulated and are cell and stimulus dependent. For instance insulin has been shown to stimulate p38α MAPK in 3T3-L1 adipocytes (Sweeney et al., 1999), but down-regulates p38 MAPK activity in chick forebrain neuron cells (Heidenreich and Kummer, 1996). Furthermore, the activation of p38 MAPK isoforms can be specifically controlled through different regulators and co-activators (low molecular weight binding proteins) and combinations of upstream regulators (Enslen et al., 1998; Hu et al., 1999). Since the activation of the MAPK is transient, de-phosphorylation by various MAPK phosphatases (MKP) also seems to regulate the deactivation of the p38 MAPK family members. For example, several members can de-phosphorylate p38α and β, however, p38γ and p38δ MAPK are resistant to all known MKP family member (Zarubin and Han, 2005). Moreover, there are two important structural requirements for selective activation of p38 MAPK isoforms. These are the upstream kinases common docking sequence in the N-terminal of the MKK and isoform-specific sequence of the p38 MAPK isoforms within the activation loop (Enslen et al., 2000). Taken together, these findings suggest a mechanism by which p38 isoforms are differentially regulated (Zarubin and Han, 2005).
Thr\textsuperscript{180} and Tyr\textsuperscript{182} are located on a flexible “activation loop” that guards the active site. Dual phosphorylation of these two amino acids in response to exotoxin, cytokines, physical stress (such as hyper-osmolarity) and chemical oxidant stress such as hydrogen peroxide (Gum et al., 1998; Han et al., 1994; Raingeaud et al., 1995; Rouse et al., 1994) is thought to cause the activation loop to refold and move out of the peptide-binding channel. This movement is then thought to exert a “crank-handle” effect on the overall tertiary structure of the kinase reorienting the N- and C- terminal lobes so that Lys\textsuperscript{53} and Asp\textsuperscript{168} move towards one another by 2.5-5Å. This alters the conformation of the catalytic site enabling the cooperation necessary for ATP binding and allowing substrate access (Diskin et al., 2004; Wilson et al., 1996). The docking grooves used by substrates and activators consist of two regions, the C-region and the docking region (Tanoue et al., 2000). The C-region is part of a shallow groove formed by the acidic residues Asp\textsuperscript{313}, Asp\textsuperscript{316}, Glu\textsuperscript{81}, and the aromatic residues Phe\textsuperscript{129} and Tyr\textsuperscript{311}. The docking region is part of a deeper groove formed by residues 159-163 at one side and residues Gln\textsuperscript{120}, His\textsuperscript{126} and Phe\textsuperscript{129} at the opposite side (Haar et al., 2007) (Figure 1-8). It is believed that these two binding regions facilitate activator (M KK3) and substrate (MAPKAPK2 and MEF2) binding (Chang et al., 2002; Haar et al., 2007).
Figure 1-8 Crystal structure of p38 MAPK with SB203580 occupying the ATP binding site.

Crystal structure of p38. The Thr^{106} residue (4), which is important for binding of pyridinyl imidazole inhibitors, and the 2 residues within the activation loop that are phosphorylated (Thr^{180} (2) and Try^{182} (1)) are highlighted. Tyr^{323} (3), which has been implicated in TCR-mediated activation of p38 is also shown. SB203580 is shown in green. The activation loop is shown in orange. Activator (ED) and substrate (CD) binding regions are highlighted. The C-terminal extension that forms the L16 loop bridging the domains is also indicated (Clark et al., 2007).

1.5.1 Mechanisms of p38 MAPK activation:

1.5.1.1 Mitogen-activated protein kinase kinases (MKKs)

Although the intracellular activation cascade for p38 MAPK under most physiological conditions is still unclear, several upstream MAP kinase kinases (MKKs) have been identified from in vitro analysis, including MKK3 and MKK6 (Derijard et al., 1995; Han et al., 1996). MKK4 is predominately involved in JNK activation but is able to activate p38 MAPK, at least, in vitro (Deacon and Blank, 1997). Using MKK-targeted mouse lines, it has been shown that, in response to most stress stimuli, MKK3 and -6 are the principal MKKs activating p38α and β MAPK respectively (Figure 1-9). MKK3 and -6 are in turn activated by phosphorylation by a MAPK kinase kinase (MKKKs). The MKKKs responsible for
activation of the p38 MAPK cascade appears to be cell type and stimulus specific and several have been implicated (Chang et al., 2002; Gallo and Johnson, 2002; Haar et al., 2007; Hutchison et al., 1998; Ichijo et al., 1997).

However, p38 MAPK activation is not limited to this traditional phospho-relay signalling cascade. Since SB203580 (the most widely used p38 MAPK inhibitor) occupies the catalytic site, without inhibiting upstream MKKs, it should only inhibit the phosphorylation events downstream of p38 MAPK without inhibiting the dual-phosphorylation of p38 MAPK itself (Young et al., 1997). Certain conditions such as myocardial ischaemia cause a SB203580-sensitive form of p38 MAPK dual phosphorylation. Two mutually exclusive explanations for these observations are i) that p38 MAPK is able to auto-phosphorylate its activation loop or ii) that SB203580 inhibits a kinase upstream of p38 MAPK involved in its activation, by trans-phosphorylation, during ischaemia.

1.5.1.2 Auto-phosphorylation

Ge and co-workers elegantly reported that auto-phosphorylation of p38 MAPK can occur, facilitated by an interaction with the non-enzymatic adaptor protein transforming growth factor-β Activated protein kinase-1 (TAK1) Binding protein-1 or TAB1 (Ge et al., 2002). TAB1 is known to perform a similar function by inducing the auto-phosphorylation of TAK1 which in turn activates MKK3/6. In vitro co-expression experiments have shown that the interaction of TAB1 and p38α MAPK leads to phosphorylation of the TGY activation motif. TAB1-dependent p38α MAPK activation appears to play a role in the injury response during myocardial ischaemia (Fiedler et al., 2006; Tanno et al., 2003), myocyte-derived dendritic cell maturation (Matsuyama et al., 2003), and peripheral T-cell anergy maintenance (Ohkusu-Tsukada et al., 2005). The interpretation of the TAB1-p38 MAPK interaction was, however, complicated by Cohen’s group who demonstrated that the phosphorylation of TAB1 on Ser^{423} and Tyr^{431} was p38 MAPK-dependent and hence prevented by SB203580. The authors proposed a feedback control mechanism of TAK1 activity, whereby p38 MAPK activity inhibits TAK1, through the phosphorylation of TAB1. Inhibition of p38 MAPK activity (by SB203580) abolishes this feedback control of TAK1, causing unopposed activation of the parallel JNK pathway (Cheung et al., 2003).

Using MKK3/6 double knock out and MKK4/7 double knock out mouse embryonic fibroblasts (MEFs) Kang et al have shown that peroxynitrite-induced phosphorylation of
p38α MAPK is associated with an ~85kDa disulfide complex in wild type MEFs (Kang et al., 2006). This association was diminished in MKK3/6 knockout MEFs (Kang et al., 2006). The authors suggested that phosphorylation of p38 MAPK mediated by TAB-1 can be modulated by a yet unknown binding partner(s) in a manner dependent on a disulfide complex (Kang et al., 2006).

In addition to TAB-1-mediated activation, p38 MAPK can also auto-phosphorylate through an alternative pathway in response to T cell antigen receptor (TCR) activation (Dong et al., 2002; Rincon and Pedraza-Alva, 2003). In this pathway, activation of TCR leads to recruitment of a Syk family kinase, ZAP-70, which directly phosphorylates p38 MAPK on Tyr^{323} (Rouse et al., 1994). In a recent study it was shown that the phosphorylation of p38 MAPK on Tyr^{323} can be blocked in the presence of the DNA damage inducible gene Gadd45a, an autoimmune suppressor. Absence of Gadd45a has been shown to result in chronic phosphorylation of p38 MAPK, T cell hyper-proliferation and autoimmunity (Salvador et al., 2005). Following ZAP-70 phosphorylation of p38 MAPK an auto-phosphorylation event similar to that induced by interaction with TAB-1 occurs resulting in dual phosphorylation, and activation of the kinase (Figure 1-9).

However, regulation of p38 MAPK kinase activity in vitro, at least, is not solely dependent on upstream kinases and binding partners. Diskin and co-workers using an in vitro mutation approach, made intrinsically active p38 MAPK isoforms based on activating mutations previously found in the yeast MAPK kinase p38/Hog1 (Bell et al., 2001). Single and multiple point corresponding mutations of human p38α MAPK resulted in high intrinsic activity independent of activation by dual phosphorylation.

Structural analysis of these p38 MAPK mutants has identified a hydrophobic core stabilised by three aromatic residues, Tyr^{69}, Phe^{327} and Trp^{337}, in the vicinity of the L16 Loop region. It is believed that the hydrophobic core is an inherent stabiliser that maintains the low basal activity level of un-phosphorylated p38 MAPK (Diskin et al., 2004). Upon activation, however, a segment of the L16 Loop, including Phe^{327}, becomes disordered allowing ATP and substrate binding. The mutation of these amino acids, involved in the hydrophobic core, results in the conformational changes imposed naturally by dual phosphorylation, namely destabilising the hydrophobic core and locking the kinase in a constitutively active state. In addition, in this active state p38 MAPK is able to auto-phosphorylate in an in vitro kinase assay (Diskin et al., 2004). More recently p38β, p38γ and p38δ MAPK mutants were
similarly constructed (Askari et al., 2007). In these mutants, a highly conserved aspartic acid located in the activation loop (Asp$^{170}$ in Hog-1, Asp$^{176}$ in p38α, p38β and δ MAPK and Asp$^{179}$ in p38γ MAPK) was mutated. The spontaneous kinase activity of p38β, p38γ and p38δ MAPK appeared to be lower than the dual phosphorylated wild type isoforms whereas the p38α MAPK isoform presented the highest spontaneous activity. Therefore, it is apparent that modifications of the amino acids in the hydrophobic core along with the mutations of Asp$^{176}$ are capable of activating p38 MAPKs (Askari et al., 2007), the former likely explaining the mechanism by which ZAP-70 induces auto-activation (Mittelstadt et al., 2005). In addition, these mutants provide a tool to delineate isoform-specific downstream signalling (Askari et al., 2007).
Chapter 1

General Introduction

Figure 1-9 Mechanisms of p38 MAPK activation.

Classical activation by MKK3/MKK6 is depicted as mechanism ①. TAB1-induced auto-activation is depicted as mechanism ②. TCR-mediated Tyr323 phosphorylation by ZAP70 is depicted as mechanism ③. TAB1 activates TAK1, which in turn activates MKK3/MKK6. In addition, TAB1 is a p38 MAPK substrate. PhosphoTAB1 is less able to activate TAK1. Pharmacological inhibition of p38 MAPK diminishes p38 MAPK dual phosphorylation and phosphoTAB-1. Heavy lines represent an interaction; dotted lines represent a modification (phosphorylation); open arrows represent a multi-element pathway (Clark et al., 2007).
1.5.2 Downstream targets and physiological roles of p38 MAPKs

Once activated, p38 MAPK isoforms can regulate various physiological processes by targeting a diverse range of downstream substrates. Many p38 MAPK targets localise both in the cytoplasm and in the nucleus, which indicates that multiple cellular functions are under their control. Although p38 MAPKs are proline-directed kinases, the specificity of the substrates is determined by the targeted amino acids as well as the specific substrate binding motif in the p38 MAPK. Thus, although the substrates of p38 MAPK largely overlap, there are some differences between p38α and p38β MAPK vs. p38γ and p38δ MAPK (Table 1-1).

Table 1-1 Characteristics and downstream substrates of p38 MAPK isoforms.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>α</th>
<th>β</th>
<th>γ</th>
<th>δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site of expression</td>
<td>Global</td>
<td>lung, thymus &amp; brain (heart?)</td>
<td>skeletal muscles, brain (heart?)</td>
<td>kidney, pancreas &amp; small intestine (heart?)</td>
</tr>
<tr>
<td>Structural homology in relation with p38α</td>
<td>75%</td>
<td>62%</td>
<td>61%</td>
<td></td>
</tr>
<tr>
<td>Pharmacological inhibitors</td>
<td>SB203580, BIRB0796</td>
<td>SB203580, BIRB0796</td>
<td>BIRB0796</td>
<td>BIRB0796</td>
</tr>
<tr>
<td>Substrates</td>
<td>MAPKAP-K2, MAPKAP-K3, Glycogen synthase, ATF2, ELK-1 SAP1 (English et al., 1999; Ono and Han et al., 2000; Nagarkatti and Ramadan et al., 1998).</td>
<td>MAPKAP-K2, MAPKAP-K3, Glycogen synthase (English et al., 1999; Ono and Han et al., 2000; Nagarkatti and Ramadan et al., 1998).</td>
<td>α1-syntrophin, SAP90/PSD95 SAP97 hD1g &amp; Tau (Hasegawa and Cahill, 2004; Sabio et al., 2005).</td>
<td>Stathmin (regulation of MT dynamics) &amp; Tau (Parker et al., 1998; Goedert et al., 1997).</td>
</tr>
</tbody>
</table>

The physiological substrates of p38α and β MAPK include transcription factors, other protein kinases (involved in phosphorylation of other kinases), cytoskeletal proteins and translational components, metabolic enzymes, glycogen synthase or cytosolic phospholipase A2 (Cohen, 1997; Kyriakis and Avruch, 2001). p38α MAPK is shown to be present in the nucleus and the cytoplasm of the quiescent cells (Raingeaud et al., 1995). However, it translocates into the nucleus where it regulates expression of various transcription factors.
p38γ and δ MAPK also share typical p38 MAPK substrates such as transcription factors ATF2, Elk-1 or synapse associated protein -l (SAP) (Goedert et al., 1997a; Knebel et al., 2001). In contrast, they are unable to phosphorylate MAPKAPK2 or MAPKAPK3, which are phosphorylated by p38α and p38β MAPK. In addition, unlike the other isoforms, p38γ MAPK isoform is able to bind to a variety of scaffold proteins through its short terminal sequence-KETXL, an amino acid sequence ideal for binding PDZ domains in proteins (Hasegawa et al., 1999). These scaffold proteins are phosphorylated by p38γ MAPK include α-syntrophin, SAP90/PSD95 and SAP97/hD1g. Under stress conditions p38γ MAPK binds to and phosphorylates the scaffold protein. This will target the scaffold protein to the plasma membrane cytoskeleton at specialised sites such as the neuromuscular junction and gap junctions through protein-protein interactions (Sabio et al., 2005). For instance phosphorylation of SAP97 by p38γ MAPK provides a mechanism dissociating SAP97 from the cytoskeleton (Sabio et al., 2005). It has also been reported that this isoform is able to translocate into the mitochondria and phosphorylate the mitochondrial protein, Sab, an in vitro substrate of JNK (Court NW et al., 2004).

p38δ MAPK has been reported to phosphorylate the cytoplasmic protein stathmin which has been linked in the regulation of microtubule dynamics (Parker et al., 1998). Other substrates of p38δ MAPK also include tau, a microtubule associated protein (Fujita et al., 2001; Goedert et al., 1997b). In addition, p38δ MAPK also phosphorylates eukaryotic elongation factor 2 (eEF2) kinase (Knebel et al., 2001; Knebel et al., 2002). It has also reported that this isoform regulates gene expression through the transcription factor CHOP (Dashti et al., 2001).

Considering such diverse downstream targets it is not surprising that p38 MAPKs regulate various physiological processes as described below.

1.5.2.1 Cellular differentiation

A number of studies have established a key role for the p38 MAPK pathway during myogenic progression and differentiation of microtubules from myoblasts (Cuenda and Cohen, 1999; Li et al., 2000). Myogenic differentiation is controlled by p38 MAPK at multiple levels: transcription factor activity, chromatin remodelling and turnover of mRNAs encoding certain regulators of muscle differentiation. The contribution of the different p38 MAPK isoforms to the differentiation process has recently been examined using transgenic mice lacking one of the four isoforms (Perdiguero et al., 2007). In this study it was shown...
that myoblasts from mice lacking p38α MAPK, but not those lacking p38β and δ MAPK, fail to differentiate to multinucleated myotubes, highlighting a central role for this kinase in myogenesis.

Various reports have suggested that both the α and β isoforms phosphorylate and enhance the translational activity of monocyte enhance factor (MEF)2, MEF2A and MEF2C. P38γ MAPK on the other hand, only phosphorylates MEF2A and C in vitro (Han et al., 1997) with little effect in vivo. p38δ MAPK however, does not seem to be able to phosphorylate any of them (Marinissen et al., 1999). p38γ MAPK had shown to play a cardinal role in skeletal muscle differentiation. This is consistent with exceptionally high expression of this isoforms in skeletal muscles compared with other tissues. Indeed, p38γ MAPK deficient myoblasts show attenuated fusion in vitro although no major alteration was detected in neonatal or adult muscle (Perdiguero et al., 2007). An important role of p38δ MAPK has been reported in the differentiation of keratinocytes. Activation of exogenously expressed p38δ MAPK correlates with increased activity of transcription factors; AP1 and Sp1 (Efimova et al., 2003).

Other differentiation processes involving either positive or inhibitory roles of p38 MAPKs include osteoclastogenesis, (Huang et al., 2006), adipogenesis (Aouadi et al., 2006), intestinal epithelial cell differentiation (Houde et al., 2001), neuronal plasticity (Butler et al., 2004) and stem cell differentiation (Seeger et al., 2005).

1.5.2.2 Cell migration

Numerous studies have reported a role for p38α MAPK in the regulation of angiogenesis, cell motility and invasion. p38α MAPK has been shown to be involved in cell migration by relaying a chemotactic signal in numerous systems (Gee et al., 2009). These include migration of smooth muscle cells in response to platelet-derived growth factor (PDGF), TGF-β, IL-β, neutrophil migration by C5a, mast cells treated with stem cell factor and invasion of human heart epithelia cells by H-Ras (Gee et al., 2009). In addition, p38α MAPK deficient mice demonstrate a partial loss of pro-angiogenic (for instance vascular endothelial growth factor (VEGF)) signalling leading to increased cell migration, which could partially explain the embryonic lethality. Interestingly, it is also known that p38α MAPK is activated by hypoxia which can then result in VEGF expression leading to defective angiogenic signalling seen in p38α MAPK-deficient mice (Rousseau et al., 1997). These observations can delineate a signalling pathway in which p38α MAPK regulate angiogenesis.
The role of p38 MAPK in cell migration has been linked with the cytoskeleton rather than with gene expression. It has been reported that p38α MAPK regulate actin polymerisation by phosphorylating MAPKAPK2 and subsequent phosphorylation of heart shock protein 27 (HSP 27) (Hedges et al., 1999; Rousseau et al., 1997). Un-phosphorylated HSP27 has been shown to block actin polymerisation and acts as an actin-cap-binding protein in vitro which is modulated by phosphorylation (Benndorf et al., 1994; Hedges et al., 1999). Initial studies revealed that inhibition of p38 MAPK activity and subsequent phosphorylation of HSP27 by MAPKAPK2 can result in actin reorganisation necessary for cell migration.

Another way by which p38α MAPK can regulate cell migration is by regulating MMPs such as MMP-9. For instance, inhibition of p38α MAPK with SB203580 has shown to block MMP-9 expression in phorbol 12-myristate 13-acetate (PMA) treated hepatocellular carcinoma (Yu et al., 2009).

1.5.2.3 Inflammation

p38α MAPK was first recognised for its role in pro-inflammatory cytokine production such as TNF-α and IL-1β in endotoxin stimulated myocytes (Salituro et al., 1999). It is also involved in regulating the production of IL-8 in response to IL-1 or osmotic shock and IL-6 in response to TNF-α (Beyaert et al., 1996). The involvement of p38α MAPK has also been implicated in post-translational regulation of TNF-α in response to LPS, possibly via phosphorylation of downstream substrates such as MAPKAPK2 (Kontoyiannis et al., 1999).

1.5.3 Tools for investigating role of p38-MAPK isoforms and manipulating their expression

1.5.3.1 Pharmacological inhibitors

Early efforts in drug discovery of small molecule inhibitors of kinases were met with scepticism that selectivity could ever be accomplished, due to the high degree of structural similarity in the adenosine binding pocket among the entire kinome. Thus, it was somewhat of a surprise when SB203580, the first reported p38 MAPK inhibitor, emerged showing selectivity over the closely related JNK and ERK MAP kinase families (Lantos et al., 1984). The pyridinyl imidazole anti-inflammatory agents were soon shown to be highly selective p38 MAPK inhibitors and the bi-cyclic pyridinyl imidazole SKF-86002 was the first compound reported to inhibit LPS-stimulated cytokine production (Lee et al., 1988; Lee et
al., 1994). It was not long before investigators explored dual 5-lipooxygenase/cyclooxygenase (LO/COX) and cytokine inhibition as potential mechanisms for the potent anti-inflammatory activity of these compounds (Lee et al., 1993) and subsequently, SB203580 was used as a pharmacological inhibitor to study the cascade of kinases (via p38 MAPK) involved in cytokine production (Gallagher et al., 1997).

The crystal structures of pyridinyl imidazole-p38α MAPK complexes have recently become available and suggest that SB203580 binds to the active site of both phosphorylated (active) and un-phosphorylated (inactive) p38 MAPK in an ATP-competitive manner. These inhibitors bind to an aryl-specificity pocket behind the site which is normally occupied by the adenine ring of ATP. The interaction occurs between the 4-pyridinyl group (analogous to the N-1 adenine of ATP) and the N-H of Met109. In addition, studies have also implicated Thr106 as a key residue conferring selectivity (Gum et al., 1998; Wilson et al., 1996). The two adjacent residues, His107 and Leu108, along with Thr106 lie at the back of the ATP pocket and are identical in p38α and p38 β MAPK, but are different in p38γ and p38δ MAPK (Met106, Pro107 and Phe108 respectively) which are insensitive to SB203580 inhibition.

In addition to novel pyridinyl imidazole compounds, a new group of selective p38 MAPK inhibitors are the aryl-pyridyl-heterocycles and non-aryl-pyridyl-heterocycles such as triazanaphthalenones, N,N-diary ureas, benzophenones, pyrazole ketones, indole amides, diamides and quinazolinones (Cirillo et al., 2002) (Figure 1-10).
Figure 1-10 Structure of representative classes of p38 MAPK inhibitors.

Inhibitors can be divided into 2 groups dependent upon their mode of binding to p38 MAPK; active site inhibitors, such as SB203580 and RJW-67657, bind to the ATP site of the enzyme whereas others bind remotely and interfere with ATP binding indirectly (such as BIRB-796) (Clark et al., 2007).
Unlike the imidazole-based p38α MAPK inhibitors, the urea-containing inhibitors, such as BIRB-796 act in a non competitive manner. These inhibitors bind to at a site remote from ATP pocket, and induce a significant movement of Phe169, such that this residue fills the ATP pocket, preventing ATP binding (Pargellis et al., 2002). Thus, inhibitors of p38 MAPK can be divided into two groups dependent upon their mode of binding to p38 MAPK; these are i) active site or “gate keeping” inhibitors (such as SB203580) and ii) those which bind remotely and interfere with ATP binding indirectly (Figure 1-11) (such as BIRB-796).

Figure 1-11 Binding of two major groups of p38 MAPK inhibitor.

p38 MAPK inhibitors can be divided into 2 groups dependent upon their mode of binding to p38 MAPK; active site inhibitors, such as SB203580 and RJW-67657, bind competitively to the ATP site of the enzyme whereas others bind remotely and interfere with ATP binding indirectly (such as BIRB-796).

1.5.3.2 Genetic mutations

Using point mutagenesis a mutant form of p38α MAPK which is insensitive to ATP binding pocket inactivation, and yet in distinguishable from wild-type p38α MAPK in terms of its activation and kinase activity was developed by Eyers and co-workers (Eyers et al., 1999). In the mutant form the Thr106-His107-Leu108 residues, essential for SB203580 – but not ATP binding, were changed to Met, Pro and Phe, respectively (T106M, H107P, L108F). This mutation results in inherent resistance to SB203580 in the same manner as the p38γ and δ
MAPK isoforms that share the 106M, 107P and 108F consensus (Eyers et al., 1999). In a similar manner, it is also possible to render p38 MAPK resistant to activation by substitution of the Thr\(^{180}\)-Gly\(^{181}\)-Tyr\(^{182}\) (dual phosphorylation motif) with Ala\(^{180}\)-Gly\(^{181}\)-Phe\(^{182}\) (Raingeaud et al., 1995). Since MAPKs may homodimerise on activation or MKK detachment is MAPK phosphorylation dependent, these non-phosphorylatable p38 MAPK mutants act in a dominant negative manner.

Modulating the activation of p38 MAPK is possible through the use of constitutively active MKK3 (Derijard et al., 1995) and MKK6 (Han et al., 1996) by replacing the sites of phosphorylation in these kinases by glutamic acid (Ensen et al., 2000) which mimics the action of phosphorylated residues, thus enabling constitutive and specific activation of p38 MAPK in cells in the absence of a specific stimulus. At present, the majority of pharmacological inhibitors of p38 MAPK are selective for the α and β isoforms of the kinase. It is clear, from the published data, that during prolonged ischaemia the α isoform plays an important role in the progression of dysfunction. Perhaps a more rational approach to inhibit p38 MAPK in a site- and condition-specific manner might be to target the activation of the kinase pathway upstream of p38 MAPK itself, such as TAB1 or MKK3/6. Using a model of coronary artery ligation in a mkk3-targetted mouse line we have recently demonstrated that removing MKK3 does not alter pathological remodelling and progression to ventricular dysfunction after myocardial infarction (Clark et al., 2007). May be this is not surprising considering the multitude of pathways involved in ischaemia and inflammation but it does, perhaps, highlight the potential importance of other pathways which warrant further investigation. Although there are many structural similarities between the four isoforms (α, β, γ and δ), they differ in important respects such as inhibitor sensitivity and substrate specificity. It also appears that the activation of particular p38 MAPK isoforms is cell type and cell stimulus dependent and different upstream pathways may well be targeted differently. Despite continued interest in the p38 MAPK pathway few studies to date have addressed the role of p38 MAPK isoforms other than p38α MAPK during ischaemia. Furthermore, since there are no isoform-specific pharmacological inhibitors of p38 MAPK activity, the contribution of each isoform to the documented physiological effects remains unclear.
1.5.3.3 Transgenic mice

As discussed above, currently there are no isoform-selective pharmacological inhibitors available. Therefore, one powerful tool to investigate the physiological roles of each isoform is to use transgenic mice lacking one or more isoform. Mice lacking p38α MAPK are not viable and die due to early embryonic lethality (Adams et al., 2000; Allen et al., 2000). Recently, a cardiac myocyte restricted p38α MAPK null mouse was described (Otsu et al., 2003). However, the floxing of p38α MAPK was incomplete and the phenotype in response to pressure overload was unexpected with the null mice hypertrophying normally but with enhanced cardiac myocytes apoptosis. The mice lacking other isoforms are viable with no apparent phenotype.

Dr Simon Arthur (Dundee, UK) has reported the generation of mice with targeted disruption of p38β MAPK (p38β KO) (Beardmore et al., 2005a) and p38γ and δ MAPK (p38γ/δ KO) (Sabio et al., 2005) and used them to identify p38 MAPK isoform-dependent events. In each of these mice there was no discernable alteration in the abundance or pattern of activation of the remaining p38 MAPK isoforms as a result of gene targeting.

1.5.4 Involvement of the p38 MAPK isoforms in myocardial dysfunction

p38 MAPKs phosphorylate a number of known transcription factors to alter their transactivating potential influencing gene expression. However, the immediate downstream targets of p38 MAPK that aggravate myocardial injury are still, largely, unknown. One downstream substrate of p38α MAPK, MAPKAPK2 can confer a number of protective effects by phosphorylating HSP27 (Kim et al., 2005). In addition, phosphorylation of MAPKAPK2 can also result in phosphorylation of factors that transactivate cytokine genes such as TNF-α, a cytokine implicated in chronic heart failure. Interestingly, TNF-α also activates p38 MAPK and thus p38 MAPK has been considered as the keystone in an auto-amplifying cytokine cascade by most investigators and an attractive target for anti-inflammatory drug development (Kuma et al., 2005; Lee et al., 2000).

The role of p38 MAPKs in cardiac hypertrophy has been investigated by studies using over-expressed active forms of MMK3 and 6. In these experiments, overexpression of MKK3 in cardiac myocytes leads to an increase in apoptosis (Wang et al., 1998). Using a transgenic mouse model overexpressing MKK3 and MKK6 subjected to a permanent ligation of the LAD, it was found that p38 MAPK signalling can contribute to the loss of contractility and
myocardial stiffness and promotes specific changes in ventricular mass (Liao et al., 2001). This suggests that this pathway *in vivo* is not sufficient to induce hypertrophy (Liao et al., 2001). Similarly, it was also shown that p38 pathway can have an anti-hypertrophic effect by restraining calcineurin-mediated hypertrophy though NFAT transcription factors in mice expressing dominant negative forms of M KK3, M KK6 and p38α MAPK in the heart (Braz et al., 2003).

Glembotski’s laboratory have demonstrated chronic activation of p38 MAPK through overexpression of M KK6 in the heart can result in improved functional recovery from ischaemia and myocardial infarction (Martindale et al., 2005). In cardiac myocytes, activation of p38α MAPK-MAPKAP-2 by M KK6 results in phosphorylation of αB-crystalline, another member of the small heat shock protein family. αB-crystalline provides protection against stress-induced apoptosis (Hoover et al., 2000). This may support the protective consequence of M KK6 overexpression. An interesting explanation between these observations was suggested by a study where p38 MAPK mediated F-actin reorganisation stimulated apoptosis but conversely protected against osmotic-derived necrosis in cardiomyocytes (Okada et al., 2005). A dual role of p38α MAPK was further demonstrated in another independent investigation. In this study, treatment with SB203580 aggravated myocytes necrosis but also revealed a cardioprotective role for the inhibition of p38α MAPK activity as it blocked contractility during reperfusion (Sumida et al., 2005). However, it is very likely that the activation of p38 MAPK isoforms at different time point accounts for the opposing responses observed in these experiments. Indeed, there is evidence to suggest that p38 MAPK isoforms are differentially activated in response to the same stimuli and confer distinct and possibly opposing effects. It is also thought that p38 MAPK isoforms may have potential protective function which may lead to possible adverse effect of prolonged p38 MAPK inhibition in the heart (Saurin et al., 2000; Wang et al., 1998).

There is increasing evidence from pre-clinical investigations that inhibition of p38 MAPK during prolonged ischaemia slows the rate of infarction/death and inhibits the production of inflammatory cytokines such as TNF-α, IL-1 and -8 which aggravate ischaemic injury (Adams et al., 2001). It was first demonstrated as early as 1996 that p38α and β MAPK are activated in response to ischaemia and reperfusion in the heart (Bogoyevitch et al., 1996). Since then, using gene transfer techniques, the α isoform has been implicated in myocyte apoptosis, consistent with the findings that this isoform alone contributes to cell death.
following ischaemia (Martin et al., 2001; Saurin et al., 2000). Inhibition of p38α MAPK activation during prolonged ischaemia, but not β, resulted in an increase in cell viability (Saurin et al., 2000). This strongly supported Wang et al who suggested that p38α MAPK activation in cardiac myocytes is sufficient to cause apoptosis whereas activation of the β isoforms leads to protection and hypertrophy (Figure 1-12) (Wang et al., 1998).

![Figure 1-12 The proposed role of p38 in cardiac hypertrophy and heart failure in response to hemodynamic stress.](image)

The p38α and p38β MAP kinases are placed as components of the molecular response to haemodynamic stress in pathological signalling pathway for cardiac hypertrophy and heart failure (Wang et al., 1998).

This is also consistent with the finding which suggests that the β isoform is activated in response to preconditioning. Furthermore, a pro-apoptotic role for p38α and/or β MAPK during myocardial ischaemia is suggested by protection of cardiac myocytes from ischaemic damage using a selective p38α/β MAPK isoform inhibitor, SB203580 (Wang et al., 1998). Using adenoviral-mediated expression of p38α and β MAPK in rat neonatal cardiomyocytes our group have previously shown that after 2.5 h simulated ischaemia p38α MAPK was activated, whereas p38β MAPK activation was significantly inhibited (Saurin et al., 2000). This perhaps explains why pharmacological inhibition of p38α and β MAPK during
preconditioning blocks protection, (since p38β MAPK is the dominant isoform activated), whilst during lethal ischaemia the same inhibitor, at the same concentrations, cause protection (since the p38α MAPK is perhaps the dominant isoform activated) (Marais et al., 2001; Nagarkatti and afi, 1998; Sanada et al., 2001).

The protective role of p38β MAPK has also been investigated in a recent study by Kim and co-workers who have shown that activation of p38β MAPK by carbon monoxide (CO) promotes the nuclear translocation of heat shock factor-1 (HSF-1), which regulates the expression of cytoprotective HSP70 in cells and tissues (Kim et al., 2005). HSF-1 can also serve as a negative regulator of pro-inflammatory genes, including IL-1β, and TNF-α (Xie et al., 2002). These observations are supported by CO-mediated protection of endothelium against endotoxic shock being abolished in p38β knockout mice (Kim et al., 2005). It has also been reported that CO activates p38 MAPK via M KK3, M KK6 signalling pathways as well as PI3K-Akt (Kim et al., 2005; Otterbein et al., 2003; Zhang et al., 2005). The PI3K-Akt signalling pathway shown to be implicated in cardioprotection (Tong et al., 2000) and is a pro-survival pathway involved in oestrogen mediated cardioprotection. This was shown to be through suppression of p38α MAPK and activation of p38β MAPK (Kim et al., 2005; Wang et al., 2005).

There is little information in the literature regarding the roles of either the γ and δ isoform of p38 MAPK in the myocardium during ischaemia. Conserved cardiac expression of p38γ MAPK amongst several different species suggests that this isoform may play an important role in the heart and therefore is unlikely to be functionally redundant (Court NW et al., 2002).

p38γ MAPK is localised in the cytoplasm of the cardiac myocyte and is reported to have a punctate distribution (Court NW et al., 2002), and p38δ MAPK mRNA is broadly expressed in a wide variety of mouse and human tissues including the heart (Hu et al., 1999). The C-terminal tail of p38γ MAPK allows its interaction with PDZ domains of its substrate protein(s) and association with α1-syntrophin and SAP90/PSD95 in skeletal muscle and neuronal synapses respectively (Hasegawa and Cahill, 2004; Sabio et al., 2004).

It has been shown that p38γ MAPK catalysed phosphorylation of hDlg (the mammalian homologue of the Drosophila tumour suppressor Dlg) triggers its dissociation from the cytoskeleton, indicating that, this may regulate the integrity of intercellular-junction
complexes, cell shape, volume and cell polarity in response to many kinds of external stimuli. Parker et al identified a novel p38δ MAPK substrate as stathmin, a cytoplasmic protein that was previously reported to be a substrate of several intracellular signalling kinases which have been linked to regulation of microtubule (MT) dynamics in a phosphorylation dependent manner (Parker et al., 1998). This may suggest that a common theme in p38 MAPK pathway activation may be the re-organisation of the cytoskeleton framework to enhance cell survival in times of stress such as ischaemia (Parker et al., 1998). Moreover, both p38γ and p38δ MAPK phosphorylate the MT-associated protein Tau in neurons in vivo. Hyper-phosphorylated Tau is the major component of the paired helical filaments, which constitute one of the main neuropathological hallmarks of many neurodegenerative disorders (Sabio et al., 2005).

Nuclear staining of p38γ MAPK in cardiac myocytes overexpressing p38γ MAPK has shown that this isoform regulates gene expression by stimulating phosphorylation of activating transcription factor (ATF)-2 and myocytes enhancer factor (MEF) - 2A (Marinissen et al., 2001), which are up-regulated during myocardial hypertrophy (Marinissen et al., 2001).

Recently, it was shown that constitutively active PKN, a Rho regulated protein kinase, potently stimulates p38γ MAPK, possibly by acting though MAP4K (Marinissen et al., 2001). In yeast, the Rho-Pkc1 signalling pathway homologous to the mammalian RhoA-PKN pathway is activated by the osmotic stress, thereby regulating the downstream MAPK pathway, consisting of Bck1-mkk1/2-Mpk1 (Marinissen et al., 2001). Therefore the RhoA-PKN-X-MKK3/6-p38γ MAPK pathway may be a mammalian homologue of the yeast osmosensing MAPK pathway. This may potentially play an important role during hyperosmotic conditions such as ischaemia.

Although there is, only circumstantial evidence to support a role for p38γ and δ MAPK isoforms in the heart this is likely to be an emerging area of research. In the absence of isoform-selective pharmacological inhibitors this is, to some extent, aided by the availability of a number of p38 MAPK isoform-targeted mouse lines (Beardmore et al., 2005b;Sabio et al., 2005) and spontaneously active mutants (Askari et al., 2007).
Inhibition of one or more harmful isoform(s) using current available pharmacological inhibitors may result in a positive outcome. However, the non-selectivity of the same inhibitors, under certain conditions, can also inhibit the activation of the protective isoform(s). This will consequently result in the manifestation of detrimental effects. In the third scenario, a balanced inhibition of isoforms with opposing effects can result in neutral effect.

**1.6 Aims and scope of the thesis**

From the existing evidence, it appears that p38 α and β MAPK are differentially regulated during myocardial stresses and that the consequence of activation of each isoform may differ by cell type. This highlights the likelihood that different members within a single kinase
family can play distinct roles in the heart during ischaemia. Despite continued interest in the p38 MAPK pathway few studies to date have addressed the role of the p38 MAPK isoforms other than p38α MAPK during acute (ischaemia and ischaemia-reperfusion injury) and chronic myocardial stresses (hypertrophy and remodelling following MI). Furthermore, since there are no isoform-specific pharmacological inhibitors of p38 MAPK activity, the contribution of each isoform remains unclear.

Understanding the physiological roles of each p38 MAPK isoform and identifying their mechanisms of activation and potential substrates are important avenues that would lead to pharmacological inhibitors with greater circumstances selectivity thereby avoiding the potential pitfalls of chronic systematic inhibition (Figure 1-13).

The aims and objectives of this thesis are to characterise the individual expression of endogenous p38 MAPK isoforms in murine myocardium and delineate their functional contributions in response to various myocardial stresses. Using transgenic mice lacking p38β knockout (p38β KO) and p38γ/δ (p38γ/δKO) isoforms in ex vivo and in vivo models, we aimed to address the following questions:

- Is there any difference in the functional responses between isoforms in response to ischaemia and reperfusion?
- Does the p38β MAPK isoform play any role in preconditioning?
- Is there a specific stress that can target and activate p38γ and δ MAPK isoforms and if so what are the downstream substrates?
- Do p38γ and δ MAPK isoforms play any major role in ventricular remodelling in chronic myocardial stresses such as cardiac hypertrophy and remodelling post infarction in vivo?
2 MATERIALS AND METHODS

Details of methodology relevant to more than one chapter are given here. Details of specific methodology and experimental groups are provided within the specific methods of each chapter.

2.1 Laboratory Reagents

Unless otherwise stated, all analytical-grade laboratory reagents were purchased from Sigma Chemical Company (Poole, UK).

2.2 Animal housing and handling & breeding strategies

Out-bred adult male c57/b16 (20-25g) and colonies of p38 wild type (WT), p38β knock out (KO) and p38γδ knockout (KO) mice were maintained in the Biological Services Animal Care facility of King’s College London. Between 4 and 6 mice were housed in each cage allowing free access to food and water. The containment room was maintained at 25°C with a 12 h light/dark cycle. All studies were performed in accordance with the Home Office Guidelines on the Operation of the Animal Scientific Procedures Act (1986) published by Her Majesty’s Stationary Office, London.

Up to 6 breeding pairs were set up for each mouse colony depending upon the requirement for litters. Transgenic mice were derived from breeding homozygous pairs of either p38β KO or p38γδ KO mice. The knock-out mice were generated by targeting embryonic stem (ES) cells by using targeting vectors designed for the gene of interest (Beardmore et al., 2005b; Sabio et al., 2005). For generating p38β KO, targeting vectors were constructed to generate a deletion of exons 2 to 7 of p38β MAPK. For p38γδ KO, targeting vectors designed to delete exon 3 of p38γ MAPK gene and from second half of exon 4 to middle of exon 7 of the p38δ MAPK gene. The deleted exons were replaced with a neomycin-resistant gene (Neo). ES cells were electroporated, cultured, selected and screened by PCR and Southern hybridisation (Beardmore et al., 2005b; Sabio et al., 2005). Targeted ES cells were injected into C56B1/6J –wild type blastocysts and the resulting chimeric males were used to establish a colony (Figure 2-1).
2.3 Isolation of adult mouse ventricular myocytes (AMVM)

Adult mouse ventricular myocytes (AMVM) were isolated from mouse hearts by a collagenase-based enzymatic digestion technique. Male c57/bl6 mice were anaesthetised with intraperitoneal (i.p.) sodium pentobarbital (Sagatal; Rohon Merieux) injection (50 mg/kg) and heparin (150 I.U.; Leo Laboratories Ltd). The abdominal cavity was opened just below the sternum and the heart exposed by removing the diaphragm and cutting laterally either side of the rib cage. The chest wall was reflected and the heart gently lifted and rapidly excised with the aorta intact and immersed in cold Tyrode solution at 4°C (see Table 2-1 for all solution compositions). After removal of excess thymus and fatty tissue, the aorta was
tied to a 23G blunt and grooved stainless cannula using 4/0 surgical silk (Johnson & Johnson).

**Table 2-1 Composition of solutions used in AMVM isolation. All chemicals were of analytical grade or above and obtained from VWR International unless otherwise stated.**

<table>
<thead>
<tr>
<th></th>
<th>Tyrode solution</th>
<th>Calcium-free</th>
<th>Enzyme solution</th>
<th>Stabilising buffer</th>
<th>Storage buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mM)</td>
<td>(mM)</td>
<td>(mM)</td>
<td>(mM)</td>
<td>(mM)</td>
</tr>
<tr>
<td>NaCl</td>
<td>130</td>
<td>130</td>
<td>130</td>
<td>130</td>
<td>130</td>
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<tr>
<td>KCl</td>
<td>5.4</td>
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</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.75</td>
<td>-</td>
<td>100</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>MgCl$_2$</td>
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<td>1.4</td>
<td>1.4</td>
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<td>1.4</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>HEPES</td>
<td>4.2</td>
<td>4.2</td>
<td>4.2</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Taurine</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>EGTA</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Collagenase</td>
<td>-</td>
<td>-</td>
<td>1 mg/mL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Creatine</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>BSA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1%</td>
<td>-</td>
</tr>
<tr>
<td>pH at 37°C</td>
<td>7.3</td>
<td>7.3</td>
<td>7.3</td>
<td>7.3</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Hearts were perfused in the Langendorff mode at 37°C for four sequential periods as follows: with i) Tyrode solution 1 for 4 min, ii) calcium free solution for 3 min, iii) enzyme solution 1mg/mL collagenase (Worthington Biochemical Corporation Type II) for 8 min. The coronary flow rate was maintained at 3.5 mL/min using a peristaltic pump (Gilson Minipuls 3, France). All solutions were maintained at 37 °C and gassed with 100% O$_2$.

After the perfusion protocol, the heart was removed from the cannula, and the arterial and non-ventricular part carefully removed. The ventricles were then chopped into smaller fragments into enzyme solution using sterile scissors. The tissue fragments were mixed by
pipetting and gently bubbled with 100% O₂ for 5 min to facilitate cell dispersion before being filtered through a nylon mesh (200 µm). The cells were allowed to settle for 10 min before being washed with stabilising buffer (Table 2-1) containing 0.5 mM CaCl₂ and 1% bovine serum albumin (BSA). Next, the supernatant was removed and the pellets were immediately re-suspended and incubated for a further 20 min with storage buffer (Table 2-1) containing 1 mM CaCl₂. The cell suspension (>70 % rod shape as estimated by light microscopy) was maintained in culture media at room temperature for 20 min before plated onto laminated culture wells as described below.

2.3.1 Cell viability

Viable AMVM were recognized as rod-shaped rectangular cells. Healthy myocytes have defined edges and square ends. Only isolations with viable cells in excess of 70% were used in experiments.

2.3.2 Culture of AMVM for Confocal microscopy

Laminin (1 mL of a 15 µg/mL solution in de-ionised water) was added to individual culture dishes (Nunc A/S, Germany), which were stored at room temperature for 1 h. The laminin was then aspirated off and wells washed with sterile M199 medium (contains Hanks salts, 25 mM HEPES, L-glutamic acid and L-amino acids; Invitrogen Life Technologies). The cell suspension in storage buffer (Table 2-1) was allowed to settle for 20 min at room temperature. The cell pellet was then re-suspended in sterile M199 medium (to wash the cells), before being allowed to settle again at room temperature and re-suspended in sterile M199 as above. To each well of the laminated dish, 2 mL of cell suspension was added and the dishes subsequently maintained in a 5 % CO₂ incubator at 37° C. After 1 h, the medium was aspirated off, leaving only adherent cells. The cells were then re-suspended in fresh culture medium.

2.3.3 Preparation of AMVM for confocal microscopy (fixation, permeabilisation and blocking)

After 1 h the culture media was removed and cells were washed with phosphate buffer saline (PBS) and fixed by 4% w/v paraformaldehyde solution (4 g powdered paraformaldehyde was dissolved in 100 mL of sterile PBS through constant stirring at 65 °C for 2 h) in PBS for 15 min. The paraformaldehyde was then aspirated off and the wells were washed three times
with sterile PBS (3× 1 mL) for 5 min. The cells were next permeabilised with 0.2% v/v Triton X-100 in PBS for 5 min. This was followed by three 5 min washes with sterile PBS as before. The cells were then blocked with 100 µL/dish of 5% normal goat serum (Calbiochem).

2.4 Immunocytochemistry

p38 MAPK antibodies total (pan-isoform) p38, monoclonal mouse p38β (Zymed), p38γ (R&D Systems) and p38δ MAPK (R&D Systems) were diluted 1:50 plus either a primary mouse α actinin (diluted 1:500 for polyclonal rabbit Total p38 antibody) or with a primary rabbit-α actinin (diluted 1:200 monoclonal mouse p38β, p38γ and p38δ antibodies) in buffer containing Trizma 20 mM, NaCl 155 mM, EGTA 2 mM, MgCl₂ 2 mM, pH 7.5 and 1% w/v BSA. The dishes were placed into a humid chamber and incubated overnight at 4°C. The following day, cells were washed in PBS as before and incubated over night at 4°C with secondary antibodies; mouse Cy3a diluted 1:500 (ML Jackson Stratech Scientific), rabbit Cy2a diluted 1:100 (ML Jackson Stratech Scientific), and 4’,6- Diamide-2- phenyindole Dilactate (DAPI) diluted 1:100. Cells were then washed as before and mounted with a droplet of Mounting Medium (Tris 0.1M, 35 mL Glycerol and 2.5g n-propylgallate and covered with cover slips (VWR 30 mm diameter, Science Warehouse). The edge of each dish was removed with a hot wire and cover-slips sealed with nail varnish. Each dish was then glued onto a microscope slide and kept at 4°C for 24 h.

2.5 Confocal Microscopy

In laser scanning confocal microscopy, a point light source (laser) is projected through an objective onto a specific object plane of a fluorescent specimen. To generate an image of the whole object plane, the specimen is scanned point- by- point and line-by-line through the use of a computer-controlled x-y axis light deflection system. The resulting sequence of florescent light points are captured by the same objective lens and focused onto a photomultiplier (converting the optical signal into electrical signals) via a dichroic mirror (Figure 2-2). Any reflected excitation light captured by the objective is deflected away by this dichroic mirror, ensuring that only emitted light can reach the photomultiplier. The electrical output from the photomultiplier is relayed to the computer and is built into the image that is displayed on the monitor.
Figure 2-2 Diagram illustrating the optics of a confocal microscope.

Emitted light from the laser passes through the illuminating aperture and is reflected onto the objective lens by the dichroic mirror. Light omitted by the sample is collected by the objective lenses and passes through the dichroic mirror however, only the light from the focused plane is able to pass through the confocal aperture and out-of-focus is filtered. Before reaching the photomultiplier light passes through a confocal aperture filtering out light emitted from outside the object plane, greatly reducing out-of-focus information (from both above and below the objective plane) and ensuring an in-focus image from a specific plane. Defined focussing along the z-axis can be employed to image any objective plane, and the ability of the computer software to stack 2-dimensional images (of 1μm optical slices) allows a 3-dimensional image of a specimen to be constructed. The image was created by Dr James Clark.
2.6 Langendorff perfusion of isolated mouse hearts

2.6.1 Background

The first preparation capable of maintaining the isolated heart was described by Oscar Langendorff in 1897. In Langendorff mode perfusion the aorta is tied to a cannula through which perfusate enters the aortic root. As long as the aortic valves remain intact and the pressure inside the left ventricle is less that in the aortic root, the perfusate is forced through the right and left coronary arteries situated in the coronary sinuses. After passing through the coronary arteries and myocardial capillaries, perfusate drains into the right atrium and ventricle and exits through the pulmonary artery. Providing the perfusate contains essential substrates and the flow is sufficiently high to maintain supply and remove metabolites, the heart can remain viable and continue to contract for many hours.

2.6.2 Isolation and cannulation of the heart

Male animals were anaesthetised and anti-coagulated with i.p. injection of pentobarbital (300 mg/kg) and heparin (150 units) respectively. Hearts rapidly excised from the chest and placed in ice-cold Krebs buffer (118.5 mM NaCl, 25 mM NaHCO3, 4.7 mM KCl, MgSO4.7H2O, 1.2 mM KH2PO4, 11 mM glucose mixed well, bubbled with 95% O2 + 5% CO2 for 5 min then add 1.4 mM CaCl2.2H2O). After removal of thymus and fatty tissue, the aorta was cannulated with a 23G blunt and grooved stainless steel needle. The heart was retrogradely perfused with Krebs buffer at a constant pressure of 80 mmHg (108 cm H2O). The Krebs buffer was pre-filtered using a 0.8 µm micro-filter (Whatman, UK) and constantly gassed with 95% O2/ 5% CO2.

2.6.3 Perfusion of the heart, monitoring and maintenance

Following isolation, the heart temperature was maintained at 37.0°C by immersing the heart and cannula in Krebs buffer kept at 37.0°C in a water-jacket chamber. A fluid filled compliant balloon was inserted into the left ventricle to measure left ventricular developed pressure (LVDP), end systolic (ES) and end diastolic (ED) pressure. The balloon was attached to a pressure transducer, which was coupled to a 4S Powerlab (AD Instruments, UK). The balloon was inserted gradually inflated until the end-diastolic pressure reached between 1 and 5 mmHg. Hearts were then paced at 580 bpm by a silver wire (Advent, UK)
placed through the right ventricular wall into the right ventricular wall apex. The wire and aortic cannula were attached to an SD9 stimulator (Grass Instruments, USA) delivering square wave pulses of 5 ms duration at 1 V amplitude. The coronary flow was directly measured as the overflow rate of effluent from the lower reservoir (mL/min) (Figure 2-3).

**Figure 2-3 Langendorff Isolated Heart Apparatus.**

The heart is perfused with Krebs buffer at 37°C bubbled with 95% O₂, 5% CO₂ at constant pressure of 80 mmHg (determined by the adjusted height of the upper buffer chamber). After cannulation, the hearts were submersed in the lower Krebs’ reservoir to maintain the temperature at 37°C. A balloon was inserted into the left ventricle connected to a pressure transducer to measure contractile function. The output from the transducer is amplified and recorded with Powerlab (Ad Instruments) Chart Software present on a computer.
2.6.4 Exclusion criteria

The following hearts were excluded: Hearts that took longer than 3 min to cannulate from the first skin incision, hearts with left ventricular developed pressure less than 60 mmHg after the stabilisation period or with a coronary flow outside the range of 2.5-4.5 mL/min.

2.6.5 Tetrazolium staining for Infarct Size measurement

At the end of the perfusion experiment, hearts were perfused for 2 min with 5 mL 1% (w/v) triphenyl-tetrazolium chloride (TTC) in phosphate buffer (Na₂HPO₄) 45.1 mM, NaH₂PO₄ 3.3 mM, pH 7.8). Hearts were then removed from the cannula and placed in an identical TTC solution at 37°C for 20 min. The heart was mounted in 5% agarose (w/v in phosphate buffered saline) with the apex uppermost. The agarose-embedded hearts were then sectioned from apex to base into 700 µm thick slices using a Vibratome (Agar Scientific). The sections were placed in 6-well plate containing 1 mL of PBS according to order that they were sliced and kept at 4°C until scanning. For scanning, the heart slices were pressed, apical aspect uppermost) between Perspex plates. Dimensions of the LV, such as free wall and septal wall, were calculated using a computer-aided analysis (Sigma Scan, SPSS) following high-resolution digital capture.

2.6.6 Harvesting heart tissue for protein extraction

At the end of experiments hearts were removed from the cannula and snap frozen in liquid nitrogen using a set of pre-cooled freeze clamps. Frozen heart samples were weighed and homogenised on ice in lysis buffer (1000 µL/1g tissue) containing 20 mM Hepes, 150 mM NaCl, 50 mM NaF, 1 mM DTT, 1 mM EGTA, 1 mM Na₃O₄ and 0.5% v/v Triton X-100 and 1 “complete” protease inhibitor tablet per 50 mL (11 6974900, Roche) using a glass homogeniser. Following homogenization, hearts were placed in 2xsample loading buffer containing 10% (v/v) β-mercaptoethanol (in 1:1 ratio) and boiled for 5-10 min. 1-2 µL of bromophenol blue dye was then added and the samples centrifuged for 2 min at 13,000 rpm in a micro centrifuge prior to loading on sodium dodecyl sulphate (SDS) polyacrylamide gels.
2.7  Polymerase Chain Reaction (PCR)

2.7.1  RNA extraction and reverse transcription of p38 MAPK isoforms

Heart, brain, skeletal muscle and kidneys were removed and snap frozen in liquid nitrogen. mRNA was extracted from frozen tissues. Total RNA purification from fibrous tissues, such as skeletal muscle, heart, and aorta tissue, can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. To isolate total RNA from heart tissue, we used a RNase Fibrous tissue (QIAGEN, catalogue number 75742) Kit. The technology applied in this kit simplifies total RNA isolation by combining the stringency of guanidine-isothiocyanate lysis with the speed and purity of silica-membrane purification. The protocol integrates proteinase K and RNase-free DNase digestion steps during RNA isolation to break down proteins and genomic DNA. Tissue samples were first lysed (30 mg into 300 μL of lysis buffer containing β-mercaptoethanol) and then diluted before being treated with proteinase K. Debris were pelleted by centrifugation (8,000 rpm for 5 min), and the supernatant was removed by pipetting into a clean RNase-free tube. The supernatant was mixed with ethanol and then centrifuged through an RNeasy spin column, where RNA binds to the silica membrane. DNA that might have been co-purified with the RNA were removed by further DNase treatment on the silica membrane. DNase and any contaminants were efficiently washed away, and total RNA was eluted in RNase-free water. To elute total RNA, 2 mL of RNase-free water was added directly onto the silica membrane. The samples were then centrifuged at 8,000 rpm for 5 min at 25°C.

RT reactions were performed by mixing 500 ng of mRNA with oligo-dT (500 μg/mL, Promega), 10 mM dNTPs (dATP, dCTP, dGTP, dTTP, Promega) and DNase free water (Promega) to make a 20 μL reaction volume in a nuclease free microcentrifuge tube (Table 2-2). Samples were incubated for 65°C for 5 min followed by a quick chill on ice and the contents of the tubes were collected at the bottom of the tube by brief centrifugation. The samples were then mixed with 5x first strand buffer (Invitrogen) (containing 250 mM Tris-HCL, pH 8.3 at room temperature; 375 mM KCl; 15 mM MgCl2) and 0.1 M DTT (Invitrogen) and gently mixed by pipetting and incubated at 42°C for 2 min. The first strand of cDNA was synthesised by adding 200 units of SuperScript™ II (Invitrogen) to the samples and incubating the mixtures at 42°C for 50 min. The reactions were terminated by heating the samples at 70°C for 15 min.
Table 2-2 Reverse transcription mixture

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount per tube (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo(dT)15 Primer</td>
<td>1</td>
</tr>
<tr>
<td>RNA</td>
<td>1-2 (depending on the tissue)</td>
</tr>
<tr>
<td>dNTP’s mix (10 mM)</td>
<td>1</td>
</tr>
<tr>
<td>DI Water</td>
<td>Make up to 20 with the above</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>2</td>
</tr>
<tr>
<td>5x first strand Buffer</td>
<td>4</td>
</tr>
<tr>
<td>Superscript 2</td>
<td>1</td>
</tr>
</tbody>
</table>

2.7.2 PCR Primers

Gene-specific primers for PCR were synthesised (MWG) according to the sequence recently designed and published by Natale et al. (2004) for p38α, β, γ and δ MAPK on available mouse full length and EST nucleotide sequence in Gene Bank nucleotide database (Natale et al., 2004). PCR primers were designed to regions of nucleotide conserved across the species. The primer sets designed are reported in Table 2-3. The primer sets designed for GAPDH gene were used to assess the efficiency of the RNA extraction. Positive (tissue cDNA; brain, skeletal muscle and kidney for p38β, γ and δ MAPK) and negative control (no cDNA templates) samples were included for each primer set in each experiment (Table 2-3).
Table 2-3 Nucleotide sequence for polymerase chain reaction.
The primers used for PCR amplification of p38α, β, γ, δ MAPK and GAPDH (shown in the 5'-3' direction) are shown below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38α</td>
<td>5’</td>
<td>AGGCCATGGTGCTGTGTGT</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>AGTAGCTGGAGGAGGAGGAG</td>
<td></td>
</tr>
<tr>
<td>P38β</td>
<td>5’</td>
<td>GGCTGCATCATGGCTGAACCT</td>
<td>544</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>TGAGGGGCGCTTTCTTGAGGA</td>
<td></td>
</tr>
<tr>
<td>p38γ</td>
<td>5’</td>
<td>TCTCAGCTTCAAGCCTCCTA</td>
<td>402</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>TCCTAGCTCTGCTCAGCTCT</td>
<td></td>
</tr>
<tr>
<td>p38δ</td>
<td>5’</td>
<td>GTCTTTGGTGTCATCATGG</td>
<td>630</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>GGATCTCTTGAGTGAGGT</td>
<td></td>
</tr>
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<td>GAPDH</td>
<td>5’</td>
<td>GGGGAGCCAAAGGGTCATCATCT</td>
<td>452</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>GACGCCCTGCCTACCACCTTCTT</td>
<td></td>
</tr>
</tbody>
</table>

2.7.3 PCR Amplification

Polymerase chain reaction (PCR) was conducted as previously described (Natale et al., 2004), using 1 μg cDNA in a 50 μL PCR reaction (Table 2-4) consisting of 0.2U of AmpliTaq Gold (Applied Biosystems), 1X PCR Gold buffer (containing 150 mM Tris-HCL, pH 8.0, 500 mM KCl, Applied Biosystems), 2.0 mM MgCl₂, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP, Promega), and 1.0 μM of each PCR primer using a thermal cycler (Eppendorf). AmpliTaq Gold was activated by an initial incubation at 95°C for 10 min followed by 40 cycles of amplification; 95°C for 30 s, 56°C for 60 s, 72 °C for 30 s, with final extension of 10 min at 72°C.
Table 2-4 PCR reaction mixture.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Amounts per tube (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR Gold Buffer</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂ (25 mM stock)</td>
<td>4</td>
</tr>
<tr>
<td>dNTP’s mix (10mM each)</td>
<td>1</td>
</tr>
<tr>
<td>Primer mixture</td>
<td>5</td>
</tr>
<tr>
<td>c-DNA</td>
<td>3-4</td>
</tr>
<tr>
<td>AmpliTaq Gold</td>
<td>0.25</td>
</tr>
<tr>
<td>DI H₂O</td>
<td>Make up to 50μL in total</td>
</tr>
</tbody>
</table>

2.7.4 Agarose gel electrophoresis

25 μl of PCR products were resolved on 2.0% (w/v) agarose gels, stained with 0.5 μg/mL ethidium bromide. The gel was then visualized using a UV dual-intensity transilluminator (UVP, Genetic Research Instrumentation Ltd., Essex, UK) and photographed with a Polaroid DS34 instant camera with a EP H-7 hood (Polaroid UK Ltd., Hertfordshire, UK) PCR reactions were repeated a minimum of three times using cDNA prepared from WT, p38β KO and p38γδ KO hearts.

2.8 Protein detection and quantification by Western blotting

2.8.1 Preparation of SDS- gels

Protein samples were subjected to SDS-PAGE using the Mini Protean II gel electrophoresis system (Bio-Rad). Polyacrylamide gels were poured between glass plates separated by 1 mm spacers. The gels were formed of a lower “resolving gel” and an upper “stacking gel” into which wells are set to allow sample loading. The gel matrix consisted of the resolving gel (containing 12% v/v acrylamide (Protogel; National Diagnostics) and a 2 cm layer of stacking gel (containing 3.5 % v/v acrylamide) the constituents of which are summarised in
79. First, the resolving gel was prepared and the polymerisation induced by addition of ammonium persulphate (APS) and NNNN-tetramethylethlenediamine (TEMED). Water saturated butanol was overlaid whilst the gel polymerised to prevent drying. Once the lower gel had polymerised, the butanol was washed off and the upper stacking gel poured. The gel was polymerised by addition of APS and TEMED and poured on top of the resolving gel. Toothed combs were inserted and removed after polymerisation to leave rectangular indentations to load samples.

2.8.2 Dimensional separation in SDS-gels

Electrophoresis was performed in reservoir buffer (Tris-base, 0.025 M; glycine, 0.192 M; SDS, 0.1 % w/v) at 90 V (~15 min) for the protein to migrate through the stacking gel, and at 120 V for protein migration through the resolving gel layer. Molecular weight markers (Rainbow™ coloured protein molecular weight markers, Bio-Rad), containing proteins ranging from 8 to 180 kDa in molecular weight, were run in a parallel to the samples on every gel, to aid determination of transfer efficiency and to estimate the molecular weight of the protein of interest.

Table 2-5 Composition of the stacking gel and three resolving gels.

<table>
<thead>
<tr>
<th></th>
<th>Stacking Gel</th>
<th>Resolving Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10%</td>
</tr>
<tr>
<td>Buffer</td>
<td>125 mM Tris</td>
<td>0.375 mM Tris</td>
</tr>
<tr>
<td>0.1% SDS pH 6.8</td>
<td></td>
<td>0.1% SDS pH 8.7</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>3.5%</td>
<td>10%</td>
</tr>
<tr>
<td>Ammonium Persulphate (w/v)</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>TEMED (v/v)</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

2.8.3 Semi-dry electrophoretic transfer

Semi-dry electrophoretic transfer was performed using a semi-dry transfer unit (Bio-Rad, UK). Novablot electrode paper (Amersham Biosciences, UK) was cut to the size of the resolving gel (85 mm x 55 mm, 2 sets of 6 per gel) and soaked in transfer buffer (Tris-base,
25 mM; glycine, 150 mM methanol, 20% v/v adjusted). A current of 0.25 mA/gel was applied for 30 min/gel allow transfer of negatively charged proteins onto polyvinylidene fluoride (PVDF) membrane.

2.8.4 Western blotting

Following transfer, membranes were blocked against non-specific binding of antibodies by incubating in blocking solution (5% (w/v) dried skimmed milk powder, 0.1% (v/v) Tween-20, in TBS) for 1 h with gentle shaking at room temperature. Following a brief wash in TBS, containing 0.1% (v/v) Tween-20 and 0.1% (w/v) milk powder (TBST), membranes were incubated with the relevant primary antibodies. For specific conditions of primary antibody incubations see the specific methods category for the relevant chapter. Following antibody binding, membranes were washed in TBST for 10 min, three times. The membranes were then incubated with horseradish peroxidase conjugated secondary antibody. For specific conditions of secondary antibody incubation see the specific methods category of the relevant chapter. Following four further washes for 10 min in TBST, membranes were developed using an enhanced chemiluminescence technique (Amersham, UK).

2.9 Immunoprecipitation

Frozen heart samples were weighed and homogenised on ice in lysis buffer (1mL/100mg tissue) containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 100 mM NaF, 10 nM calyculin A, 1 mM DTT, 0.5% v/v Triton X-100 and 1 “complete” tablet of cocktail inhibitor (one tablet per 50 mL of lysis buffer). Crude homogenate was boiled at 100°C for 5 min to denature proteins in order to optimise the binding between p38 MAPK and the pull down antibody. The samples were then spun at 14,000 rpm for 15 min at 4°C and the supernatant kept and subjected to immunoprecipitation.

Crude homogenate (200 μL) was mixed with 5 μL of the relevant monoclonal pull down antibody plus 50 μL of Protein G-Sepharose beads (Amersham, UK) and incubated overnight at 4°C. Negative control were included which contained the crude homogenate plus the beads and no antibody or lysis buffer plus the pull down antibody and the protein beads. The next day the samples were spun at 14,000 rpm at 4°C and washed three times with 500 μL of lysis buffer containing 0.1% SDS w/v and 0.5% v/v Triton X-100. After the
final wash the pellet was solubilised in 30 µL of SDS Page sample buffer and heated at 95°C for 5 min and analyzed by western blotting as described as above.

2.10 Enzymatic Linked Immuno-Sorbent Assay (ELISA) for Phospho-p38γ MAPK

2.10.1 Principal of the Assay

A sandwich ELISA kit (R&D Systems, UK) was used to measure phospho p38γ MAPK (T<sup>183</sup>/Y<sup>185</sup>) in heart tissues treated with 10 min of ischaemia. An immobilised capture antibody specific for p38γ MAPK binds both phosphorylated and unphosphorylated p38γ MAPK. After washing away unbound material, a biotinylated detection (phospho-specific-pan) antibody is used to detect the dually phosphorylated p38 MAPK at T<sup>183</sup> and Y<sup>185</sup>, utilizing a standard Streptavidin-HRP format.

2.10.2 Sample collection

Frozen heart samples were homogenised in lysis buffer (1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, 10 µg/mL Leupeptin, 10 µg/mL Pepstatin, 100 µM PMSF, 3 µg/mL aprotinin, 2.5 mM sodium pyrophosphate, 1 mM activated sodium orthovanadate in PBS, pH 7.4)

2.10.3 Generation of a standard curve for phospho-p38γ MAPK (T183/Y185)

Recombinant human phospho-p38γ MAPK (reconstituted in 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea in PBS, pH 7.4) was immediately diluted (1:6) before use. Further serial dilutions (1:2) were made to generate a seven point standard curve.

A 96- well microplate was coated over night with 100 µL of capture antibody (2.0 µg/mL in carrier free PBS) at room temperature. The next day the wells were aspirated and washed three times with 400 µL of wash buffer (0.05% v/v Tween -20 in PBS, pH 7.4). After each wash, any remaining wash buffer was aspirated completely by inverting the plate and blotting it against clean paper towels. After the final wash, the plate was incubated for 2 h at room temperature with 100 µL of sample or standard (in Diluent #3 containing 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 1 M urea in PBS, pH 7.4). The plate was washed three times as before and further incubated for two h with 100 µL of the detection antibody...
(4.0 µg/mL in 1% BSA in PBS, pH 7.2). The aspiration/wash step was repeated as before and the plate was incubated with 100 µL of the streptavidin-HRP (R&D Systems, UK) for 20 min at room temperature wrapped in aluminium foil. This was followed by addition of 50 µL of stop solution to each well and gently tapping the plate to ensure thorough mixing. The optical density of each well was determined immediately using a microplate reader set to 450 nm.

2.11 Surgical Models

2.11.1 Hypertrophy induced model using osmotic pump implant

This model involved subcutaneous implantation of an Alzet osmotic pump containing hypertrophy inducing agent, DL-isoproterenol hydrochloride (ISO) at a concentration of 30mg/kg/day.

2.11.1.1 Alzet osmotic pumps, principle of drug delivery

Alzet pumps operate by an osmotic pressure difference between a compartment within the pump, called the salt sleeve, and the tissue environment in which the pump is implanted. The high osmolarity of the salt sleeve causes water to flux into the pump through a semi permeable membrane which forms the outer surface of the pump. As the water enters the salt sleeve, it compresses the flexible reservoir, displacing the test solution from the pump at a controlled, predetermined rate. As the compressed reservoir cannot be refilled, the pumps are designed for single use only. The rate of delivery of the pump is flow moderated thus, the size and weight of the compound or its physical and chemical properties, has no bearing on its rate of delivery.

In all of the hypertrophy studies, we used 14 day Micro-osmotic pumps (model 1002) (Figure 2-4) with a pumping rate of 0.25 µL/h (± 0.05 µL/h) and reservoir volume of 90 µL (± 10 µL).

2.11.1.2 Filling Micro-osmotic pumps

Hypertrophy inducing agent isoproterenol (ISO) 30mg/kg/day was prepared in saline (0.9 % NaCl w/v). The pumps were filled either ISO or vehicle (saline) using a 1.0 mL syringe and the blunt-tipped 27 gauge filling tube provided by the manufacturer. The syringe was filled
with the fluid and the filling tube was attached. Care was taken to prevent any air bubbles, as any air bubbles trapped in the pump reservoir could result in unpredicted pumping rate fluctuations. With the flow moderator removed, the pump was held in an upright position and the filling tube inserted though the opening at the top of the pump until it reached the bottom of the pump reservoir. The pump was held in an upright position and filled slowly. The filling was stopped when the solution appeared at the outlet and the tube gently removed. The excess solution was wiped off and the flow moderator inserted so that the white flange was resting at the top of the pump.

2.11.1.3 Implantation of the Micro Osmotic pump in vivo

Animals were anaesthetised with 4% isoflurane (in 100% oxygen by inhalation) and the skin was shaved between the scapulae. A small incision was made and a pocket was formed by separating the subcutaneous connective tissues apart using blunt dissection. The pumps containing either ISO or vehicle were inserted into the pocket with the flow moderator pointing away from the incision. The skin was closed using a 5/0 silk suture. The mice recovered and kept alive for two weeks.
Figure 2-4 A 14 days osmotic pump (Alzet, Model 1002).
Total volume 0.5 mL and weight (empty) 0.4 g. An empty reservoir is filled with the drug. Due to the presence of a high concentration of salts surrounding the reservoir (but isolated from an impermeable layer), water enters the pump though the semi-permeable layer. The entry of water increases the salt chamber, causing compression of the flexible reservoir and drug delivery.

2.11.2 Permanent LAD ligation

2.11.2.1 Surgical preparations and intubation
The mice were anaesthetised with 4% isoflurane (Forene; Abbott, Baar, Switzerland) in 100% oxygen (by inhalation). Mice were placed on a preheated warming pad and a rectal temperature probe inserted. To maintain a constant temperature of 37°C, a thermal controller (Clark and Bell MKI, St Thomas’ Hospital) was used. The mice were secured in the supine position on the operating table by attaching stirrups constructed of PE-50 tubing abducting the legs. The upper limbs were taped across the shoulders and the neck was extended via a plastic tube behind the front teeth. The animal was shaved over the anterior neck, upper chest and abdomen.

A midline skin incision was made from the sternal notch to just below the mandible. The mice were intubated using a 19 gauge cannula (Figure 2-5 A) stub attached via a plastic T-
connector to a mini-vent ventilator (Hugo Sacs Mini-vent 845, Linton Instruments, UK). The salivary glands and the muscle overlying the trachea were bluntly divided and retracted to allow visualisation of the cannula as it was placed in the trachea (Figure 2-5 B). The tongue was slightly retracted, and the cannula was inserted through the larynx and into the trachea until a tight seal was achieved. Care was taken not to puncture the trachea or other structures in the pharyngeal region. Ventilation was started with 2% isoflurane in 100% oxygen with a respiratory rate of 110 breaths per min and a tidal volume of 230 μL.

### 2.11.2.2 Tying the left anterior descending (LAD) coronary artery

A lateral skin incision was made (Figure 2-6 A) followed by removal of the abdominal and the chest muscles to allow the visualisation of the first and the second ribs (Figure 2-6 B). To open the chest cavity and gain access to the heart, the innermost intercostal muscles were dissected using a cautery tool. The ribs were retracted apart to allow access to the heart (Figure 2-5 C). Ligation proceeded with a 8/0 nylon suture (Ethilon, Johnson and Johnson, UK) with a tapered needle passed underneath the LAD approximately 1-2 mm left of the edge of the left atrium (Figure 2-5 D). The suture was tied to invoke ischemia, which was visually confirmed by a change of colour of the LV from red to a purple/grey colour (Figure 2-6 E and F).

![Figure 2-5 Mouse tracheotomy intubation.](image)

(A) An intubating cannula. A midline incision was made just below the chin and the salivary gland retracted to allow the visualisation of the intubating tube. The tongue was retracted to allow the insertion of the cannula into the trachea (B).
Figure 2-6 A permanent LAD ligation model.

(A) A lateral incision was made on the skin followed by a lateral thoracotomy (B). Ribs were retracted apart to gain access to the heart (C). An 8/0 nylon suture with a tapered needle was passed from 1-2 mm left from the edge of the left atrium, underneath the LAD (D & E). Area of ischemia (black arrow) appeared purplish/grey on the LV following the ligation of the LAD (F).
2.11.2.3 **Recovery from anaesthesia and post-operative care in remodelling model**

In the remodelling recovery model, the chest was closed after the LAD ligation by using interrupted sutures (5/0 silk, Johnson and Johnson) joining the two ribs together. Care was taken to close the chest properly to prevent pneumothorax. This was followed by closing the overlying skin both at site of surgery and the thoracotomy using a 5/0 silk suture (Johnson and Johnson). The anaesthetic was turned off and the animals were left under pure oxygen until they fully recovered consciousness. The mice were rehydrated by an i.p. injection of sterile saline on recovery, in addition to an i.p. injection of 100 µL of analgesic (Buprenorphine, 0.3 mg/mL- diluted 1:20 in sterile water). The animals were left in a hot box at a temperature of 28 °C for 24 h until recovered. The animals were kept alive for 4 weeks to allow remodelling before being sacrificed.

2.11.3 **Assessment of LV function using the pressure volume analysis**

Pressure-volume relationships are the gold-standard for evaluating contractility in the intact heart. Recently, a miniature conductance catheter system has been developed which allows the measurement of both pressure and volume, rapidly and in real time (Figure 2-7). The volume electrodes are separated by a pressure sensor, and the device is mounted on a catheter and placed in the longitudinal axis of the LV. The catheter can be inserted into the LV cavity from the carotid artery via the ascending aorta or by apical puncture of the LV apex following a subcostal incision (Figure 2-8). The electrodes produce a potential difference, which is inversely proportional to the conductance, and thus blood volume in the LV. The volume measurements derived from the conductance catheter are reproducible between animals of a similar size but are not necessarily absolute measures of volume in the left ventricle. This can be controlled for using an ultrasonic flow probe positioned on the ascending aorta just above the aortic root to accurately measure LV ejection and therefore act as an internal calibration for each experimental animal. Each cardiac cycle generate a pressure volume loop which can be measured by the catheter (Figure 2-9).
Figure 2-7 Photograph of the miniaturized conductance-micromanometer catheters.

(A) Millar 1.4F used for this study. The catheter is 0.4 mm in cross-sectional diameter. The two electrodes (distal and proximal) are used to generate volume signal. The distal electrode is positioned to lie at or just above the aortic valve. A pressure transducer is located between the electrodes and falls within the mid-cavity of left ventricle (LV). (B) Scisense 1.2F PV catheter used for measurement of pressure-volume data by admittance.

Pressure-volume relationships can be measured, using the conductance catheter under normal conditions or during altered pre- or after-load achieved by transiently occluding the inferior vena cava (IVC) or aorta, respectively (Figure 2-10). To generate a pressure volume loop for the LV, the LV pressure is plotted against LV volume at a high sampling frequency (1000Hz) during a single cardiac cycle (Figure 2-11). The transient occlusion of IVC, yields successive cardiac cycles from which the following data are obtained; end-systolic pressure-volume relation (ESPVR) slope, end-systolic elastance (Ees) and stroke work (SW), end-diastolic volume (EDV) relation, preload recruitable stroke work (PRSW).
Figure 2-8 A PV catheter in the LV.
A pressure transducer is mounted between the two pairs of conductance electrodes. The excitation (outermost) electrode generates an electrical field while the sensory electrode (inner pair) measures volume dependent voltage signal. The voltage signal changes during each cardiac cycle as the LV fills with, and ejects blood. Conductance can be derived by considering Ohm’s Law. $V=IR$, where $V=\text{voltage}$, $I=\text{Current}$ and $R=\text{resistance}$.

The combination of conductance catheter and real-time analysis software (Chart v5.2, AD Instruments) generates haemodynamic measurements such as end-systolic pressure and volume, end diastolic pressure and volume, stroke volume, ejection fraction, cardiac output, maximum work (maximum force of contraction by LV) and $dP/dt_{\text{max}}$ (the rate of change of pressure with time). The latter is an index of ventricular contractility (Little and O’Rourke, 1985) and is independent of afterload as it measures isovolumic contraction, i.e. prior to opening of the aortic valves (Figure 2-10).

Of particular relevance among the different data obtained by the conductance catheter are the stroke work and the elastance. These are measures of contractile function which are used in determining the LV function. Stroke work is the external work performed by the LV and is
defined as the area contained within the pressure-volume loop. It is chamber size independent and has dimensions of force and provides data that may be directly compared across the species.

![Image of pressure-volume loop and cardiac cycle](image)

**KEY**

- A. Diastolic Filling
  - M: Mitral Valve Closes
  - A: Aortic Valve Opens

- B. Isovolumic Contraction

- C. Ejection
  - A: Aortic Valve Closes

- D. Isovolumic Relaxation
  - M: Mitral Valve Opens

**Figure 2-9 The Cardiac Cycle.**

(A) Left ventricular pressure (LVP) and volume (ESV) are integrated by the catheter to produce a pressure-volume loop from which various parameters can be measured (B). The key describes various stages during the cardiac cycle. Isovolumic contraction (B) proceeds after closure of the mitral valve until the ventricle encounters its afterload, the aortic pressure. After the aortic valve opens pressure first rises then falls during ejection (C). When systole ends, ventricular pressure and volume come to lie on the end-systolic pressure volume relationship (EDPVR). After aortic valve closure, the pressure in the ventricle decreases and relaxation begins under isovolumic conditions (D). When the pressure in the ventricle falls below the left atrium, the mitral valve opens and the atrium empties into the ventricle (A).
Figure 2-10 Positing of a PV catheter in vivo.

X-ray photograph of mouse on its side with the catheter positioned in the LV and ascending aorta (arrow indicates position of catheter). (B) time-series tracings of LV volume, LV pressure, and thoracic aortic flow at both steady state and during transient inferior vena cava (IVC) occlusion. Arrow, onset of IVC occlusion. $V_0$, offset term. Adapted from (Georgakopoulos et al., 1998).

Figure 2-11 A screen capture of pressure volume loops.

The window on the left shows left ventricular (LV) pressure-volume loops during successive cardiac cycles. The window on the right shows various haemodynamic parameters such as LV developed pressure (red trace) and LV volume (blue trace), heart rate and body temperature. The readings from both, the developed pressure (80-100 mmHg) and LV volume (20-35 µL) were used to locate the optimal catheter position within the LV.
2.11.3.1 Haemodynamic measurements

Steady state: After instrumentation, LV pressure-volume signals were acquired over 10 min (Figure 2-11) in steady state to quantify baseline haemodynamic conditions: Heart rate, stroke volume, cardiac output, end-diastolic volume, end diastolic pressure, and end-systolic pressure were assessed. These parameters were derived from the pressure-volume loops by the manufacture’s software (PVAN v3.5 Cardiac Pressure Volume Analysis Software, Millar instruments, Houston, Texas).

Pressure-volume relationship: To obtain load-independent indices of systolic and diastolic LV function, pressure-volume relations were determined by recording pressure-volume loops during a gradual preload reduction. This was achieved by transiently occluding the IVC
using forceps through the midline incision in the abdomen. By this procedure, the systolic pressure reduced approximately by 40 mmHg within 2s.

2.11.3.2 Insertion of the Conductance Catheter

In most of our studies, hearts were catheterised by an apical approach. In the remodelling study (final chapter), we utilised a carotid approach. LV remodelling is associated with a significant thinning of the ventricular wall and is prone to rupture. In addition, scarring following MI results in misalignment of the heart in the chest cavity. Therefore, we adopted a carotid approach to insert the catheter into the LV in the remodelling study. The two methodologies are described below.

2.11.3.2.1 Apical Approach

Animals were intubated (Figure 2-12 A). The abdomen was opened through a lateral laparotomy just below the diaphragm to obtain access to the chest. A vertical incision was also made along the line of the sternum. The overlying chest muscles (external oblique) were removed using blunt dissection. The diaphragm was cut carefully in the middle at its central anterior insertion to obtain access to the chest. A left field thoracotomy was performed to expose the heart and the lungs using cautery forceps (Figure 2-12 B). The area was cleaned and the pericardium removed carefully from around the heart (Figure 2-12 C). A small puncture was made in the LV apex using a 25 gauge needle until there was a flashback of blood in the needle hub. The micro conductance catheter was inserted into the LV along the cardiac longitudinal axis with the distal tip placed in the aortic root and the proximal electrode just within the endocardial wall of the apex (Figure 2-12 D).

2.11.3.2.2 Carotid Approach

After intubation, the muscles of the neck on the right were further blunt dissected to expose the right common carotid artery. The vessel was occluded at the most proximal accessible point with a 4 mm vessel clip. The most distal accessible part of the artery was ligated using 5/0 silk suture and a second suture was placed loosely just above the vessel clip. A small transverse incision in the artery was then made, as distal as possible (Figure 2-13 A). The proximal side of this incision was held open and the catheter was gently introduced into the artery and was passed caudally. The loose suture was then placed under slight tension, with the tip of the catheter at the point of vessel occlusion by the clip. The clip was removed and the catheter advanced swiftly into the ascending aorta. At this point, pressure readings from
the catheter provided information on the site of the catheter tip. The catheter was further advanced until the tip passed across the aortic valve, evidenced by changes in the shape of pressure traces and obtaining volume readings. Once the catheter was in position, the most proximal suture was tightened to maintain the catheter in position (Figure 2-13 B).

Figure 2-13 Insertion of a PV Catheter into the hearts via the Carotid Artery.
The right carotid artery was exposed and cleaned following intubation and sedation. A small arteriotomy (A) was made in the proximal carotid and a PV catheter carefully inserted into the vessel (B).
2.11.3.3 **Calibration of the conductance catheter**

The volume was calibrated using a cuvette calibration method as described in the User’s Guide provided by the manufacturer. The cuvette calibration method uses known volumes of blood and takes into account the resistivity of blood and electrode spacing. To derive absolute volumes from the conductance catheter, the signals must be calibrated for parallel conductance ($V_p$). This was determined by the hypertonic saline method (Baan et al., 1984). At the end of the procedure, parallel conductance was determined by intravenous injection of 10 µL 15% w/v NaCl into the right jugular vein.

However there are two major drawbacks with the classical conductance system 1) Field correction factor alpha 2) parallel conductance estimation (Kottam et al., 2006).

1) **Field correction factor alpha:** This is associated with the non linear relationship between blood and conductance and volume due to no linear (inhomogeneous) shape of stimulating electrical field. In the classical conductance, it is often assumed that the electrical field generated is homogenous. This results in a volume underestimation when converting the measure raw signal into blood volume.

2) **Parallel conductance estimation:** As described previously, the parallel conductance is determined by injection of hypertonic saline bolus into the right jugular vein at the end of the experiment. However, this technique is essentially an approximation and user dependent. In addition, this approach defines a dynamic variable as a constant (Kottam et al., 2006).

To overcome the above drawbacks and obtain more accurate data, we utilised a new Admittance technique recently developed by Scisense (Canada).

2.11.3.4 **Assessment of pressure-volume by complex admittance**

Unlike the conductance system, the admittance technique measures both conductive and capacitive properties of blood and muscle. This allows separation of the admittance of the muscle from admittance of blood, using electrical field theory. This is achieved by employing an improved conductance-to-volume conversion equation in which Wei’s equation replaces Field Correction Factor alpha. The new equation corrects for the
inhomogeneous nature of the catheter electrical field distribution thus improving accuracy over a wider range [164].

The new admittance system (ADVantage™) also allows true volume measurements in real time by using a surface probe to measure specific myocardial properties (conductivity and permittivity). The system then uses a conductance catheter to measure a combination of admittance magnitude and phase angle signal from the ventricle. The signals are processed to provide true ventricular blood volume (Kottam et al., 2006).

### 2.11.4 Determining the ischemic risk zone volume in a non-recovery model

The risk zone volume is the volume of myocardial tissue that is ischemic and therefore at risk of infarction. The risk zone is determined by infusing Evans blue into the coronary circulation at the end of the study. The dye penetrates perfused myocardium whilst leaving non-perfused myocardium (risk zone) unstained. At the end of the experiment, a bolus of 0.5 mL Evans Blue dye (2% v/w in sterile filtered saline, 0.9% NaCl w/v in water) was slowly infused into the LV cavity via the apex (Figure 2-14 A). The needle was then gently withdrawn and the heart carefully excised and fixed in a solution of 10% paraformaldehyde (w/v in phosphate buffered saline) for 24 h at 4°C. After fixation, the heart was mounted in 5% agarose (w/v in PBS) with the apex uppermost. The agarose-embedded hearts were then sectioned from apex to base into 700 μm thick slices using a vibratome (Agar Scientific) (Figure 2-14 B).
Figure 2-14 Infusion of the heart with Evans blue.

(A) The ischemic region of the LV remained unstained following the injections. The perfused region was stained with Evans blue. (B) Representative slices of the hearts from mice after LAD ligation used for morphometric analysis. The ischemic regions appear white (red lines) and become more concentric towards the apex.

Immediately after sectioning, heart slices were pressed, apical aspect uppermost between glass plates. In each slice the risk zone was identified as white/pink regions whereas the non-risk areas were stained with Evans blue (Figure 2-15 A). Dimensions and ischaemic volume were calculated using a computer-aided analysis (Sigma Scan, SPSS, UK) following high-resolution digital capture (Figure 12-15 B).

\[
\text{LV Area} = \text{Total heart area} - \text{Right ventricle}
\]

\[
\%\text{AAR} = \left( \frac{\text{AAR}}{\text{LV Area}} \right) \times 100
\]

Figure 2-15 Analysis of area at risk (AAR) using Sigma Scan.

An Evans blue-stained heart as a raw image (A) and a Sigma Scan (B) output image with total heart (red), right ventricle (blue) and area at risk (AAR, green) areas highlighted in overlays, with the equations used to calculate LV area and percentage AAR of LV (%AAR).

2.11.5 Morphometric analysis

Hearts were excised from the chest, cannulated and perfused with saline (0.9% w/v NaCl) to flush out any remaining blood in the LV. A fluid filled balloon attached to a cannula was
inserted into the LV and inflated under a pressure of 10 mmHg. The hearts were perfused with 1 mL of 10% v/v formaldehyde in distilled water. At the same time, the heart (still attached to the cannula) was immersed for 10 min in an Eppendorf tube containing 10% v/v formaldehyde to allow the fixation of the outer surface of the myocardium as well as the inside. The balloon was then deflated hearts were removed from and further fixed in 10% formaldehyde overnight at 4°C.

After fixation, the heart was mounted in 5% agarose (w/v in phosphate buffered saline) with the apex uppermost. The agarose-embedded hearts were then sectioned from apex to base into 700 μm thick slices using a vibratome (Agar Scientific). The sections were placed in 6-well plate containing 1 mL of PBS according to order that were sliced and kept at 4C° and scanned as described in the previous section.

2.12 Echocardiography

An electrocardiogram (echo) is a clinical examination of the heart that uses ultrasound waves to create both still and moving images. The images are obtained using a transducer gently applied to the surface of the chest wall. In our studies we used the VisualSonics Vevo770 high-resolution murine ultrasound which operates in the very high frequency range (centre frequencies of 25-55 MHz) to visualise and quantify LV function.

2.12.1 System Configurations

The main components of the Vevo770 consist of a computer monitor located on top of the cart, a RMV (707B model) scanhead system (Figure 2-16 A) and an animal handling and physiological monitoring system (Figure 2-16 B). The Vevo770 also includes the following software packages; an analytic software package for B-mode (2D) image capture and analysis, Cine loop capture, advanced measurements and annotations and physiological Data on-screen trace. The animal handling and physiological monitoring system is used to secure the animal and monitor heart rate, ECG as well as core temperature (Figure 2-16 C). In addition, it allows manipulation of the angle at which the animal is placed to achieve an optimal imaging plane.
2.12.2 The RMV Scanhead System

The RMV component includes a rail, a mount and an RMV (707B-model) scanhead with maximum frame rates up to 200 frames/s. It, therefore, provides greater sensitivity and depth of penetration with higher resolution for small animal imaging.

The transducer within the RMV scanned is used to transmit an ultrasound pulse into the animal through a coupling medium such as ultrasound gel. The transducer is a microphone-like device that detects the ultrasound waves from the animal. The echo is used to generate a single line of the digital images. These images may be one-dimensional (M-mode), displayed graphically over time, two dimensional (B-Mode), or three-dimensional representations, displayed in a repeating single heart beat format known as cine-loop.
Figure 2-16 The Visualsonics Vevo770 murine ultrasound system.

The Vevo770 system (A). The system consists of a scanhead, an animal physiological system unit and a temperature, heart rate and ECG monitoring system (B).
2.12.3 Ultrasound Imaging

2.12.3.1 Animal preparation

Mice were anaesthetised using inhalation anaesthetic. Following induction, the mice were moved to the physiological monitoring system with the anaesthetic mask placed over the snouts. A small amount of corneal lubricant was carefully placed onto each eye. A small drop of ECG cream (Parker Laboratories, USA) was placed on each metal ECG connector and the animal’s paws were secured to each of the ECG connector contact pads with dermatological tape. A rectal probe covered with lubricating gel (Parker Laboratories, USA) was slowly inserted into the rectum. As successful ultrasound imaging requires direct contact of the gel to the skin, any hair must be removed as any hair left on the area of interest traps air bubbles and hinders image quality. Mice were shaved first and the remaining hair was removed by gently applying depilatory cream (Nair hair removal cream, Curch & Dwight Canada Crop, Canada) onto the chest using a cotton swap. The ultrasound gel (Anagel, UK) (pre-warmed at 37°C prior to scanning) was gently placed onto the chest. The monitoring unit was slowly moved until the scanhead was situated just above the animal. The scanhead was then lowered, watching the screen at the same time, the positioning controls were used until the desired image of the heart was brought into view.

2.12.4 Scanning using different imaging modes

2.12.4.1 B-mode image

To create a B-mode image, the transducer acquires multiple lines while scanning the image field. The lines then are processed and combined to generate a two-dimensional image. This mode is often used to determine the location of the heart for measurements in other modes. Figure 2-17 describes the features of the mode window in B-mode.

Different positioning of the scanhead allows various anatomical visualisation of the LV. In our studies we used parasternal long axis (Figure 2-17 A), parasternal short axis (Figure 2-17 B) and Aortic arch view (Figure 2-17 C) to access the LV size and function.
Figure 2-17 Positioning of the scanhead used for visualisation of the left ventricle in different anatomical views.

The scanhead was placed vertically to in relation to the chest to generate a parasternal long axis (A). Positioning the scanhead at 90° allows visualisation of the LV in a short axis view (B). An Aortic arch view can be obtained by tilting the unit to the right and placing the probe at a sharp angle to the side of the chest wall to generate a 60° angle (C).
2.12.4.2 M-mode imaging

M-mode imaging is often used to evaluate cardiovascular function, studying the differences between normal and transgenic or diseased hearts. The images can also be used to determine the chamber dimensions at different points throughout the cardiac cycle in long and short axis (Figure 2-18 A and 2-18 B respectively). M-mode imaging displays the motion of tissue detected by one line of the B-mode image over (Figure 2-18 A and B). The image in this mode is constructed by the backscatter data that is acquired by rapidly pulsing the transducer at a single point.

![A](image1.png)  
![B](image2.png)

**Figure 2-18 An M-mode image of parasternal long (top) and short (bottom) axis.**

An m-Mode image of parasternal long (A) and short axis (B) allows measurements of various LV parameters such as septal wall thickness (IVS), interior left ventricular dimension (LVID) and left ventricular posterior (LVPW) wall thickness during systole and diastole (blue vertical lines) and anterior left ventricular thickness (LVAW). By taking an LVID trace (blue wave-like pattern), various haemodynamic parameters such as cardiac output (CO), ejection fraction (EF), stroke volume (SV) and fractional shortening (FS) can be obtained.
2.12.5 PW imaging

Using the PW Doppler mode imaging, the transducer can also examine the velocity of blood flow within a region of interest (Figure 2-19).

![Figure 2-19 A PW-image of Aortic arch view.](image)

An M-mode image of PW scanning allows various velocity time integral (VTI) measurements. These measurements are indicator of the volumetric blood flow through the aorta with each ventricular contraction, represented by a distance which is equivalent to stroke volume.

2.12.6 Ending the imaging sessions

All the ultrasound gel was removed from the animal and the animal was cleaned with dampened gauze. The temperature probe and the securing tape were gently removed. For recovery, the anaesthetic was turned off and animals were left under 100% oxygen for a few min. The animal was left under a heat lamp until fully recovered.

2.13 Statistical Analysis

Functional data in Langendorff perfusions including data for functional recovery at the end of reperfusion and haemodynamic parameters measured by PV and echocardiography were analysed by either one-way ANOVA or two-way ANOVA followed by Tukey post test with Graphad Prism. A value of p<0.05 was considered significant. All values are expressed as mean ± SEM.
In the characterisation study (Chapter 6, section 6.4.1) using PV and echocardiography, haemodynamic parameters from each animal were normalised as a % age of baseline. Data were pooled and linear regression analyses were carried out to determine the relationship between the changes in each parameter and the ischaemic zone (% LV muscle volume) measured. Data are presented as scatter plots where each point is an individual experiment. Linear regression and 95% confidence intervals are represented by a single solid and a pair of dashed lines, respectively. All analysis was performed using GraphPad Prism. R^2 (goodness of fit) and linear regression equation are shown in the figure only where the slope of the regression were considered by the analysis to be significantly non-zero. A value of p<0.05 was considered statistically significant.
3 CHARACTERISATION OF P38 MAPK ISOFORMS IN THE MURINE HEART

3.1 Introduction

To date, four isoforms (α, β, γ and δ) have been identified which show cell type and stimuli specific activation. p38 MAPK isoforms share structural similarities, but differ in sensitivity to pharmacological inhibition by pyridinyl imidazole molecules such as SB203580 and non-aryl pyridyl heterocycles such as BIRB-796 and substrate specificity (Cirillo, et al., 2002). From the multitude of studies investigating the role of p38 MAPK isoforms during myocardial ischaemia, it is evident that p38 MAPK isoforms are differentially regulated; p38α MAPK has shown to have detrimental consequences by promoting apoptosis whereas p38β MAPK has shown to have protective effects by having anti-apoptotic roles (de Winter et al., 2005; Sugden and Clerk, 1998).

In order to define the value of p38 MAPKs in the clinical setting, clearer insight into differential expression and activation of p38 MAPK isoforms in the heart is warranted. However, since there are no isoform-specific inhibitors available, the contribution of each isoform in the heart remains unclear. One powerful tool to study the role of p38 MAPK isoforms is to use transgenic mice either lacking or over-expressing each isoform in combination with pharmacological inhibitors against p38α/β MAPK isoforms, such as SB203580. Mice lacking the p38α MAPK isoform are not viable and die due to severe developmental abnormalities, however, global knock out of p38β, γ and δ MAPK isoforms are viable and fertile (Bogoyevitch et al., 1996; de Winter et al., 2005; Luss et al., 2000; Ono and Han, 2000; Ping and Murphy, 2000).

3.2 Aims

The first step to delineate the function and contribution of each isoform is to characterise the relative expression of each isoform in the heart. Using commercially available isoform-specific antibodies and transgenic mice lacking either p38β isoform (p38β KO) or p38γ and δ isoforms (p38γ/δ KO), the current study was designed to characterise the abundance of endogenous p38 MAPK subtypes (at the transcription and translational level) in the murine heart.
3.3 Specific Methods

3.3.1 Langendorff perfusion and sample collection

Mice were anesthetized by intraperitoneal injection of pentobarbital (300 mg/kg) and heparin (150 units) (to prevent emboli formation). Hearts were rapidly excised and placed in ice cold Krebs buffer. After cannulation, hearts were perfused under a constant pressure of 80 mmHg for 40 min through an aortic cannula delivering warmed buffer (37°C). At the end of the perfusion, hearts were removed and snap frozen in liquid nitrogen and later homogenized (1 mL/100 mg tissue) on ice in lysis buffer (20 mM Hepes, 150 mM NaCl, 50 mM NaF, 1 mM DTT, 1 mM EGTA, 1 mM Na3O4 and 0.5% v/v Triton X-100 and protease inhibitors, Roche). Crude homogenates were centrifuged at 10,000 rpm at 4º C for 15 min and the supernatant kept for immunoprecipitation followed by western blotting.

3.3.2 Testing the selectivity and sensitivity of commercially available antibodies for p38 MAPK isoforms

To test the selectivity of the isoform-specific antibodies, crude homogenates were "spiked" with 5 ng of GST-tagged recombinant of p38α, β, γ or δ MAPK (Cell Signaling Biotechnology). To test the sensitivity, crude heart homogenates were spiked with serially-diluted GST tagged recombinant p38 MAPK proteins (246.0-0.4 ng). GST-tagged recombinant proteins are made by fusion of a construct expressing full length human protein kinase with an amino acid terminal (GST) using a baculovirus expression system. The samples were solubilised in 2X sample loading buffer (20% v/v glycerol; 6% v/v sodium dodecyl sulphate (SDS), β-mercaptoethanol 10% v/v and bromophenol blue), in a 1:1 ratio, heated for 5 min at 95°C and analysed by immunoblotting (Chapter 2, section 2.3 for specific conditions) using the commercially available antibodies as listed in Table 3-1.
Table 3-1 Commercially available p38 isoform-specific antibodies. IP: Immunoprecipitation; ICC: Immunocytochemistry; WB: Western blotting

<table>
<thead>
<tr>
<th>Antibody against:</th>
<th>Manufacturer (code)</th>
<th>Type</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38α</td>
<td>Cell Signaling (#9218)</td>
<td>Rabbit pAb</td>
<td>IP, WB</td>
</tr>
<tr>
<td>p38β</td>
<td>Zymed Laboratories (# 33-8700)</td>
<td>Mouse mAb</td>
<td>IP, WB, ICC</td>
</tr>
<tr>
<td>p38γ</td>
<td>R&amp;D Systems (# MAB1347)</td>
<td>Mouse mAb</td>
<td>IP, WB, ICC</td>
</tr>
<tr>
<td>p38δ</td>
<td>R&amp;D Systems (# MAB1519)</td>
<td>Mouse mAb</td>
<td>IP, WB, ICC</td>
</tr>
<tr>
<td>Total p38</td>
<td>Cell Signaling (#9212)</td>
<td>Rabbit pAb</td>
<td>WB, IP, ICC</td>
</tr>
<tr>
<td>p38α</td>
<td>Cell Signaling (#9217)</td>
<td>Mouse mAb</td>
<td>IP, ICC</td>
</tr>
<tr>
<td>Phospho p38 (Thr 180/Tyr 182)</td>
<td>Cell Signaling (#9211)</td>
<td>Rabbit pAb</td>
<td>WB</td>
</tr>
</tbody>
</table>

### 3.3.3 Western blotting conditions

Samples in SDS sample loading buffer were run on a 12.5% (v/v) SDS gel at 100V for 2 h. Proteins were transferred to a PVDF membrane (Amersham Biosciences), using a semi-dry electrophoretic transfer system, at 0.25 mA (per mini gel) for 30 min per gel (Biorad) as described in section 2.8, Chapter 2. The membranes were incubated with rabbit p38α, mouse p38β MAPK, mouse p38δ MAPK (all diluted 1:1000 dilution), rabbit total p38 and mouse p38γ MAPK (1:2000 dilution) (Table 3-1) overnight at 4°C in TRIS-buffered saline (TBS; 50 mM TRIS-HCl; 150 mM NaCl) containing 0.1 % v/v TWEEN 20 (TBS-T) and non-fat dry milk (5 % w/v; Marvel) with constant gentle shaking on a Rota-test shaker (Luckham Ltd). The following day, membranes were washed (3x 10 min washes) in TBS-T containing dry milk (1% w/v), before being incubated with the corresponding horseradish peroxidase conjugated secondary antibody diluted (1:2000) in TBS-T containing 0.1% w/v dry milk, at room temperature with gentle shaking for 60 min. The membranes then underwent further washing in TBS-T and were developed using an ECL detection kit (Amersham Bioscience, UK).
3.3.4 Immunoprecipitation of total p38 MAPK from murine mouse heart

To immunoprecipitate (IP) total p38 MAPK from heart homogenates (input), 5 µL of monoclonal pan p38 antibody (p38 α MAPK), Cell Signaling # 9217 was added to 185 µL of crude homogenate and rotated on a orbital rotator (Stuart SB3) at 4ºC overnight. A negative control was included which contained lysis buffer (200 µL) plus the pull down antibody. After 18 h, 50 µL of Protein G-Sepharose beads (Amersham) was added to the antibody and antigen complex and the negative control tubes. The samples were re-placed on the rotator for a further two h at 4ºC. Protein G-Sepharose beads/antibody-antigen complex were washed three times with 250 µL of lysis buffer used for homogenisation (without Triton X-100). For each wash the samples were centrifuged at 2000 rpm for 30 s at 4ºC and supernatant removed. The beads were then re-suspended in 250 µL of fresh lysis buffer. On the final wash the supernatant was removed, care was taken not to disturb the pellet which contained the protein beads bound to p38 MAPK antigen-antibody complex. Finally the pellet was solubilised in 30 µL of SDS sample loading buffer and heated at 95ºC for 5 min and analyzed by western blotting as described before. Proteins were detected by using rabbit polyclonal p38 MAPK primary antibody (Cell Signaling #9212).

3.3.5 Optimising IP conditions

Using the above protocol failed to efficiently pull down total p38 MAPK from crude heart homogenate. This was probably due to poor binding between the pull down antibody and p38 MAPK which was confirmed by abundant p38 MAPK protein in the supernatant, obtained after the incubation period with Protein G-Sepharose beads.

To optimise the pull down protocol we tried to enhance the binding by adapting the following methods; (i) increasing the concentration of detergent (ii) doubling the volume of the pull down antibody (iii) using a polyclonal (rabbit total p38, Cell Signalling #9212) antibody and (iv) denaturing approach. The latter involved boiling of the crude homogenate in a lysis buffer containing a stronger detergent (1% w/v SDS instead of 0.5% v/v NP40).

3.3.6 Increasing the concentration of detergent

Heart samples were weighed and homogenised on ice in lysis buffer (1 mL/100 mg tissue) containing 0.5% v/v Triton X-100 (more stringent detergent and enhances antibody-antigen
3.3.7 Doubling the volume of IP antibody

Crude heart homogenates were mixed with 8 µL instead of 4 µL of mouse monoclonal pan p38 IP antibody and subjected to IP as in section 3.3.4.

3.3.8 Using a polyclonal antibody for IP

Heart homogenates were also subjected to IP as described in section 3.3.4. However, in place of the monoclonal mouse antibody, we used a polyclonal rabbit antibody against total p38 (Cell Signalling # 9212). This approach was adapted to increase the chance of antigen-antibody interaction.

3.3.9 Denaturing approach

Heart samples were weighed and homogenised on ice in modified lysis buffer (1 mL/100 mg tissue) containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 100 mM NaF, 10 nM calyculin A, 1 mM DTT, 1% w/v SDS and 1 “complete” tablet of cocktail inhibitor (one tablet per 50 mL of lysis buffer). Crude homogenate (input) was boiled at 95°C for 5 min to denature proteins in an attempt to optimise the binding between p38 MAPK and the pull down antibody. The samples were then spun at 14,000 rpm for 15 min and the supernatant kept and subjected to IP. The input (200 µL) was mixed with 5 µL of monoclonal pan p38 MAPK plus 50 µL of protein G-Sepharose beads and incubated overnight at 4°C. As before, a negative control was included which contained the crude homogenate plus the beads and no antibody. The next day the samples were spun at 14,000 rpm and washed three times with 500 µL of lysis buffer containing 0.1% SDS w/v and 0.5% v/v Triton X-100. The samples were prepared as before in 30 µL of SDS-PAGE loading buffer, divided into two parts and run on a single 12.5% SDS-polyacrylamide gel. After transferring, each membrane was cut into two halves so that each half contained the same set of samples. One half of the membranes was probed for total p38 MAPK using a polyclonal rabbit total p38 MAPK antibody (Cell Signalling #9212) and the other half for either p38α, p38β, p38γ or p38δ MAPK (Table 3-1).
3.3.10 IP of p38β, γ and δ using isoform-specific antibodies

Heart homogenates (input) (200 µL) were mixed with 5 µL of either monoclonal p38β MAPK (ZYMED Laboratories), monoclonal p38γ MAPK (R&D Systems) or monoclonal p38δ MAPK (R&D Systems) plus 50 µL of Protein G-Sepharose beads and incubated overnight at 4°C. Some of input was also "spiked" with 5 ng of human recombinant p38β, γ and δ MAPK. The "spiked" input was divided into 200 µL and 50 µL aliquots. The larger aliquot was subjected to IP as described before (section 3.3.9 in this Chapter). The rest of the "spiked" input analysed by western blotting along with "spiked" and IP samples on a 12.5% gel as described above. Each isoform was stained with the same isoform-specific antibodies (1:1000 dilutions) used for IP.

3.3.11 RNA extraction, reverse transcription and PCR amplification of p38 MAPK isoforms

Heart, brain, skeletal muscle and kidneys were removed from WT and transgenic mice and mRNA was extracted from frozen tissues using the RNeasy for Fibrous Tissue Kit (Quiagen). Tissue samples were first lysed (30 mg into 300 µL of lysis buffer containing β-mercaptoethanol) and then diluted before being treated with proteinase K. Debris were pelleted by centrifugation (8,000 rpm for 5 min), and the supernatant was removed by pipetting into a clean RNAse-free tube. The supernatant was mixed with ethanol and then centrifuged through an RNeasy spin column, where RNA binds to the silica membrane. DNA that might have been co-purified with the RNA was removed by further DNase treatment on the silica membrane. DNase and any contaminants were efficiently washed away, and total RNA was eluted in RNase-free water. To elute total RNA, 2 mL of RNase-free water was added directly onto the silica membrane. The samples were then centrifuged at 8,000 rpm for 5 min at 25°C.

Reverse transcription (RT) reactions were performed by mixing 500 ng of mRNA with oligo-dT (500 µg/mL, Promega), 10 mM dNTPs (dATP, dCTP, dGTP, dTTP, Promega). The first strand cDNA was synthesised by adding 200 units of SuperScript™ II (Invitrogen) (Chapter 2, section 2.7.1) to the samples and incubating the mixtures at 42°C for 50 min. The reactions were terminated by heating the samples at 70°C for 15 min.
Polymerase chain reaction (PCR) was conducted, using 1 μg cDNA (from heart, brain, kidney and skeletal muscle) in a 50 μL PCR reaction consisting of 0.2 U of AmpliTaq Gold (Applied Biosystems) (see Chapter 2, section 2.7.2 to 2.7.4 for details). The reaction was started by an initial incubation at 95°C for 10 min followed by 40 cycles of amplification; 95°C for 30 s, 56°C for 60 s, 72 °C for 30 s, with final extension of 10 min at 72°C. PCR products were resolved on 2.0% agarose gels containing 0.5 μg/mL ethidium bromide. PCR reactions were repeated a minimum of three times using cDNA prepared from WT, p38β KO and p38γδ KO hearts.

3.3.12 Immunocytochemistry

Freshly isolated cardiac myocytes were prepared as described in Chapter 2 (section 2.3.2) and cultured on laminin-coated dishes for one h in culture media. The media was removed and cells were washed with PBS and fixed and permeabilised as described in Chapter 2, Materials and Methods (section 2.3.3). Cells were then blocked with 100 μL/dish of 5% normal goat serum (NGS). The same p38 MAPK antibodies (used in immunoprecipitation studies); Total (pan) p38, p38β, p38γ and p38δ MAPK were diluted 1:50 plus either a primary mouse α actinin (diluted 1:500 for polyclonal rabbit Total p38 antibody) or with a primary rabbit-α actinin (diluted 1:200 for monoclonal mouse p38β, p38γ and p38δ antibodies) in buffer containing Trizma 20 mM, NaCl 155 mM, EGTA 2 mM, MgCl2 2 mM, pH 7.5 and 1% BSA. The dishes were placed into a humid chamber and incubated overnight at 4°C. The following day the cells were washed in PBS same as before and incubated over night at 4°C with secondary antibodies; mouse Cy3α diluted 1:500 (ML Jackson Stratech Scientific), rabbit Cy2α diluted 1:100 (ML Jackson Stratech Scientific), and DAPI diluted 1:100. The cells were then washed as before and mounted with a droplet of Mounting Medium (Tris 0.1 M, 35 mL Glycerol and 2.5g n-propylgallate and covered with cover slips (VWR 30 mm diameter, Science Warehouse). The edge of each dish was removed with a hot wire and cover-slips sealed with nail varnish. Each dish was then glued onto a microscope slide and kept at 4°C until analysis.
3.4 Results

3.4.1 Mono-selectivity and sensitivity of isoform-specific antibodies

Since the assessment of p38 MAPK isoform abundance, and ultimately functional analysis, is wholly dependent on the use of selective antibodies against the different isoforms which are assumed not to cross react with other p38 MAPK isoforms, we first ascertained isoform selectivity of the antibodies chosen for this study. When GST-tagged active human recombinant p38β, p38γ or p38δ MAPK (68 kDa expected molecular weight) were added to crude heart homogenates and blotted and probed with antibodies against each of these isoforms, mono-selective binding was observed (Figure 3-1). This was based on the observation of a single band at the expected molecular weight for p38α, p38β, p38γ and δ MAPK isoforms. The antibodies were also able to detect endogenous p38α and γ in the spiked samples. No endogenous β nor δ were detected in these samples. The data suggested that there was little or no cross-reactivity between isoform-specific antibodies. The pan isoform-specific antibody (total p38) was able to detect all recombinant p38 MAPK isoforms except for p38δ MAPK (Figure 3-1). In addition we tested the sensitivity of isoform-specific antibodies against serially diluted (0.4-246 ng per 0.05 mg total protein per well) recombinant p38 MAPK proteins to ensure sensitivity (Figure 3-2). The data showed that all antibodies are able to detect recombinant proteins as low as 6-16 ng per 0.05 mg total protein per well (p38α, β and δ MAPK antibody) and 1 ng per 0.05 mg per total protein per well (p38γ MAPK antibody). However as before, only endogenous p38α and γ MAPK were detected in crude heart homogenates (Figure 3-2 A-D).

Next, we attempted to quantify the relative expression of p38 MAPK isoforms in the heart. To do this, the density of the bands in Figure 3-2 for the GST-tagged and the endogenously expressed protein (where applicable) were determined by densitometry (Bio-Rad GS800). The band density values for the GST-tagged proteins (α, β, γ and δ) were plotted against each concentration 73.6 nmoles/mg to 0.0118 nmoles/mg using Prism (Figure 3-3 A-D). The fitted curves were used to estimate the relative concentration of the endogenous α and γ isoforms in each well based on the relative units (RU) obtained by the densitometric analysis (Figure 3-3A and C). We were able to determine that the α isoform is most abundantly
expressed in the heart (11.8 nmoles/mg) followed by the $\gamma$ isoform (2.6 nmoles/mg) (Figure 3-3A and C). As the endogenous p38 $\beta$ and $\delta$ MAPK were not detectable, we were unable to estimate the expression of these isoforms (Figure 3-3 B and D). However, from Figure 3-2 B and D, we can postulate that these isoforms are expressed less than 1.8 nmoles/mg.

Figure 3-1 Representative immunoblots showing the selectivity of all p38 MAPK isoform.

Crude heart homogenate were spiked with GST- tagged (68 kDa) recombinant proteins (240 ng/well) and probed with isoform-specific monoclonal antibodies and a total (pan) antibody. The arrows indicate the endogenously expressed p38$\alpha$ (38 kDa) and $\gamma$ (~42 kDa) also detected in the crude heart homogenate. No endogenously expressed p38$\beta$ or $\delta$ was detected.
### Chapter 3

**Characterisation of p38 MAPK isoforms in the heart**

<table>
<thead>
<tr>
<th>GST Tagged recombinant proteins (ng/well)</th>
<th>246</th>
<th>99</th>
<th>39</th>
<th>16</th>
<th>6</th>
<th>2</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous p38α (38 kDa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST-p38α (68 kDa)</td>
<td>82</td>
<td>62</td>
<td>48</td>
<td>32.5</td>
<td>25</td>
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<tr>
<td>GST-p38β (68 kDa)</td>
<td></td>
<td></td>
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<tr>
<td>Endogenous p38β (38 kDa)</td>
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<td>62</td>
<td>48</td>
<td>32.5</td>
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<tr>
<td>GST-p38γ (68 kDa)</td>
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<tr>
<td>Endogenous p38γ (42 kDa)</td>
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<td>62</td>
<td>48</td>
<td>32.5</td>
<td>25</td>
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</tr>
</tbody>
</table>

Figure 3-2 Representative immunoblots showing the sensitivity testing of all p38 MAPK isoforms-specific antibodies.

Crude heart homogenate were spiked with GST tagged recombinant proteins serially diluted (246-0.4 ng/well and probed with isoform-specific monoclonal antibody. Upper bands indicate the spiked GST-tagged recombinant protein and the lower bands indicate the expected molecular weight for endogenously expressed isoform.
3.4.2 IP of total p38 MAPK

We were able to detect p38 MAPK in the crude homogenate which was confirmed by including a positive control (COS cells transfected with plasmid expressing p38α). However, the protocol used to pull down total p38 MAPK failed to IP p38 MAPK from the heart samples efficiently (Figure 3-4 A). Only a faint band which corresponded to total p38 was
observed (Figure 3-4 A). These results suggested that, although the homogenisation and sample collection steps were optimal for the extraction of p38 MAPK protein from the heart, the binding between the antibody and the protein G-Sepharose was weak resulting in a poor pull down. Doubling the concentration of the monoclonal p38 MAPK pull down antibody from 4 to 8 µL did not further enhanced on the efficiency of IP (Figure 3-4 B). This was supported by detection of p38 MAPK protein in the supernatant. Increasing the stringency of the detergent, by using 0.5% v/v of Triton X-100 instead of 0.5% v/v NP40 only slightly enhanced the binding between the antigen-antibody complex and protein G-Sepharose (Figure 3-4 C). Using a polyclonal p38 MAPK antibody also failed to further improve the efficiency of the IP (Figure 3-4 D)

Figure 3-4 Representative immunoblots for IP of total p38 MAPK from heart homogenate.

Hearts were homogenised in lysis buffer and subjected to immunoprecipitation. Monoclonal p38 MAPK was used to pull down p38 MAPK from Input (crude homogenate) (A). Doubling the concentration of the antibody from 4 µl to 8 µl did not have any significant effect on the efficiency of IP (B). Using a stronger detergent slightly enhanced the efficiency of Pull down (C). The heart samples were subjected to IP as before, by using a polyclonal rabbit to IP the total p38 protein. This approach did not further improve the quality of the IP (D) +ve: positive control (COS cells transfected with plasmid expressing p38α). (-): negative control containing only beads and antibody in lysis buffer were also included. The IPs for each condition was repeated at least 4 times.
3.5 IP of total p38 and p38 MAPK isoforms using denaturing protocol

The denaturing protocol significantly enhanced the binding between the antibody-antigen complex and the protein beads. Pulled down total p38 was detected by a specific band in the IP samples (Figure 3-5). This band (38kDa) is absent in the negative IP samples which does not contain any antibody. The data suggests that denaturing the heart samples in 1% w/v SDS followed by boiling the samples (at 100 °C for 5 min) as well as increasing the incubation period, increased the efficiency of the IP. P38α MAPK isoform was also successfully precipitated from the crude homogenate. This was confirmed by the absence of a specific band in the negative IP (Figure 3-6 A).

![Figure 3-5 Representative immunoblot of IP for total p38 MAPK using denaturing approach.](image)

Monoclonal p38 antibody (5μL) was use to pull down total p38 (crude homogenate (Input) and probed using a p38 (polyclonal rabbit) antibody. (-): negative control contained beads and antibody in lysis buffer. The IP was repeated at least 6 times.
However, no specific band corresponding to other isoforms of p38 MAPK was observed (Figure 3-6 B-D). Poor efficiency of p38\(\beta\), p38\(\gamma\) and p38\(\delta\) MAPK pull-downs was confirmed by presence of p38 protein in the supernatant of these samples. Failure to IP p38\(\beta\) and \(\delta\) could be explained by the relative low expression of these isoforms in the myocardium. It has been reported that the \(\alpha\) isoform is the most abundant in the heart (Lim et al., 1998).

Although the specificity of the pull down monoclonal p38 MAPK antibody was tested against the GST-tagged recombinant proteins, it was possible that this antibody was unable to detect endogenous expressions of this isoform. Therefore, the next approach was to IP p38\(\beta\), \(\gamma\) and \(\delta\) MAPK isoform using isoform-specific p38 MAPK antibody which were previously shown to be selective in recognising each specific isoforms.

### 3.6 IP of p38\(\beta\), \(\gamma\) and \(\delta\) MAPK isoforms using isoform-specific antibodies

#### 3.6.1 IP of p38\(\beta\) MAPK isoform

To increase the efficiency and selectivity of IP, we used mouse monoclonal anti p38\(\beta\) MAPK antibody for both IP and also immunoblotting. The efficiency of pull down was confirmed by successfully IP the GST-tagged p38\(\beta\) MAPK recombinant protein (expected 68 kDa) from the crude homogenate (highest band in IP with GST-tagged p38\(\beta\) MAPK) (Figure 3-7). However, we were not able to detect any endogenous p38\(\beta\) MAPK in the crude heart homogenate which was also reflected in the negative IP result (Figure 3-7). To date only one study has shown the expression of this isoform in the mouse myocardium (Beardmore et al., 2005). In this study we were not able to detect this isoform in WT mouse hearts.
Figure 3-6 Representative immunoblot of IP for p38 MAPK isoforms using denaturing approach.

Total p38 antibody (5μL) was used to pull down total p38 from crude heart homogenate (Input). The membranes were cut and probed with p38 (polyclonal rabbit) antibody (as a positive control for immunoprecipitation) and monoclonal p38 α (A), p38β MAPK (B), p38γ MAPK (C) and p38δ MAPK monoclonal antibody (D). The IP for each experiment was repeated at least 4 times.
Figure 3-7  Representative immunoblots for IP of p38β MAPK from heart homogenates using isoform-specific antibody.

A monoclonal p38β antibody was used to IP p38β from heart homogenate (Input) of wild type or p38β knock out (p38β KO) and heart homogenates of WT spiked with 5ng of recombinant p38β protein (Input + GST tagged β). Negative controls (-): sample + beads (no pull down antibody) and lysis buffer + beads + antibody were included for the non-spiked IP samples. The (-) in the spiked samples contained lysis buffer + beads only. The second (-) for the spiked samples contained lysis buffer + beads + antibody to ascertain result specificity. The IP was repeated at least 6 times.
3.6.2 IP of p38γ MAPK isoform

A monoclonal antibody against human/mouse p38γ was used for IP and immunoblotting of p38γ MAPK. We were able to pull down both, endogenous p38γ (42kDa) and "spiked" p38γ MAPK (GST-tagged, molecular weight of 68kDa) from crude homogenate and "spiked" crude homogenate respectively. Interestingly, a significant level of endogenous p38γ was observed in crude heart homogenate (Figure 3-8 A) and crude homogenate "spiked" with the recombinant protein p38γ MAPK. These data suggest that p38γ MAPK is abundant in the heart. This was confirmed by including a negative IP control as well as an IP for p38γ MAPK from p38γ/δ KO. The specific band indicating the pulled down p38γ MAPK is absent from both the KO mouse (Figure 3-8 A) and the negative IP which only contains the sample plus beads, thus supporting the selectivity of this antibody.

3.6.3 Immunoprecipitation of p38δ MAPK isoform

A monoclonal antibody against p38δ was used to IP p38δ MAPK. Figure 3-8 B shows successful pull down of GST-tagged p38δ MAPK (68kDa) from the "spiked" crude homogenate. Although this confirms that the pull down of GST-tagged p38δ MAPK (68kDa) was successful, no endogenous p38δ could be detected in crude heart homogenate or in the IP (Figure 3-8 B). This is in keeping with other studies which suggest there is a very small amount if any p38δ MAPK present in the myocardium (Lim et al., 1998).

3.7 Detection of mRNA encoding p38 MAPK isoforms in mouse myocardium

Following RT-PCR we were able to detect mRNAs encoding all four p38 MAPK isoforms (α, β, γ and δ) and GAPDH in murine myocardium from out-bred mice (Figure 3-9 A-D). The transcript for β, γ and δ was absent in DNA isolated from transgenic mice lacking p38β MAPK and p38γ/δ MAPK isoforms respectively. In all cases, the size of each product was identical to known murine cDNA from positive control.
Figure 3-8 Representative immunoblots for IP of p38γ and δ MAPK from heart homogenates using isoforms specific antibodies.

Monoclonal p38γ and δ MAPK antibodies were used to IP p38γ (A) and δ (B) MAPK from input of wild type or p38γδ knock out (p38γδ KO) and input spiked with 5ng of recombinant of p38γ or δ MAPK protein. The first supernatant following IP was also included to assess the efficiency of IP (for p38γ pull down (A) only). Two negative controls (-) which contained sample + beads (no pull down antibody) and beads + antibody (No input) were included to ascertain specificity. The IP for each experiment was repeated at least 6 times.
Figure 3-9 Detection of transcripts encoding p38 MAPK isoforms in mouse myocardium.

Total RNA was extracted from wild type (A-D), p38 β KO (B) and p38γ KO (C). RT-PCR products encoding p38α (A), 320bp; β (B), 544bp; γ (C), 402bp; δ (D), 630bp were detected in mouse heart. GAPDH was included to assess the efficiency of the RNA extraction. Positive (tissue cDNA; skeletal muscle, brain, and kidney) and negative control (no cDNA template) were included. The PCR reaction was repeated in 3 independent experiments.
3.8 Subcellular localisation of p38 MAPK isoforms in isolated mouse myocytes

We next investigated the subcellular localization and expression of these isoforms using the same isoform-specific antibodies used in our immunoprecipitation studies. The confocal analysis of isolated mouse cardiac myocytes showed that total p38 MAPK (corresponding mainly to p38α MAPK isoform) is present in the cytoplasm and in the nucleus (Figure 3-10). p38β MAPK is also localised in the cytoplasm and shows a striated pattern (Figure 3-11) p38γ MAPK presents a punctuate distribution in the cytoplasm of mouse cardiac myocytes (Figure 3-12) and, interestingly, p38δ MAPK expression was observed at the intercalated discs as well as the cytoplasm of cardiac myocytes (Figure 3-13), which may suggest a distinctive role compared with other isoforms which are mainly expressed in the cytoplasm. The specificity of the signal was confirmed by using cardiac myocytes from p38β KO and p38γ/δ KO hearts and a negative control (no primary antibody).

![Figure 3-10 Expression and subcellular distribution of Total p38 MAPK (corresponding mainly to p38α MAP) in isolated mouse cardiac myocytes.](image)

Fresh ventricular cardiac myocytes were isolated from wild type and subjected to immunofluorescence using a total p38 rabbit antibody as well as a mouse α-actinin and DAPI. Negative control contained no primary antibody.
Figure 3-11. Expression and subcellular distribution of p38β MAPK isoforms in isolated mouse cardiac myocytes.

Fresh ventricular cardiac myocytes were isolated from wild type and p38β KO subjected to immunofluorescence using isoform-specific antibodies as well as a rabbit α-actinin and DAPI. Negative control contained no primary antibody.
The data presented here is interesting, as unlike the IP and western blotting experiments, we were able to detect all four isoforms by immunocytochemistry in isolated myocytes. This could be due to the nature of the methods used. In immunocytochemistry, in order to ensure free access of the antibody to the target epitopes, the cells must be fixed and permeabilised. Fixation by cross linking reagents (such as paraformaldehyde) forms intercellular bridges, normally through free amino group, thus creating a network of linked antigens whilst, permeabilisation step allows access of the antibody to the specimen. However unlike immunocytochemistry, the epitopes on the target proteins are hindered by various other proteins in the heart homogenate samples used in western blotting. This could result in poor antibody binding during immunoblotting and consequently a weak signal. This is discussed in more details in the discussion in this chapter. Please refer to section 3.9.3.
Figure 3-13 Expression and subcellular distribution of p38δ MAPK isoform in isolated mouse cardiac myocytes.

Fresh ventricular cardiac myocytes were isolated from wild type and p38γ/δ KO and subjected to immunofluorescence using isoform-specific antibodies as well as a rabbit α-actinin and DAPI. Negative control contained no primary antibody.
3.9 Discussion

This study demonstrates differential expression of p38 MAPK isoforms in murine heart. In our hands, endogenous p38α MAPK and p38γ MAPK were found to be the most abundant isoforms in murine heart and are detected both at protein and RNA transcript levels, whereas p38β and p38δ MAPK were only detected at transcript levels and in confocal analysis of isolated mouse cardiac myocytes.

3.9.1 Selectivity and sensitivity of commercially available p38 isoforms

The selectivity test of the isoform-specific antibodies against GST-tagged recombinant p38β, p38γ or p38δ MAPK revealed a mono-selective binding (Figure 3-1). This suggests that there was little or no cross-reactivity between isoform-specific antibodies. The pan isoform-specific antibody (total p38) was able to detect mainly the α and β and only weakly stained the γ isoforms. This antibody seems to not be able to detect the δ isoforms (Figure 3-1 C). The sensitivity test of the same antibodies against serially diluted (246-0.4 ng per 0.05 mg total protein per well) data, showed that all antibodies were able to detect recombinant proteins as low as 16-6 ng/0.05 mg total protein per well (p38α, β and δ MAPK antibody) and 1 ng per /0.05 mg total protein per well (p38γ MAPK antibody) however, only endogenous p38α (0.59 nmoles/well) and γ (0.13 nmoles/well) were detected in crude heart homogenates (Figure 3-2 A and D) and are most abundantly expressed compared to the β and δ isoforms (Figure 3-3 A-D). Although the monoclonal antibodies are, as expected selective for each isoform, the pan polyclonal p38 MAPK antibody appear to be selective against the α, β and γ isoforms. Overall, the monoclonal antibodies are best used in immunoprecipitation protocol or any other experiments investigating a specific isoform as opposed to the pan p38 MAPK which appear to have selectivity mainly against the α and β isoforms.

3.9.2 Optimising the IP protocol

Various conditions were examined in order to increase the efficiency of the binding between p38 MAPK and the monoclonal p38 MAPK antibody. In these experiments a more stringent detergent was used; 0.5% v/v Triton X-100 instead of 0.5% v/v NP40. This partially increased the efficiency of the IP (Figure 3-4 A). Increasing the concentration of the pull down antibody however, did not have any effect (Figure 3-4 C) on the outcome. In addition,
we used a polyclonal rabbit antibody for the pull down to increase the probability of antigen-antibody interactions and hence the efficiency of the IP. This approach also failed to result in a successful pull down (Figure 3-4 D). Therefore, we tried a “denaturing” approach which required homogenising the hearts in lysis buffer containing 1% w/v SDS followed by boiling the homogenates at 100°C for 5 min and centrifugation at a higher speed, 14,000 rpm instead of 2,000 rpm to remove insoluble cell debris. We also increased the incubation period for the antigen-antibody complex and Protein G beads from 2 h to 18 h overnight at 4°C. This approach appeared to be successful and was adopted in our future experiments (Figure 3-5).

3.9.3 Distinct protein and gene expression of p38 MAPK isoforms in murine myocardium

Northern blot and dot blot analysis of m-RNA encoding the four isoforms have shown a wide tissue distribution of p38 MAPK isoforms in human and rodent tissues (Cuenda et al., 1997b;Goedert et al., 1997a;Jiang et al., 1996;Jiang et al., 1997;Li et al., 1996;Wang et al., 1997). p38α and β MAPK are ubiquitously expressed (Jiang et al., 1996) whereas p38γ and δ MAPK are differentially expressed in different tissues (Cuenda et al., 1997a;Cuenda et al., 1997b;Lechner et al., 1996;Li et al., 1996). The m-RNA expression of p38γ MAPK has been shown to be highly expressed in skeletal muscle (Mertens et al., 1996) and is thought to play a role on skeletal muscle differentiation (Lechner et al., 1996). However, apart from lower level of p38γ MAPK mRNA in microarray analysis of Duchene muscular atrophy patients’ skeletal muscle samples, little is known about its function (Chen et al., 2000). Northern blot hybridization of human p38δ m-RNA has shown that this isoform is enriched in lung, kidney small intestine, testis and pancreas (Jiang et al., 1997;Kumar et al., 1997). In a recent report, quantitative real-time RT-PCR of human CF cultured from biopsies of right atrial appendage revealed m-RNA expression of all four isoforms (Turner et al., 2008). The data demonstrated that p38α MAPK m-RNA was expressed at the highest level in CF followed by the γ and δ isoforms. The β isoform was expressed at the lowest level (Turner et al., 2008).

The protein expression and activation of endogenous p38 MAPK isoforms remains poorly characterised with limited studies being performed in immortalised cell lines (Conrad et al., 1999;Wang et al., 2000). In one study, it was shown that when p38 MAPK isoforms are over-expressed in various human cell lines, they demonstrate different patterns of expression as detected by western blotting. For instance p38 α and β MAPK can be detected in Jurkat,
293 fibroblasts, epG2, U937 and Hela cell lines. However, ectopically expressed p38γ is only detected in 293, HepG2, U937 and Hela cells whereas p38δ MAPK is only detected in Jurkat, 293 and HepG2 cell lines (Jiang et al., 1997). The protein expression of p38 MAPK isoforms varies in the myocardium across species. To date, the dominant protein expression of p38α MAPK has been confirmed by numerous studies in the mammalian myocardium in human as well as rodents’ heart. However, there are contradictory studies regarding the expression of p38β MAPK isoform and the presence of this isoform in the myocardium has not yet fully been confirmed (Beardmore et al., 2005; Lemke et al., 2001). The cardiac expression of the p38γ endogenously expressed in mouse, rat, human pig and dog heart was recently investigated (Court NW et al., 2002). In the same study, the localisation of this isoform in cultured neonatal rat cardiac myocytes over-expressing the γ isoform (Court NW et al., 2002) demonstrated a punctuate distribution. The characteristics of exogenously over-expressed p38γ MAPK (introduced by transient transfection) have also been studied at protein level in C2C12 myoblasts and in human epithelial KB cells (Cuenda and Cohen, 1999; Lechner et al., 1996). The protein expression of endogenous p38δ MAPK isoform in isolated mouse cardiac myocytes has shown that this isoform is barely detectable (Nishida et al., 2004).

To date p38α MAPK has attracted the greatest level of scientific interest since it is the most abundant. However, since there are no isoform-specific pharmacological inhibitors of p38 MAPK activity, the contribution of each isoform in the heart remains unclear. Therefore, in order to elucidate the net effect of activation of p38 MAPK isoforms, characterisation of relative abundance and localisation of these isoforms in the heart is essential.

Using the optimised IP protocol in this Thesis, we were able to detect total (pan-isoform) p38, endogenous p38α and p38γ MAPK isoforms from the perfused crude heart homogenate. Despite the ability to pull down recombinant tagged p38β and p38δ MAPK, we were not able to pull down the endogenous form of these isoforms (Figure 3-7 and Figure 3-8 B). However, contrary to our pull-down observations, we were able to detect mRNA encoding p38α and p38γ MAPK as well as p38β and δ MAPK in mouse heart (Figure 3-9 A-D). The immunocytochemistry data presented demonstrates that total p38 (pan) MAPK isoform is localised in the cytoplasm and the nucleus (Figure 3-10), p38β MAPK is also localised in the cytoplasm and the nucleus and presents a slight striated pattern (Figure 3-11) whereas p38γ
MAPK presents a punctate distribution (Figure 3-12) with p38δ MAPK localised in the cytoplasm as well as the intercalated discs (Figure 3-13).

The contradicting data obtained in gene and protein expression of p38 MAPK isoforms in the IP, western blotting, RT-PCR and the immunocytochemistry of the isolated cardiac myocytes can be interpreted by taking the following into account; 1) the types of methods (western blotting vs. RT-PCR vs. immunocytochemistry) 2) the models (whole heart tissue vs. isolated myocytes in immunocytochemistry) and finally 3) the apparent differential level of expression of p38 MAPK isoforms. The data in this chapter suggests that p38β and p38δ MAPK are relatively expressed at low levels compared with the α and γ isoforms (Figure 3-3 A-D). This can be confirmed by their relatively low protein abundance (less than 1.8 nmol/mg in murine myocardium which may well be below the detection level of the antibodies. This can be observed in the antibody sensitivity test (Figure 3-1 B) where we showed that the antibodies used to detect p38β and δ MAPK can detect recombinant proteins at a concentration of greater than 1.8 nmol/mg (Figure 3-1 B & C and Figure 3-3 B & C).

In general, the proteins (p38β and p38δ) that are expressed endogenously at very low levels present fewer numbers of epitopes and are therefore more difficult to detect by an antibody. In addition the epitopes may become lost by denaturation by SDS during the process of western blotting. Unlike immunoblotting, in immunocytochemistry, a perfect fixation of the target cells would immobilise the epitopes whilst maintaining the authentic cellular and subcellular architecture and permit unhindered access of the antibodies to all cells and subcellular compartments. Under these circumstances, the antibodies can access and bind the epitopes, despite the low number of available epitopes. It can, therefore, be postulated that our inability to detect the β and δ isoforms, in western blotting and IP experiments, might have been due to the low number of available epitopes and poor antibody access.

Another key fact to also consider is that, although a negative control (containing no cDNA) was included in our PCR reaction, we did not include a sample without the RT-enzyme to ascertain the absence of genomic DNA contamination in our PCR reaction. Although we did include heart samples from the null mice and also used a DNase step during RNA isolation, it is possible that the band we observed for p38β and δ MAPK in the PCR reaction be due amplification of genomic DNA not mRNA.
3.9.4 Different subcellular localisations of p38 MAPK isoforms in isolated cardiac myocytes

The diverse expression of p38 MAPK isoforms observed in ventricular mouse myocytes can suggest different functions. For instance, localisation of p38α and β MAPK in the cytoplasm may suggest that these isoforms control multiple cellular functions. p38α MAPK has previously been shown to be present both in the nucleus and the cytoplasm of quiescent cells, but upon stimulation, some studies suggest that it translocates from the nucleus into the cytoplasm (Raingeaud et al., 1995). The substrate specificity of p38 MAPK isoforms is determined by the targeted amino acids, specific docking domains present on the substrate protein and by specific substrate binding motif in the p38 MAPK (Eyers et al., 1999). p38α and β MAPK share most structural homology compared with p38γ and δ MAPK. As a result there are some differences in p38α and β versus p38γ and δ MAPK with regard to substrate selectivity of these kinases. Some of the physiological substrates of p38α and β MAPK have been shown to be transcription factors, other kinases, cytoskeletal proteins and translational machinery components and other proteins such as glycogen synthase or cytosolic phospholipase A2 (Braz et al., 2003; Pfeffer and Braunwald, 1990).

Intracellular analysis of p38γ MAPK in isolated cardiac myocytes revealed that this isoform is localized throughout the cytoplasm (Figure 3-12) which is in keeping with the previous findings of Court and co-workers in cardiac myocytes over-expressing p38γ MAPK (Court NW et al., 2002). p38γ MAPK is the only MAPK that has a PDZ domain binding sequence (Hasegawa et al., 1999). The presence of this PDZ domain in p38γ MAPK gives this isoform a unique characteristic among all MAPK family members enabling it to dock with PDZ domains of different proteins in the cytoplasm. This is reflected in its relatively large abundance in the cytoplasm of isolated cardiac myocytes as shown by our confocal analysis.

Presence of p38γ MAPK isoform in the cytoplasm may therefore allow this isoform to form multiple complexes which can then be targeted to the cytoskeleton whereby it can regulate the integrity of intercellular-junctional complexes, cell shape and volume in response to various stimuli such as osmotic stress (Sabio et al., 2005). Conserved cardiac expression of p38γ MAPK amongst several different species together with its considerable protein expression (almost as abundant as p38α MAPK isoform), suggests that this isoform may play an important role in the heart and therefore is unlikely to be functionally redundant. Thus,
identifying the PDZ domain containing proteins that binds to p38γ MAPK will provide an important clue to elucidate the specific function of this isoform in cardiac myocytes.

On the other hand p38δ MAPK has been reported to phosphorylate stathmin, a cytoplasmic protein, which has been shown to regulate microtubule dynamics (Parker et al., 1998). In addition, our data demonstrates that p38δ MAPK is mainly localised at the interacted discs (Figure 3-13) which serves to anchor actin filaments, to bind adjacent ventricular myocytes to each other, and to communicate between cells. These findings may therefore suggest a common theme in p38 MAPK pathway activation in the re-organisation of the cytoskeletal framework to enhance cell survival in times of stress such as ischaemia.

In conclusions, we have demonstrated that all p38 MAPK isoforms are expressed in the murine heart with p38α and γ MAPK being the most abundant. p38β and δ MAPK seemed to be expressed at lower abundance detected only in isolated cardiac mouse myocytes by confocal microscopy. The transcripts for all of the p38 MAPK isoforms were detected in murine heart.

To date, the immediate downstream targets of specific p38 MAPK isoforms that aggravate myocardial injury are still largely unknown. Characterising the expression and localisation of p38 MAPK isoforms in the heart can provide an insight in the physiological roles of each isoform and identify their mechanism of activation and potential substrates. These findings will be of great clinical interest as they will provide important avenues that may lead to pharmacological inhibitors with greater selectivity and avoid the potentials drawbacks of chronic systematic inhibition.
4 INVESTIGATING THE FUNCTIONAL ACTIVATION OF P38 MAPK ISOFORMS IN RESPONSE TO CYTOKINES AND CELLULAR STRESS IN ISOLATED PERFUSED HEART

4.1 Introduction

Pro-inflammatory cytokines (such as TNF-α, IL-1) as well as osmotic stress play important roles in the pathogenesis and pathophysiology of ischaemic heart disease. In both patients and experimental animal models, the induction of cytokines is closely associated with interstitial fibrosis and the progression of cardiac remodelling (Yin et al., 2008). Inflammatory stimuli activate many intracellular pathways including MAPKs (p38, ERK, JNK) signalling. Among them, p38 MAPK is considered to be a central regulator of inflammation (Zhang et al., 2007b).

A substantial amount of research has been dedicated to exploring the role of inflammatory cytokines such as TNF-α and IL-1 during various myocardial stresses. Clinical studies have demonstrated a strong correlation between plasma TNF-α levels and the progression of left ventricular remodelling and heart failure (Deswal et al., 2001;Torre-Amione et al., 1996;Zhang et al., 2007b). Moreover, in animal models, chronic infusion or cardiac-specific overexpression of TNF-α results in LV dysfunction and heart failure (Bozkurt et al., 1998;Bryant et al., 1998). Recently it was also demonstrated that administration of the p38 MAPK inhibitor, SB203580, in rats with myocardial ischaemia (MI) (injected i.p. once every three days following MI) decreased TNF-α production, attenuated pathologic LV remodelling and dysfunction (Yin et al., 2008). As described previously, IL-β (IL-1) induction is mainly elevated in the infarcted myocardium. IL-1 alone or in combination with interferon γ and TNF-α, induces cardiomyocytes apoptosis, associated with activation of Bak and Bcl-xL through pathways involving nitric oxide (NO) (Ing et al., 1999). Furthermore, IL-1 induces cardiomyocytes hypertrophy (Palmer et al., 1995), upregulating arterial natriuretic factor and suppressing the expression of calcium regulatory gene (Thaik et al., 1995). IL-1 inhibits contractility in isolated heart (Schulz et al., 1995) and cardiac myocytes (Gulick et al., 1989;McTiernan et al., 1997) and stimulates proinflammatory cytokine
expression in human cardiac myoFb (Turner et al., 2009). IL-1 inhibits β-adrenergic agonist mediated increase in cardiac myocytes contractility and cAMP accumulation which can result in poor cardiac function (Gulick et al., 1989). Beyond its pro-inflammatory and fibrogenic properties, IL-1 also promotes ECM remodelling by enhancing CF MMP-3, -8 and -9 expression (Siwik et al., 2000). In a study by Cuenda et al., it was shown that p38γ MAPK is also strongly activated in vitro (HEK293 cells) in response to sorbitol, TNF-α and IL-1 (Cuenda et al., 1997a). IL-1 is also capable of modulating fibroblast phenotype and activity. It diminishes the capacity of mitogen-stimulated fibroblasts to synthesize DNA and exerts its effects at the G1/S interphase by altering the expression of CFs cyclins and cyclin-dependent kinases (Koudssi et al., 1998). IL-1 directly enhances fibrous tissue deposition by up-regulating the expression of Ang II receptor 1 on CF (Gurantz et al., 2005) and by stimulating fibroblasts migration (Mitchell et al., 2007). TNF-α and IL-1 have been shown to un-couple agonist-occupied receptors from adenylate cyclase in isolated myocytes (Gulick et al., 1989). These findings implicated guanine nucleotide binding protein (G-protein) function in the direct or indirect action of cytokines on the heart. G-protein mediated depression of cardiac myocyte L-type calcium channels by IL-1 has been reported (Liu and Schreur, 1995).

It has been shown that hyperosmotic stress induced by sorbitol rapidly stimulates apoptosis in cultured cardiomyocytes (Galvez et al., 2001) possibly by acting through aldose reductase. Aldose reductase (AR) is the first enzyme in the polyol pathway, which helps to promote resistance of cells to anisotonic perturbations. AR catalyzes the formation of sorbitol from glucose using NADPH as a cofactor (Srivastava et al., 1984). Activation of AR also has been shown to play an important role in myocardial I-R injury (Ramasamy et al., 1997;Ruef et al., 2000). Although all of the p38 MAPK isoforms are activated in mammalian cells in response to hyperosmotic stress, the activation of p38γ MAPK is particularly rapid and strong compared to other p38s (Goedert et al., 1997a;Sabio et al., 2004). However, its activation in the heart in response to osmotic stress (a component of I-R) has not yet been investigated.

A number of studies in vitro have suggested a protective role for p38β MAPK. For instance recent work by Kim and co-workers have shown that activation of p38β MAPK by carbon monoxide promotes the nuclear translocation of heat shock factor-1 (HSF-1), which regulates the expression of cardioprotective HSP70 in cells (Kim et al., 2005). Using adenoviral-mediated transfection, our lab had previously shown that p38β MAPK is deactivated and p38α MAPK is activated, following 2.5 h of stimulated ischaemia.
Moreover inhibition of the α isoform, but not β, led to an increase in cell viability and protection (Saurin et al., 2000). It has been demonstrated that pharmacological inhibition of p38α and β MAPK during preconditioning blocks protection (since β is the dominant form activated), while during lethal ischaemia the same inhibitors at identical concentrations cause protection (when the α isoform is activated). These studies therefore, support the concept that ischaemic preconditioning is the result of selective activation of p38β MAPK. The protective role of p38β MAPK has also been investigated in a recent study which showed that activation of this isoform by CO promotes the nuclear translocation of HSF-1 (Kim et al., 2005). HSF-1 regulated the expression of cytoprotective HSP70 in cell and tissues (Kim et al., 2005) and can also serve as a regulator of IL-1 and TNF-α (Xie et al., 2002). These observations are supported by CO-mediated protection of endothelium against endotoxic shock being abolished in p38β KO mice (Kim et al., 2005). Interestingly, it was recently reported by our group that p38α MAPK is the important isoforms in ischaemic preconditioning (Sicard et al., 2010). In this study the drug resistant p38α (DRα) and p38β (DRβ) hearts (with the gate keeper threonine substituted with a methionine) were subjected to ischaemic pre-conditioning in presence and absence of SB203580 (Sicard et al., 2010). The data demonstrated that SB203580 abolishes the reduction in myocardial infarction and the phosphorylation of downstream substrates only in WT and DRβ mice. These effects were absent in DRα hearts. Furthermore, ischaemic pre-conditioning occurred unaltered in p38β null hearts. These findings suggest that the p38α isoform is involved in ischaemic pre-conditioning (Sicard et al., 2010). However, the role of p38β in response to pharmacological preconditioning is unknown.

4.2 Aims

Due to the lack of selective pharmacological inhibitors for each p38 MAPK isoform, the contribution of individual isoforms in the response to inflammatory cytokine, ischaemia and I-R injury have not been investigated. The aims of the present study are to examine the contribution of p38γ and δ isoforms in response to these stresses using WT and p38γ/δ KO mice. In addition, we explore the possible protective role of p38β MAPK in response to ischaemia-reperfusion injury (I.R) and preconditioning by using a novel water soluble CO-releasing molecule (CORM-3) in WT and the p38β targeted mouse line.
4.3 Specific Methods

4.3.1 Langendorff perfusion protocol

WT or transgenic hearts (p38γδ KO) were subjected to 40 min of stabilisation period with Krebs buffer under a constant pressure of 80 mmHg. The hearts were then subjected to 30 min of either ischaemia followed by 120 min of reperfusion (Figure 4-1 A) or 30 min of ischaemia alone (Figure 4-1 B).

In the preconditioning study hearts of WT and p38β KO mice were perfused with buffer containing drug (CORM-3 and/or p38 inhibitor, SB203580) following stabilisation period (Figure 4-2 A and B). The hearts were then treated with 30 min of ischaemia followed by 120 min of reperfusion (Figure 4-2). All the Langendorff perfusions using CORM-3 were performed by Dr James Clark.

To test the effects of cytokines (TNF-α and IL-1α) and osmotic stress (sorbitol) on p38γ and δ MAPK, WT and p38γ/δ KO hearts were perfused under the above conditions for a period 15 min following stabilisation (Figure 4-3 A). The control group were perfused with Krebs solution for 40 min and further 15 min with Krebs (Figure 4-3 B).

Haemodynamic parameters were continuously measured including coronary flow and left ventricular developed pressure (LVDP). At the end of the experiment the hearts were removed and snap frozen in liquid nitrogen for analysis by western blotting as described in Chapter 2, section 2.6).
Chapter 4  Response of p38-MAPK isoforms to cellular stress

Figure 4-1 Langendorff perfusion protocol for ischaemia (A) and I-R experiments (B) in WT, p38βKO and p38γδ KO.

In IR protocol, hearts stabilised for 40 min and made ischemic for 30 min followed by 120 min of reperfusion (A). The experiment was ended by removing and freezing the hearts in liquid nitrogen. In ischaemia protocol hearts were removed snap frozen after 30 min of ischaemia (B).

Figure 4-2 Langendorff perfusion protocol for preconditioning experiments in WT and p38β KO.

After 35 min stabilising, hearts were perfused for 15 min with CORM-3 without (A) and with SB203580 (B) and subjected to 30 min of ischaemia and 120 min reperfusion.

Figure 4-3 Langendorff perfusion for cytokines and osmotic stress experiments in WT and p38γδ KO.

Hearts were stabilised for 40 min before being perfused with sorbitol, TNF-α and IL-1 (A) or with Krebs buffer in control group (B).
4.3.2 Assessment of infarct size

At the end of reperfusion in the preconditioning study, hearts were perfused with 5 mL of 1% triphenyl tetrazolium chloride (TTC) in Krebs buffer at a flow rate of approximately 2 mL/min and then placed in an identical solution for 15 min at 37°C and fixed in 4% paraformaldehyde at 4°C. The following day hearts were set in 5% agarose and sliced from apex to base in 750 µm slices and scanned (Epson, UK) as before. Images were analysed using image analysis software (Sigma Scan Pro, SPSS) as described in Chapter 2 (section 2.6.5).

4.3.3 Immunoprecipitation of phospho p38 MAPK

Hearts tissues subjected to 10 min of ischaemia were homogenised in lysis buffer (1000 µL/1g tissue) in ice-cold lysis buffer containing 0.01% SDS (w/v) and 0.5% Triton x-100 (v/v). Crude heart homogenates (Input) were heated at 95°C for 5 min and spun at 14,000 rpm for 15 min at 4°C. The supernatant or input (200 µL) was removed by pipetting and placed into new tubes and subjected to immunoprecipitation by adding 5 µL of monoclonal anti-phospho p38 antibody (Cell Signaling # 9216) and 50 µL of G-protein. The samples were then incubated with rotation overnight at 4°C. The beads were washed three times with ice-cold lysis buffer containing 0.5% Triton X-100 (v/v) to remove un-bound protein, prior to re-suspension in 30 µL 2× sample loading buffer containing 10% (v/v) β-mercaptoethanol and bromophenol blue. The samples were then heated for 5 min at 95°C and loaded (20 µL) on 12.5% SDS polyacrylamide gels.

4.3.4 Western blotting

Heart samples were run on 12.5% polyacrylamide gels as described previously in Chapter 2, section 2.8.4. The PVDF membranes were incubated with polyclonal rabbit phospho p38 (T180/Y182) (Cell Signaling # 9211) polyclonal rabbit p38 (Cell Signaling # 9212) and monoclonal mouse p38γ (R&D Systems) (all at 1:1000 dilutions) overnight at 4°C. The blots were washed with TBST three times for 10 min prior to incubation with secondary antibody at a 1:2000 dilution for 2 h at room temperature. The antibody complexes were detected using a peroxidise conjugated anti-rabbit IgG or anti-mouse IgG secondary antibody (Amersham) accordingly.
4.3.5 Enzyme Linked Immuno-Sorbent Assay ELISA

Heart samples were homogenised and activity of phospho-p38γ (Pp38γ) was analysed using a commercially available ELISA kit (R&D Systems) as described in Chapter 2, section 2.10).

4.3.6 Statistical analysis

All values are expressed as mean ± SEM except for western blotting data where representative blots are shown and quantified where applicable. Functional data in Langendorff perfusions including data for functional recovery at the end of reperfusion were analysed by one-way ANOVA. A p value of less than 0.05 was considered significant.
4.4 Results

4.4.1 Development of appropriate methods in distinguishing between the activation of p38 MAPK isoforms

4.4.1.1 Immunoprecipitation

Previously, although we were able to detect all four isoforms at the m-RNA transcript level and by confocal microscopy of isolated cardiac myocytes, we were only able to specifically IP the α and γ isoforms from heart homogenates. Using the optimised protocol for immunoprecipitation of p38α and γ MAPK isoforms, we initially examined the activation of these isoforms in response to ischaemia. Using a monoclonal phospho p38 antibody, we were able to pull down phosphorylated (Pp38) from homogenates of ischaemic hearts (Figure 4-4 A). This was confirmed by immunoblotting the pull down samples using a polyclonal phospho specific antibody. When the same samples were probed with the monoclonal p38γ antibody, we observed a band which ran at the expected molecular weight (Figure 4-4 B black arrow). However, since this band was also present in the pull down samples of p38γ/δ KO and in the negative control (with no pull- down antibody), it was determined to be a non- specific band of a similar molecular weight to p38 MAPK.

These observations clearly indicated that although we can detect Pp38α in the pull down samples, we could not rely on the developed immunoprecipitation protocol to investigate the activation of p38γ MAPK. Therefore we decided to utilise a commercially available ELISA kit to elucidate the specific phosphorylation of p38γ MAPK in heart tissues.
Figure 4-4 Representative immunoprecipitation of Pp38 MAPK from ischaemic (10 min) hearts.

Following immunoprecipitation of Pp38 MAPK using a monoclonal phospho specific antibody from crude heart homogenate (input), the immunoblots (IB) were probed with anti-Pp38 (A) and p38γ (B). Negative control (-) in lane 3 of Pp38 and lane 6 of the p38γ blot contained samples plus agarose beads. Negative control (-) in lane 3 of the p38γ blot contained lysis buffer and beads only. A positive control (+ve), HEK 293 overexpressing p38α MAPK was also included. Black arrow indicates a non-specific band which was present in the negative IP and IP from a p38γ/δ KO heart.

4.4.1.2 Enzyme-Linked Immunosorbent Assay (ELISA)

We first analysed the level of p38 MAPK phosphorylation in control and ischaemic heart samples using the polyclonal pan-isoform phospho p38 MAPK antibody before subjecting them to ELISA. The immunoblots confirmed a high phosphorylation of p38 MAPK in ischaemic hearts of both WT and p38γ/δ KO compared with controls with an identical molecular weight (Figure 4-5).
Figure 4-5 Representative immunoblots showing the activation of p38 MAPK after 10 min of ischaemia in isolated heart

The phosphorylation of p38 MAPK was assessed in control and after 10 min of ischaemia in WT and p38γ/δ KO hearts using the Pp38 MAPK (polyclonal pan-isoform antibody). The blots were also probed with total p38 MAPK antibody to show equal loading in each well.

It is noteworthy to mention that the Pp38 pan antibody does not generate a doublet corresponding to p38α MAPK and p38γ MAPK (at higher molecular weight). Although the generation of a double band corresponding to different p38 MAPK isoforms was previously shown by Kuma et al (2005) and Conrad et al (1999) (Conrad et al., 1999;Kuma et al., 2005), we were unable to reproduce this observation in our experiments on crude heart homogenates using the phospho-specific antibody. We were, however, able to show the difference in the molecular weights of the two isoforms by loading the samples onto a 12.5% larger gel as opposed to the standard mini gels we used previously. Using the same immunoblotting conditions as before, we were able to resolve a higher molecular weight for p38γ MAPK compared to the total and p38α MAPK in non-ischaemic heart homogenate (Figure 4-6 A). This observation was encouraging; therefore we repeated the same technique on heart homogenates treated with ischaemia to explore the contribution of each isoform. In this experiment, samples were loaded onto a large gel and ran for 3 h as before. The samples were then probed with phospho-specific antibody (Cell Signalling # 9211), as well as p38γ MAPK and total p38 antibodies. However despite our efforts, we were unable to observe a distinct molecular weight for these isoforms in the ischaemic heart homogenate when the samples were probed with the phospho-specific antibody (Figure 4-6 B).
Figure 4-6 Immunoblots of non ischaemic (A) and ischaemic (B) heart homogenates loaded onto a big gel.

Hearts samples were loaded onto a 12.5% gel and ran for 3 h to. After the transfer, the membranes were cut into three. Non-ischaemic heart samples (A) were probed with total p38, p38γ, p38α. Ischaemic heart samples (B) were probed with total p38, p38γ and phospho-specific p38 antibody. A higher molecular weight was observed only in the non-ischaemic samples probed with total and p38α antibodies compared with samples probed with p38γ (A).

To analyse the activation of p38γ MAPK by the ELISA, first a typical standard curve used to calculate concentration of Pp38γ MAPK (pg/mL) shown in Figure 4-7. Following analysis of the data, a high level of Pp38γ MAPK was detected in control samples from p38γ/δ KO (Figure 4-8) which considered to be background and non-specific. The sandwich ELISA used in this chapter utilises a capture (p38γ MAPK specific) antibody which binds the sample antigen to form a complex. The detection (phospho-pan, Pp38) antibody is added next which then binds to the capture antibody-antigen complex, hence the name sandwich ELISA. The whole complex is detected by the addition of an enzymatic linked secondary antibody. The plate is washed between stages with a wash buffer containing a detergent to remove the unbound antigens. It is, therefore, likely that the non-specific signal observed in the present study was associated with poor washing. To eliminate this possibility, the ELISA was repeated at least three times according to the protocol provided by the kit. Despite this, we were unable to resolve this problem. It is therefore possible that, the detergent was not strong enough to remove the unbound antigens. Consequently, when the detection antibody
was added, a non-specific antigen, antibody cluster (consisting of the capture antibody, non-target sample antigen-detection antibody) was generated which was identified by the secondary antibody. Due to the high background we were unable to use this ELISA for our subsequent experiments to investigate the activation of p38γ MAPK in whole heart tissue.
Chapter 4

Response of p38-MAPK isoforms to cellular stress

Figure 4.7 A typical standard curve used to calculate the activation of p38\(\gamma\) MAPK.

A standard curve was generated for each set of samples using the phospho-p38\(\gamma\) MAPK (T183/Y185) recombinant protein (n=3).

Figure 4.8 Representative ELISA to measure activation of p38\(\gamma\) MAPK in hearts.

Hearts from WT and \(\gamma/\delta\) KO subjected to 10 min of ischaemia. Hearts were homogenised in lysis buffer and subjected to both ELISA. Values are mean ± SEM, n=3.
4.4.1.3 Immunoblotting using Phospho (pan-isoform) p38 antibody

Since neither the immunoprecipitation nor the ELISA provided a satisfactory solution to elucidate p38γ MAPK activation, we used a polyclonal phospho (pan-isoform) p38 MAPK antibody (Cell Signaling #9211) in our future experiments to examine the activation of this isoform in response to various stimuli. We examined the activation of each isoform by comparing the phosphorylation of p38 MAPK (detected by the Pp38 pan-isoform antibody) in WT against KO animals. The same antibody was also used in all the future experiments investigating the activation of other isoforms.

4.4.1.4 Effects of ischaemia on activation of p38 MAPK isoforms

Using the polyclonal phospho specific (pan-isoform) antibody, we found no difference in the level of p38 MAPK phosphorylation following 10 min of ischaemia in WT hearts compared with either p38β KO or p38γ/δ KO (Figure 4-9 A-E).

4.4.1.5 Effects of ischaemia reperfusion injury on activation of p38 isoforms

There was no significant difference in baseline parameter of either p38β KO or p38γ/δ KO compared with WT. There was a significant reduction in left ventricular pressure in response to 30 min of ischaemia followed by 120 min of reperfusion in WT, p38β KO and p38γ/δ KO (Figure 4-10 A). However, there were no significant differences in the functional parameters between either of the null mice compared with WT group. Analysis of the infarct size following the I-R also revealed no significant difference in p38β KO and p38γ/δ KO compared with WT (Figure 4-10 B). These findings, therefore, suggest that elimination of p38β, p38γ and or δ MAPK isoform has no effect on functional recovery or infarct size, following I-R injury.
Figure 4-9 Representative immunoblots of p38 MAPK activation after 10 min of ischaemia in WT and p38γδ KO in Langendorff perfusion.

Hearts were homogenised in lysis buffer and activation of p38 MAPK (Pp38) in response to ischaemia was analysed by probing the immunoblots with phospho specific (pan-isofrom) antibody in WT (A) p38β KO (B) and p38γδ KO (C). Quantification of p38 MAPK phosphorylation after treatment with 10 min of ischaemia in WT and p38β KO (D) and p38γδ KO (E) hearts. Values are mean ± SEM of 6 independent experiments.
Figure 4-10 The Analysis of LVDP and infarct size in hearts from WT, p38β KO and p38γδ KO subjected to ischaemia followed by 120 min reperfusion.

WT, p38γδ KO and p38β KO hearts were perfused in Langendorff mode according to the protocol described before (section 4.3.1). Left ventricular function (LVDP) (A) and measurement of infarct size by tetrazolium staining (B) in isolated heart subjected to 30 min global ischaemia and 120 min reperfusion. Functional values are mean ± SEM, n=6-7.
4.4.2 Effects of pharmacological preconditioning on p38β MAPK isoform

To explore our hypothesis that p38β MAPK is involved in CORM-3 mediated cardioprotection, haemodynamic parameters were continuously monitored and infarct size measured in WT and p38β KO mice subjected to an I-R protocol (described in Figure 4-2). The data revealed that after 120 min reperfusion, LVDP decreased to 25% of baseline in vehicle treated hearts whilst hearts perfused with CORM-3 showed a significantly improved recovery (52% of baseline, p<0.05) (Figure 4-11 A). The resultant infarct size measured with tetrazolium red staining at the end of the reperfusion phase correlated with the observed functional recovery, with CORM-3 reducing infarct size from 49% to 20% heart volume in WT hearts (p<0.05), whereas hearts from p38β mice had similarly sized infarcts to those of the same genotype exposed to vehicle alone (Figure 4-11 B).

These observations may suggest a functional role for the p38β MAPK isoform in CORM-mediated cardioprotection.

The experiments with CORM in this section of the Thesis were performed by Dr James Clark.
Figure 4-11 Functional recovery and infarct size in hearts subjected to ischaemia/reperfusion in the presence of CORM-3.

Left ventricular function (LVDP) (A) and measurement of infarct size by tetrazolium staining (B) in isolated hearts subjected to 30 min global ischaemia and 120 min reperfusion from WT and p38β KO mice after exposure to vehicle or CORM-3. Values represent mean ± SEM of n=6-7 independent experiments.
4.5 Effects of cytokines and osmotic stress on p38γ and δ MAPK isoforms

4.3.5.1 TNF-α and IL-1 Cytokines

There was a significant reduction (p<0.01) in left ventricular developed pressure in WT and p38γ/δ KO following treatment with TNF-α (20 ng/mL) after 10 min and 15 min (Figure 4-12 A). The reduction in the left ventricular pressure of also noted after 5 min in the WT hearts only (Figure 4-12 A). Although a similar trend was observed in the coronary flow (Figure 4-12 B) the data did not reach significant difference. Treatment with IL-1 (20 ng/mL) resulted in a significant reduction (p< 0.05) of the LVDP of the null mice after 5 min. This response was absent in the WT counterparts. The LVDP remained unchanged in both genotypes after 5 and 10 min of perfusion with IL-1 (Figure 4-14 A). There was no significant change in the coronary flow in either genotypes post treatment with IL-1 (Figure 4-14 B). When the heart samples of TNF-α and IL-1 were analysed by western blotting (using the same phospho-specific (pan) antibody), no significant difference was observed in the level of p38 MAPK phosphorylation in WT compared with transgenic group (Figure 4-13 A-C, Figure 4-15 A-C respectively).

A number of studies have shown that p38 MAPK activation is at least in part responsible for the early cardiodepressant action of TNF-α (Bellahcene et al., 2006;Li et al., 2005;Tanno et al., 2003). In one study, SB203580 attenuated TNF-α induced dysfunction in isolated mouse heart under constant flow perfusion (Bellahcene et al., 2006). In the same study, pre-treatment with 1 µM SB203580 abolished the TNF-α effect on contractility and inhibited the rise in perfusion pressure under constant flow (Bellahcene et al., 2006). In addition, SB203580 blunted p38 MAPK phosphorylation as well as HSP27 phosphorylation. The reduction in dual phosphorylation may reflect the component arising from coronary vasoconstriction (Gorog et al., 2004). It has been suggested that the ability of SB203580 to prevent coronary vasoconstriction may be due to TNF-α induced p38 MAPK activation within vascular smooth muscle which has known to cause contraction (Yamboliev et al., 2000). The involvement of p38 MAPK in the development of cardiac dysfunction has also been demonstrated in transgenic mice overexpressing the upstream activator MKK6 (Li et al., 2005). These mice have shown to display a marked elevation of cytokines such as TNF-α and IL-6. Oral administration of SB239068 in these mice resulted in a significant reduction
in the plasma level but an increase in the intracardiac accumulation of both cytokines (Li et al., 2005). These mice also displayed impaired haemodynamic function and inhibition of p38 MAPK improved cardiac performance and prolonged survival (Li et al., 2005).

Our data together with the previous findings (Bellahcene et al., 2006; Li et al., 2005; Tanno et al., 2003) imply that the cytokines released in the myocardium during stress are likely to act on the downstream targets, preferentially, by activating p38α. Therefore, it is unlikely that p38γ and or δ MAPK isoforms play significant roles in the heart in response to the above cytokines.
Figure 4-12 The functional analysis of WT and p38γδ KO hearts perfused with TNF-α.

WT and p38γδ KO hearts were perfused with TNF-α (20ng/mL) for 15 min after a 40 min stabilisation period. Left ventricular pressure (LVDP) (A) and coronary flow (CF) (B) were measured at 5, 10 and 15 min of perfusion with TNF-α (20 ng/mL). * indicates p< 0.01. Values are mean ± SEM of 6 independent experiments.
Figure 4-13 Representative immunoblots showing activation of p38 MAPK (Pp38) in WT and p38γδ KO hearts perfused with TNF-α.

Following treatment with TNF-α, the hearts were homogenised and activation of p38 MAPK (Pp38) analysed in WT (A) and p38γδ KO samples (B) using the phospho-specific (pan) antibody. Quantification of p38 MAPK phosphorylation after treatment with TNF-α (20 ng/mL) for 15 min in WT and p38γδ KO hearts (C). Values are mean ± SEM of 6 independent experiments.
Figure 4-14 The functional analysis WT and p38γδ KO hearts perfused with IL-1.

WT and p38γδ KO hearts were perfused with IL-1 (20ng/mL) for 15 min after a 40 min stabilisation period. Left ventricular pressure (LVDP) (A) and coronary flow (CF) (B) were measured at 5, 10 and 15 min of perfusion with IL-1 (20 ng/mL). * indicates p<0.05. Values are mean ± SEM of 6 independent experiments.
Figure 4-15 Representative immunoblots showing activation of p38 MAPK (Pp38) in WT and p38γδ KO perfused with IL-1.

Following treatment with IL-1α (20ng/mL), the hearts were homogenised and activation of p38 MAPK (Pp38) analysed in WT (A) and p38γδ KO hearts (B) using the phospho-specific (pan) antibody. Quantification of p38 MAPK phosphorylation after treatment with IL-1 (20 ng/mL) for 15 min in WT and p38γδ KO hearts (C). Values are mean ± SEM of 6 independent experiments.
4.5.1 Sorbitol - a hyperosmotic stimulus

There was no significant difference in the baseline parameters of WT compared with p38γ/δ KO. There was a significant reduction in left ventricular developed pressure in the WT group after 5, 10 and 15 min following treatment with sorbitol (0.1M) compared with the control group (Figure 4-16 A). Interestingly, this response was attenuated in p38γ/δ KO hearts (Figure 4-16 A). The coronary flow remained unchanged in both genotypes after treatment with sorbitol (Figure 4-16 B). The functional data was also consistent with the immunoblotting data which revealed a lower level of p38 MAPK phosphorylation in WT sorbitol treated, compared with transgenic, hearts, when probed with the polyclonal phospho p38 (pan-isoform) antibody (Figure 4-17 A-C).

These observations may suggest that hyperosmotic stress is one of the stimuli which could activate the p38γ MAPK isoform more readily than the other p38 MAPK isoforms in isolated hearts. Although we were able to observe a significantly lower level of p38 MAPK phosphorylation in the WT compared with the transgenic hearts, we were unable to categorically ascertain that the activation is associated with p38γ MAPK activation. Two key factors are implicated:

1) We were unable to observe a double band corresponding to the α and γ isoforms by using the polyclonal rabbit phospho specific (pan isoform) antibody. Previous studies have shown that this antibody can generate a double band representing the activation of different isoforms (Conrad et al., 1999; Kuma et al., 2005). However, it must be emphasised that these observations were made in vitro in cells overexpressing the flagged tagged form of p38 MAPK isotypes (Conrad et al., 1999; Kuma et al., 2005). We were only able to detect a distinct molecular weight for these isoforms when the heart samples were ran for 3 h on a larger gel and probed with the total p38 pan- antibody (Figure 4-6 A). The difference in the molecular weights by this antibody was also noted in the immunoblots of the recombinant GST-tagged p38 MAPK (Chapter 3, Figure 3-1).

2) In the characterisation chapter (Chapter 3), we suggested that all four isoforms appear to be present in the murine myocardium. Although only the α and γ isoforms were detected by immunoblotting, we were able to determine the expression of all four isoforms (including the β and δ isoforms) at m-RNA level and at protein level in isolated mouse cardiac myocytes.
Therefore, the contribution of these isoforms must also be considered when interpreting the data. Nevertheless, using immunoblotting at least, we were unable to detect the protein expression of the β and δ isoforms. This suggests that the band detected in the western blotting data with sorbitol is unlikely to be from either of these two isoforms. Furthermore, previous studies have reported that p38γ MAPK is more readily activated in response to sorbitol compared with the other isotypes. These observations together with the data presented in this section could, therefore, to some extent support our argument for implicating the γ isoform in the myocardial response to hyperosmolar stimulation induced by sorbitol.

Based on the above, we next investigated the potential downstream substrates of p38γ MAPK following its activation by hyperosmotic stress. Synapse Associated Protein 97 (SAP97), is a scaffold protein and a member of membrane associated guanylate kinase (MAGUK) protein found at the level of intercalated discs in the atrial (Godreau et al., 2002;Kuma et al., 2005) and ventricular myocardium (Murata et al., 2001). Recently it was demonstrated that SAP97 colocalizes with voltage-gated Kv1.5 (crucial for normal transmission of action potential) where it regulates the surface expression of Kv1.5 potassium channels of cardiac myocytes (Abi-Char et al., 2008). This protein has also been shown to be a physiological substrate of p38γ in vitro in HEK 293 (Sabio et al., 2005). In that study, it was shown that SAP97 becomes phosphorylated on residue Ser 158 by p38γ MAPK.

Using a phospho-SAP97 (Ser 158) antibody (donated by Anna Cuenda, Dundee), we investigated whether the same residue on SAP97 can become phosphorylated in sorbitol treated hearts. Immunoblotting the samples with this antibody, we were not able to detect a significant band, indicating that SAP97 is unlikely to be a substrate of p38γ MAPK in the heart in response to hyperosmotic osmotic stress (Figure 4-18). However, the certainty of this is diminished by the lack of a positive control.
Figure 4-16 The functional analysis of WT and p38γδ KO hearts perfused with sorbitol.

WT and p38γδ KO hearts were perfused with sorbitol (0.1M) for 15 min after a 40 min stabilisation period. Left ventricular pressure (LVDP) (A) and coronary flow (CF) (B) were measured at 5, 10 and 15 min of perfusion with sorbitol (0.1M). * indicates p<0.001. Values are mean ± SEM of 6 independent experiments.
Figure 4-17 Representative immunoblots showing activation of p38 MAPK (Pp38) in WT and p38γ/δ KO hearts perfused with sorbitol.

Following treatment with sorbitol (0.1M), the hearts were homogenised and activation of p38 MAPK (Pp38) analysed in WT (A) and p38γ/δ KO (B) hearts using the phospho-specific (pan-isoform) antibody. Quantification of p38 MAPK phosphorylation after treatment with sorbitol (0.1M) for 15 min in WT and p38γ/δ KO (C) hearts. Values are mean ± SEM of 6 independent experiments.
### Figure 4-18

A representative immunoblot testing the phosphorylation of SAP 97 (Ser 158) in WT and p38\(\gamma/\delta\) KO hearts perfused with sorbitol.

Following treatment with sorbitol (0.1 M), the hearts were homogenised and activation of phospho-SAP97 (pSAP97) was analysed in WT and p38 KO hearts using the phospho-SAP97 anti-Ser 158 antibody.
4.6 Discussion

4.6.1 Distinguishing between activation of p38α versus p38γ isoform

In the previous chapter, we showed endogenous p38α and p38γ MAPK can be pulled down from crude heart homogenate. We next aimed to examine their activations in response to various stimuli in this chapter. As discussed earlier, phospho-specific p38 MAPK antibodies do not selectively detect individual p38 isoforms. Therefore, to study the activation of p38α MAPK and p38γ MAPK, we optimised an immunoprecipitation protocol to pull-down phosphorylated p38 MAPK followed by immunoblotting using the isoform-specific antibodies. Ischaemia (10 min) was used as the prime stimulus to activate p38 MAPK in isolated heart perfusion. As expected, the α isoform was phosphorylated in IP samples of heart tissue subjected to 10 min of ischaemia. However, when the same samples were probed with anti-p38γ MAPK monoclonal antibody, a false positive result was observed. This was confirmed by the presence of a protein band at the expected molecular weight of Pp38γ MAPK (42kDa) in both the negative control and heart tissue from the p38γ/δ KO.

This led us to pursue alternative methods such as ELISA to distinguish between activation of p38α and γ MAPK. However utilising the commercially available Pp38γ MAPK specific ELISA (in our hands) also failed to provide any clear answer due to the non-specific signal generated. The high background was possibly due to poor removal of the unbound antigens by poor washing (Figure 4-4). Perhaps it would have been more appropriate to have used the ELISA on isolated ventricular myocytes as opposed to whole heart tissue. This approach would have probably reduced the background non-specific signal and allowed us to explain the data more confidently. Indeed this kit has been previously used by other investigators to work efficiently on human CF (Turner et al., 2008).

Since there is no phospho-specific antibody selective for each isoform commercially available, the other option was to use the phospho-specific pan antibody. We therefore, examined the activation of each isoform by comparing the phosphorylation of p38 MAPK (detected by the Pp38 pan-isoform antibody) in hearts from WT and KO animals.
4.6.2 Effect of Ischaemia and Ischaemia reperfusion on specific p38 isoforms

It was first demonstrated as early as 1996 that p38α and β MAPK are activated in response to ischaemia and reperfusion in the heart (Bogoyevitch et al., 1996). It has also been suggested that p38α and β MAPK play different, perhaps opposing roles, with α being pro, and β MAPK being anti, apoptotic. However, less is known regarding the roles of either p38γ or δ MAPK in isolated cardiac myocytes or in whole heart. In this study, we aimed to investigate the functional roles of endogenously expressed p38 MAPK isoforms by comparing the acute responses of wild type mice expressing all four isoforms (as detected by RT-PCR and immunocytochemistry of isolated cardiac myocytes) with transgenic mice (p38β KO and p38γδ KO).

Langendorff perfusion of WT and transgenic mice (both p38β KO and p38γδ KO) treated with 30 min of ischaemia followed by 2.5 h of reperfusion showed similar responses in both, haemodynamic data and infarct size in all genotypes. Biochemical analysis of p38 MAPK phosphorylation, using the phospo specific pan antibody, also revealed no difference between the different genotypes in response to either ischaemia-reperfusion or ischaemia alone. From these observations, one can conclude that the knocking out any one or more isoforms (apart from the α isoform) seems to play no significant role in the functional response of the heart following ischaemia-reperfusion or ischaemia alone.

4.6.3 Possible roles of p38β MAPK during pharmacological preconditioning by CORM-3

Previously, using adult ventricular cardiac myocytes, we had shown p38β MAPK to be activated during preconditioning whereas its activation was inhibited during ischaemia (Saurin et al., 2000). These observations led us to investigate the effects of preconditioning on this isoform expressed endogenously in the murine heart. The data presented here demonstrates that CORM-3 pre-treatment followed by a 5 min washout of hearts prior to in vitro ischaemia-reperfusion resulted in decreased infarct size and increased LV function. This effect was reversed in WT hearts in presence of the p38 MAPK inhibitor SB203580, implying that a SB203580-sensetive isoform (α and β) may be involved. This could be partly explained by a possible chemical interaction during co-perfusion of CORM-3 and SB203580. However, using a CO bioassay it has been shown that this is not the case with
respect to CO release by CORM-3 (Figure 4-19). This may therefore suggest a direct effect of SB203580 on p38 MAPK activation/activity or resulting from the pre-treatment phase prior to ischaemia. Taken together, the reduction in infarct size accompanied by an improved functional recovery in WT compared with p38β KO, perfused with CORM-3 suggest a protective role for the p38β MAPK isoform. However, the exact mechanism of cardioprotection remains to be determined. This also suggests, that p38β MAPK may be involved in other forms of preconditioning (such as ischaemic preconditioning), through a similar mechanism and this complexity needs to be further investigated.

![Graph showing CO-Myoglobin levels](image)

**Figure 4-19 Bioavailability of CO from solutions containing 0-100 µM CORM-3 in the presence or absence of 1 µM SB203580.**

Solutions of CORM-3 and SB203580 were prepared in water and then added to 100µM reduced myoglobin solution and analysed on a spectrophotometric plate reader (Molecular Devices, UK) to assess conversion of reduced-Mb to CO-Mb as previously described (Clark et al., 2003).
4.6.4 Effects of TNF-1α and IL-1α on p38γ and δ isoforms

Our hypothesis for investigating the activation of p38γ MAPK in response to such cytokines was based on the observations made by Cuenda et al., in 1997 (Cuenda et al., 1997). In this study, p38γ and δ MAPK were strongly activated in vitro in HEK 293 cells by various stimuli including TNF-α, IL-1 and sorbitol. Therefore, we aimed to investigate the endogenous activation of these isoforms in isolated mouse heart in response to the above stimuli. The present study demonstrated that TNF-α and IL-1 are unlikely to act through the γ or δ isoform, as both the haemodynamic and immunoblot data were identical in WT and p38γ/δ KO hearts.

4.6.5 Effects of osmotic stress induced by sorbitol on p38γ and δ MAPK activation

Interestingly, p38γ/δ KO hearts seemed to be protected against hyperosmotic stress induced by sorbitol which was absent in the WT hearts. This protective response was reflected both in the haemodynamic and biochemistry data.

Sorbitol accumulation is considered an adaptive response to hypertonicity that is observed in many cells (Yancey et al., 1982). Cells respond to hypertonicity in various ways including cytoskeletal rearrangement by rapid F-actin polymerisation and remodelling of the actin cytoskeleton. The cytoskeletal rearrangement could function as a cell volume sensor and offer mechanical protection against the detrimental effects of excessive shrinkage. It can also be involved in signal transmission from osmosensors to osmoregulator (Yancey et al., 1982).

A signal transmission supported by evidence that, hypertonicity induces assembly of an actin-associated protein complex containing a scaffolding protein, a GTPase and a member of a mitogen activated protein kinase such as MEK3, MKK3 and p38 MAPK.

As discussed before, p38 MAPK isoforms are activated differently in response to the same stimuli. It has been shown that the γ isoform is activated more readily by sorbitol compared with the other isoforms. It is therefore, possible that in the WT activation of the γ isoform triggers a signalling pathway with detrimental effects leading to a reduction in the haemodynamic parameters. However, in the absence of γ/δ, possibly other p38 MAPK isoforms (p38β MAPK, perhaps) involved in a protective pathway against the hyperosmotic stress dominate.
It has been shown that when SAP97 is phosphorylated (on Ser 158) by p38γ MAPK in response to osmotic stress in vitro, it dissociates from its cytoskeletal scaffold protein complex, the protein guanylate kinase-associated. This leads to a novel regulatory pathway for the adaptation of cells to hyperosmolar environment, which play a role in remodelling of cytoskeletal proteins (Chang and Goldman, 2004). Although, this antibody has been shown to work on HEK 293 cells, as shown by Sabio et al., 2005 (Sabio et al., 2005), we were unable to optimise the conditions for this antibody to work on heart homogenate samples.

In conclusion, although data from sorbitol treated hearts in WT and p38γ/δ KO mice looks encouraging, we were not able to fully investigate the potential downstream targets of p38γ MAPK in the heart in response to osmotic stress. This is mainly due to; 1) the lack of a phospho specific antibody selective for p38γ MAPK and 2) the poor quality of phospho-SAP97 (ser 158) antibody. However, it is also possible that SAP97 is phosphorylated by p38γ MAPK on the other phosphorylation sites (Sabio et al., 2005) found on this protein. In addition, despite our inability to detect p38δ MAPK (using the total p38 antibody) at protein level, we were able to detect this isoform at the mRNA transcript level. Therefore, the possible contribution of p38δ should also be considered when comparing the functional responses of WT with transgenic mice lacking both p38γ and δ MAPK isoforms. One potential way to overcome this problem and to delineate the activation of the p38γ MAPK isoform in this pathway is to raise a p38γ MAPK phospho specific antibody. However, this can prove difficult, as the phosphorylation site for p38γ MAPK overlaps with the phosphorylation of all the other isoforms.
5 INVESTIGATING THE POTENTIAL INVOLVEMENT OF P38γ AND δ MAPK ISOFORMS IN CARDIAC HYPERTROPHY

5.1 Introduction

Heart failure is defined as a deficiency in the capability of the heart to adequately pump blood in response to systematic demands. It is typically induced by a number of common diseases including chronic hypertension, myocardial ischaemia and aortic stenosis. Most of these stimuli induce a phase of cardiac hypertrophy which is associated with an enlargement of the heart in an endeavour to manage the increased hemodynamic demand. When sustained and extensive, cardiac hypertrophy can lead to maladaptation and progressive dysfunction leading to heart failure secondary to cardiomyocyte apoptosis and fibrosis (Frey and Olson, 2003).

The activation of many diverse signalling pathways has shown to regulate the process of hypertrophy, including those involving MAPKs (ERK, JNK and p38) (Garrington and Johnson, 1999). MEK1-ERK1/2 has been demonstrated to induce hypertrophy, in vivo, by enhancing the transcriptional activity of NFAT, whereas JNK and p38α have been shown to be the negative regulator of hypertrophy (Bueno and Molkentin, 2002; Nishida et al., 2004). It has been reported that transgenic mice over expressing MKK 3/6, upstream of p38 MAPK, or MKK7, an upstream activator of JNK, do not induce hypertrophy; instead they develop a cardiomyopathy, characterised by reduced functional performance, fibrosis and thinned ventricular walls (Liao et al., 2001; Petrich and Wang, 2004). It is possible that JNK and activation of one or more p38 isoforms alter the cardiac hypertrophic response as an indirect consequence of their capability to induce apoptosis and necrosis in cardiac myocytes (Heineke and Molkentin, 2006). Although, some studies have investigated the role of p38α MAPK isoform in hypertrophy, to date the role of p38γ and δ MAPKs has not yet been explored.

Recently, it was shown that p38γ MAPK isoform is selectively activated by RhoA (small binding protein) via MKK6, in NIH-3T3 fibroblast cells in response to osmotic stress (Marinissen et al., 2001). This study identified a new pathway, including RhoA, PKNα,
MLTKα (which acts as a MAPKKK for the p38 MAPK signalling cascade), MKK6 and p38γ MAPK in the regulation of gene expression and cellular transformation (Takahashi et al., 2003). In this pathway, phosphorylation of p38γ MAPK resulted in the binding of transcription factors such as ATF2 and MEF2A. These transcription factors have been shown to promote hypertrophy and cell growth by acting on the c-jun promoter through the AP-1 and MEF2 responsive elements (Takahashi et al., 2003). In addition, both p38γ and δ MAPK phosphorylate α-1 syntrophin, SAP90 and stathmin (regulator of microtubule dynamics) and Tau, all of which are involved in maintaining the integrity of cytoskeletal structure (Belmont and Mitchison, 1996; Parker et al., 1998; Sabio et al., 2004; Sabio et al., 2005). Whether these pathways exist in the mammalian heart, however, is unknown.

As discussed in the first chapter, (general introduction, section 1.4.1), diverse arrays of signalling pathways are activated in response to various initiating stimuli such as neurohormonal, biochemical, and stretch. The adrenergic signalling is one of the pathways that is elevated in patient with heart failure. There are two types of adrenergic receptors (AR); α-adrenergic (α-AR) and β-adrenergic (β-AR) (Figure 5-1). They both are GPCRs that are highly abundant in the major arteries of the body; including the aorta, pulmonary and coronary arteries. α-AR are Gq (Gq/α11) bound which are activated in response to physiological agonists such as serotonin, Ang II, catecholamines (noradrenaline and adrenaline) as well as pharmacological agonist, phenylephrine. Activation of α-AR by these agonists result in hydrolysis of phosphoinositol 4,5-bisphosphate. The product of this reaction is IP3 and DAG. DAG in turn activates PKC, an important step in the development of concentric hypertrophy- indeed inhibition of PKC abrogates GPCR-mediated hypertrophy in mice (Niizeki et al., 2007; Takeishi et al., 2000). Activation of PKC has been coupled with the development of cardiomyopathy that is characterised by left ventricular fibrosis and decreased left ventricular performance (Takeishi et al., 2000; Wakatsuki et al., 2004).

The β-AR couples to Goα (Gαs) subunit, including adenylyl cyclase activity, accumulation of cAMP and consequently activation of PKA. PKA phosphorylate proteins involved in cardiac contraction, including L-type calcium channels, ryanodine receptors, phospholamban and troponin (Marian, 2006), increasing their activity and hence cardiac contractions (Figure 5-1).
Figure 5-1 Adrenergic signalling pathway.
Activation of the α-adrenergic receptor (by Enothelin-1, Ang II and catecholamines) allows coupling of the G-protein and activation of phospholipase C (PLC). PLC promotes hydrolysis phosphoinositol 4,5-bisphosphate and the product of this reaction are IP3 and DAG. DAG in turn activates PKC and MAPK which are involved in hypertrophy and vasoconstriction. β-adrenergic receptors couple to Gs, include adenylyl cyclase and accumulation of the second messenger cAMP. The resulting PKA activity affects several aspects of the hypertrophy program, including phosphorylation and inactivation of the SERCA inhibitor phospholamban. This has the effect of increasing Ca2+ influx into the cytoplasm and increasing Ca2+ re-uptake into sarcoplasmic reticulum, causing myocyte contraction and vasodilatation (Barry et al., 2008).

The major agonists for the β-AR subtype include; adrenaline, noradrenaline and isoproterenol. There are two subtypes of β-AR receptor, β1-AR and β2-AR. It has been shown that the β1-AR are expressed on the surface of cardiac myocytes whereas CF have shown to express predominately the β2-AR (Porter and Turner, 2009). Data from reverse PCR analysis show that the m-RNA of β2-AR is highly expressed in adult rat CF and human myoFb, with little or no β1-AR mRNA detectable (Gustafsson and Brunton, 2000; Turner et al., 2003). The expression of β2-AR on CF is demonstrated to be implicated in CF proliferation and IL-6 secretion. Chronic activation of β-AR is brought about by elevated circulating catecholamines and inversely correlate with survival in patients with heart failure (Engelhardt et al., 1999; Lohse et al., 2003). The increased adrenergic drive is initially beneficial through increased contractility and heart rate, however chronic adrenergic
stimulation is thought to be ultimately detrimental (Bristow, 2000). This was demonstrated in a study using transgenic mice overexpression β1-AR. These mice displayed an increased contractility when young, but pronounced cardiac hypertrophy and fibrosis when older, which eventually lead to heart failure (Engelhardt et al., 1999). This suggests that β1-AR may indeed improve cardiac function but prolonged hyper-adrenergic drive eventually leading to pathological hypertrophy.

In this chapter we used isoproterenol (ISO), a non-selective β-AR agonist, to induce cardiac hypertrophy. Many studies have reported that ISO induces hypertrophy by β1-AR stimulation and fibrosis by β2-AR stimulation (Dudnakova et al., 2002; Liggett, 2000; Morisco et al., 2001; Turner et al., 2003). It has also been demonstrated that ISO, via β2-AR, stimulates renin angiotensin system (RAS), IL-18, and other systemic, local and immunological factors (Chandrasekar et al., 2004; Leenen et al., 2001). Together, the reactive responses to ISO results in ECM remodelling and possibly, diastolic dysfunction (Dudnakova et al., 2002).

In addition to the classical Gs-adenylyl cyclase-cAMP dependent-PKA signalling pathway, increasing evidence show that stimulation of β-AR activated MAPK. In a study by Zheng et al (2000), a novel β2-AR mediated cross talk between PKA and p38 MAPK was demonstrated (Zheng et al., 2000). In this study adult mouse cardiac myocytes expressing β2-AR, with a null background of β1 and β2-AR double KO stimulated with ISO, displayed an increased p38 MAPK activity in a time –does dependent manner. Inhibiting Gi with pertussis toxin or scavenging Gβγ with βARK-ct overexpression could not prevent β2-AR induced p38 MAPK activation. In contrast, specific peptide inhibitor of PKA completely abolished the stimulating effect of β2-AR. Furthermore, inhibition of p38 MAPK with SB203580 (10 µmol) markedly enhanced the β2-AR mediated contractile response without altering the baseline contractility (Zheng et al., 2000). Additionally, it has been suggested that p38α MAPK is one critical downstream signal for the development of cardiomyopathy following chronic β2-AR stimulation, but other kinases may be more important in ameliorating the adverse effects of chronic β-AR stimulation (Peter et al., 2007). In this study long-term (>1 year) overexpression of β2-ARs in the mouse heart eventually resulted in cardiomyopathy, the mechanisms by which β1-AR and β2-ARs mediate cardiomyopathy
seem distinct; inhibition of p38 MAPK reduced cardiac dysfunction in β2-AR overexpressed mice but not in β1-AR overexpressed ones (Peter et al., 2007).

The role of p38 MAPK was recently examined in a cardiomyocyte-specific dominant negative (CKO) in response to pressure overload and ISO induced cardiac hypertrophy. The neonatal cardiac myocytes from the CKO mice were also isolated and treated with different concentration of ISO (0.31-40 μm) for two days. The study demonstrated a correlation between an increase in apoptosis and dilatation in p38α CKO mice in response to pressure overload or ISO (Nishida et al., 2004). In a separate study by Zhang et al. in 2003 it was demonstrated that pressure over load caused a similar level of hypertrophy in dominant negative (dn) p38α mice as in controls (Zhang et al., 2003a). In contrast to these findings, Braz et al., showed that dn p38α mice with an FVB/N background developed cardiac hypertrophy following aortic banding, suggesting an anti-hypertrophic function of p38α (Braz et al., 2003). The apparent discrepancy was postulated to be associated with the developmental compensation by increased expression of p38 MAPK isoforms other than p38α MAPK. Therefore to date, the role of p38 MAPK in cardiac hypertrophy has remained controversial.

It has also been reported that in cardiac myocytes, ISO activates ERK through signal transduction pathway consisting of β-AR phosphorylation by; Gs/cAMP dependent PKA, Giβγ subunit derived from Gi, Src family tyrosine kinases, the formation of the Shc-Grb2-Sos complex and Ras and raf-1 kinase (Zou et al., 1999). The activation of Ras by ISO is intriguing, as Ras has also been shown to be a strong activator of p38γ (not p38α) MAPK in breast cancer invasion (Qi et al., 2006). Induction of p38γ MAPK by K-Ras (an isotype of Ras) has also been shown to be increased in human colon cancer (Tang et al., 2005).

5.2 Aims

Based on the observations above, we hypothesised that by using ISO we could induce a hypertrophic response which is a weak activator of p38α MAPK (perhaps through cross talk with PKA) but also activates Ras which could potentially activate p38γ MAPK isoform. We then hoped to investigate the role of p38γ and δ MAPK in cardiac hypertrophy by comparing the phenotypic and functional responses of WT and p38γδ KO. Due to the limited availability of the budget during the project, we were only able to use a p38γδ double KO for
our studies. Although we were unable to detect p38δ MAPK at protein level by western blotting, this isoform may be present in the murine myocardium (as detected in this Thesis by immunocytochemistry and PCR) and its contribution should also be considered. However, studies have shown that both isoforms are involved in cytoskeletal remodelling and possibly share a common theme by which they regulate cell volume and size (two responses are also implicated in cardiac hypertrophy) in response to stress (see Table 1-1 in section 1.5.2 of Chapter 1, general introduction).

In this chapter we used a variety of techniques such as PV analysis and echocardiography (echo) to investigate the functional responses of ISO-induced cardiac hypertrophy. Each technique is associated with certain advantages and limitations. For instance, echo enables real time visualisation of the heart and allows accurate wall measurements and cavity dimension of the ventricles. On the other hand, PV analysis offers a technique for measuring various haemodynamic and functional parameters (such as end systolic and diastolic pressure and volume and dP/dt_max) which are not provided by using echo alone. Conversely, measuring PV is invasive and requires animal surgery. As part of our aim in this chapter we were also keen to investigate, how the two techniques compare in measuring haemodynamic parameters in the same animal.
5.3 Specific Methods

5.3.1 Mini-Osmotic pump implantation

Age and weight-matched WT and p38γ/δ KO (25-30 g) mice were subjected to echocardiography one day prior to pump implantation. Mini-osmotic pumps (Model 1002, Alzet, UK) with a pumping rate of 0.25 µL/h filled with either vehicle (0.9% v/w NaCl) or isoproterenol (ISO, 30 mg/kg/day) were surgically implanted sub-cutaneously into adult WT and p38γ/δ KO mice following anaesthesia with 2% isoflurane. Animals were recovered and, after two weeks, subjected to follow-up echocardiography. A summary of the protocol is illustrated in Figure 5-2.

![Figure 5-2 Protocol for mini-osmotic pump implantation for induction of cardiac β-AR hypertrophy.](image)

Mice were subjected to echocardiography one day prior to osmotic pump implantation containing either ISO or saline. Cardiac hypertrophy was analysed by echo on day 14. The following day, the mice were subjected to PV analysis. The experiment was terminated by removal of the heart for morphological analysis.

5.3.2 Echocardiography

Mice from the WT and p38γ/δ KO colony (n=6) were anaesthetised with 4% isoflurane and prepared for scanning. Body temperature was maintained at 37°C. Ultrasound imaging was conducted using a 30-MHz probe with an imaging depth of 12.7 mm (as described previously in Chapter 2, section 2.12). M-mode images of both, parasternal long and short axis were obtained. 2D-images were used to measure LV anterior (LVAW) and posterior (LVPW) wall thickness as well as septal (LVS) wall and internal dimension (LVID) during systole and diastole at baseline and two weeks after mini pump implantation. Pulse-wave Doppler (PW) imaging was used to examine the blood flow in the aortic arch.
5.3.3 Pressure-volume analysis

One day after echocardiography, the mice were re-anaesthetised intubated and the abdomen was opened by a lateral laparotomy. The diaphragm was opened to gain access to the heart and the apex was punctured using a 27 gauge needle. A pressure-volume (PV) admittance catheter (Scisense Inc, Canada) was inserted into the LV via the puncture site and advanced until a systolic pressure of 80-110 mmHg was observed. The positioning of the conductance catheter was adjusted to maximise the size of the volume achieved by the PV loops. The system was then calibrated before recording. Following 20 min of stabilisation, the LV haemodynamic measurements during systole and diastole; end diastolic volume (EDV) and pressure (EDP), end systolic volume (ESV) and pressure (EDP), heart rate (HR), stroke volume (SV), cardiac output (CO), ejection fraction (EF) and stroke work (SW) were determined.

5.3.4 Morphological Analysis

The catheter was gently removed and hearts were relaxed in diastole by apical injection (200µL) of 0.9% (v/w) KCl in saline. The hearts were excised quickly and fixed in 4% PFA as before. The hearts were weighed, sectioned and sliced (as described in Chapter 2 section 2.11.5). For hypertrophy analysis, septal and free wall thicknesses as well as heart weight to body weight and heart weight to tibia length ratio were measured.

5.3.5 Data Analysis and statistics

Data are expressed as means ± SEM. Statistical comparisons were made by one way ANOVA followed by Tukey post test with GraphPad Prism. A value of p<0.05 was considered statistically significant. A student t-test was performed to analyse the two study groups and a p value of less than 0.05 was considered significant.
5.4 Results

5.4.1 Characterisation of in vivo ISO induced hypertrophy model

Induction of hypertrophy was associated with a significant increase in the heart weight to body weight ratio and tibia length (Figure 5-3 A and 5-3 B).

![Figure 5-3 Analysis of HW/BW and HW/TL ratios in hearts subjected to chronic infusion of ISO.](image)

Mini osmotic pump containing ISO or saline (control) were implanted in WT mice to induce cardiac hypertrophy. After 14 days, mice were weighed and sacrificed to remove hearts and tibia for analysis. Heart weight to body weight ratio (A) and heart weight to tibia length (B) was measured in ISO and control group to assess cardiac hypertrophy. Values are mean ± SEM, n=6.

Table 5-1 Physiological parameters in WT before and following treatment with ISO

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n=6)</th>
<th>After 14 days</th>
<th>Baseline (n=6)</th>
<th>After 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BW (g)</strong></td>
<td>26.5 ± 1.94</td>
<td>28.95 ± 1.57</td>
<td>25.82 ± 1.03</td>
<td>28.41 ± 1.24</td>
</tr>
<tr>
<td><strong>HW (mg)</strong></td>
<td>185.5 ± 19.7</td>
<td>230.5 ± 16.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5-1 summarizes the body weight, and heart weight at baseline and post treatment with ISO. Interestingly, treatment with ISO did not result in a reduction in total body weight. Therefore, the increase in heart weight to body weight ratio is a reflection of hypertrophy (Figure 5-3 A). Morphological analysis of hearts subjected to ISO revealed a marked increase in LV free wall and interventricular septal thickness (Figure 5-4 A and B) accompanied with LV dilation (Figure 5-4 C).

Figure 5-4 Morphological analysis of hearts subjected to chronic infusion of ISO.
Mini osmotic pumps containing ISO or saline (control) were implanted in WT mice to induce cardiac hypertrophy. After 14 days, mice were sacrificed and hearts removed, fixed and sliced. Free wall thickness (A) and interventricular septal thickness (B) were measured in ISO and saline (control) groups to assess cardiac hypertrophy. (C) Representative heart slices treated with either ISO or saline. Values are mean ± SEM, n=6.
5.4.2 Morphological analysis of LV remodelling in ISO induced hypertrophy in WT and p38γ/δ KO mice

Treatment with ISO (30mg/kg/day) resulted in hypertrophy of WT (n=6) and p38γ/δ KO (n=6) compared to control/vehicle treated hearts. This was indicated by a marked increase in heart weight to body weight ratio from 0.65 to 0.80 in WT and 0.61 to 0.81 in p38γ/δ KO (Figure 5-5 A). The body weight to tibia length ratio, however did not reach statistical significance (Figure 5-5 B).

There was a marked increase in LV free wall thickness (Figure 5-6 A) of WT (from 1.60 ± 0.15 mm to 2.24 ± 0.23 mm) and transgenic hearts (from 1.64 ± 0.22 mm to 2.15 ± 0.31 mm) measured at the papillary muscle. The interventricular septal wall, however, did not show a significant increase in either of the genotypes in response to ISO (Figure 5-6 B). Overall, morphological analysis revealed no apparent difference between WT and p38γ/δ KO hearts when subjected to ISO (Figure 5-6 A-C).
**Figure 5-5 Analysis of HW/BW and HW/TL ratios in mice subjected to chronic infusion of ISO.**

Mini osmotic pump containing ISO or saline (control) were implanted in WT mice to induce cardiac hypertrophy. After 14 days, mice were weighed and sacrificed to remove hearts and tibia for analysis. Heart weight to body weight ratio (A) and heart weight to tibia length (B) was measured in ISO and control group to assess cardiac hypertrophy. Values are mean ± SEM, n=6.
Involvement of p38-MAPK isoforms in Cardiac Hypertrophy

5.4.3 Echocardiographic analysis of LV remodelling in ISO induced hypertrophy in WT and p38γδ KO mice

Echocardiographic images in the M-mode parasternal view showed a marked increase in heart size with ISO treatment in both genotypes compared with the control group (Figure 5-7.
A-D). Interestingly, a marked increase was observed in LV systolic and diastolic volume in WT only which was absent in the transgenic hearts (Table 5-2). Analysis of hearts two weeks following pump implants revealed an increase in LV anterior wall mass in WT only (Table 5-2). In addition, the LV systolic volume was significantly greater in the WT compared with the null mice (p<0.05) after treatment with ISO (Table 5-2). A significant LV dilatation was also indicated in an increase in LVID during diastole in WT hearts only (Figure 5-8 C). This may suggest that the null mice are to some extend are protected against LV dilatation in response to hypertrophy induced by ISO. However, there were no significant changes in the LVID in either genotype during systole (Figure 5-8 D). This is also in keeping with morphological analysis shown in Figure 5-6 A-C. We observed a marked increase in LVS (Figure 5-8 A) and LVAW during diastole (Figure 5-9 A) in the WT and null hearts. Both, systolic LVS (Figure 5-8 B) and LVAW (Figure 5-9 B) remained unchanged during systole. No apparent difference was observed in the LVPW dimensions of either WT or transgenic mice treated with ISO during systole or diastole (Figure 5-9 C and D).

Table 5-2 Echocardiography measurements from M-mode images.
LV volume diastole, left ventricular volume during diastole; LV volume (systole), left ventricular volume during diastole; LV mass, left ventricular mass; LV Mass (AW), left ventricular mass (anterior wall). * p<0.05 and # p< 0.01 compared with control group. ¥ p<0.05 compared with WT ISO treated. Values are mean ± SEM, n=6.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>γ/δ KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ISO</td>
<td>ISO</td>
</tr>
<tr>
<td>LV Volume, Diastole (µL)</td>
<td>87.58± 4.51*</td>
<td>66.02± 19.38</td>
</tr>
<tr>
<td>LV Volume, Systole (µL)</td>
<td>53.51± 8.77#</td>
<td>27.01± 9.41 ¥</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>153.28± 28.23</td>
<td>139.67± 34.66</td>
</tr>
<tr>
<td>LV Mass, AW (mg)</td>
<td>138.36± 14.88*</td>
<td>113.07± 40.39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV Volume, Diastole (µL)</td>
<td>62.05± 17.68</td>
<td>65.22± 23.05</td>
</tr>
<tr>
<td>LV Volume, Systole (µL)</td>
<td>17.98± 8.43</td>
<td>13.14 ± 8.55</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>133.89± 41.90</td>
<td>135± 20.77</td>
</tr>
<tr>
<td>LV Mass, AW (mg)</td>
<td>101.17±18.32</td>
<td>108.76± 16.61</td>
</tr>
</tbody>
</table>
Involvement of p38-MAPK isoforms in Cardiac Hypertrophy

Figure 5-7 Echocardiography images at parasternal long axis view showing LV remodelling in response to ISO induced hypertrophy.

WT and p38γδ KO hearts were treated with either ISO (A and B) or saline (C and D) for two weeks. On day 14, mice were subjected to echocardiography to assess cardiac hypertrophy.

Induction of hypertrophy also resulted in a significant reduction in %EF (Figure 5-10 A) and %FS (Figure 5-10 B) of WT and p38γδ KO. Interestingly, the degree of significance observed in the% EF and the %FS of the WT was greater compared with the null mice (p<0.01 in WT compared with p< 0.05 in the transgenic mice). These observations suggest that the KO mice have a more preserved LV function following treatment with ISO. There were no differences in CO of either WT or transgenic mice with chronic infusion of ISO (Figure 5-10 C).

We also used PW analysis on the ultrasound echo to investigate turbulent flow and increased aortic outflow velocity (Figure 5-11 A-D). In this case, we observed normal outflow velocities in the WT and transgenic hearts treated with ISO infusion compared with the saline treated group (Table 5-3).
Overall, we did not observe any significant differences in the responses of WT and p38γ/δ KO subjected to hypertrophy using this model.

Figure 5-8 Echocardiographic analysis of LV morphology in WT and p38γ/δ KO mice subjected to ISO infusion from m-mode long axis parasternal view.

Mini osmotic pumps containing either ISO or saline were implanted in WT and p38γ/δ KO mice. After two weeks, the mice were subjected to echocardiography and the following parameters measured to assess the induction of cardiac hypertrophy: IVS, left ventricular septal thickness during diastole (A) and systole (B); LVID, Left ventricular interior dimension during diastole (C) and systole (D). Values are mean ± SEM, n=6.
Figure 5-9 Echocardiographic analysis of LV morphology in WT and p38γ/δ KO mice subjected to ISO from m-mode short axis parasternal view.

Mini osmotic pumps containing either ISO or saline were implanted in WT and p38γ/δ KO mice. After two weeks, the mice were subjected to echocardiography and the following parameters measured to assess the induction of cardiac hypertrophy: LVAW, left ventricular anterior wall thickness during diastole (A) and systole (B); LVPW, left ventricular posterior wall thickness during diastole (C) and systole (D). Values are mean ± SEM, n=6.
Figure 5-10 Echocardiographic analysis of LV function in WT and p38γ/δ KO mice subjected to ISO infusion from m-mode long axis parasternal view.

Mini osmotic pumps containing either ISO or saline were implanted in WT and p38γ/δ KO hearts. After two weeks, the mice were subjected to echocardiography to examine functional changes in response to cardiac hypertrophy. (A) Ejection fraction (%EF); (B), Fractional shortening (%FS) and; (C) cardiac output Values are mean ± SEM, n=6.
We observed large error bars associated with the blood turbulent and flow measurements when using the Doppler (Table 5-3). These observations could be due to the positioning of the transducer. Although we ensured that the beam angle was always placed in the same position (parallel to the direction of the flow in the aorta), it is possible that turbulent flow and small angle variations have resulted in a range of frequency shift ($\Delta f$). This can be further explained by the Doppler equation:

$$\Delta f (\text{frequency shift}) = \frac{2v f \cos \theta}{C}$$

Where $v$ is the velocity of the blood, $f$ is the transmitted frequency and $\theta$ is the angle between the direction of flow and the beam, $C$ is the velocity of ultrasound. As $\cos 0^\circ$ is 1
maximum value), the maximum velocity will be recorded when the blood is directly flowing towards the transducer. Therefore, if the beam angle is at any angle other than 0° (not parallel to the direction of the flow), the velocity will be underestimated resulting in frequency shift as indicated by large error bars in table 5-3.

Table 5-3 Echocardiographic measurements of blood flow velocity and flow using PW imaging.

Values are mean ± SEM. AO VTI, Aortic Outflow Velocity Time Integral; AV VTI, Aortic valve Velocity Time Integral peak velocity.

<table>
<thead>
<tr>
<th></th>
<th>WT (ISO)</th>
<th>γ/δ KO (ISO)</th>
<th>WT (Control)</th>
<th>γ/δ KO (Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ao VTI (VTI/cm)</td>
<td>3.53±0.67</td>
<td>3.06±0.57</td>
<td>3.58±0.71</td>
<td>2.97±0.65</td>
</tr>
<tr>
<td>Ao Mean Velocity (cm/s)</td>
<td>826.54±251.61</td>
<td>916.32±134.26</td>
<td>595±93.35</td>
<td>599.49±90.46</td>
</tr>
<tr>
<td>Ao Mean Gradient (mmHg)</td>
<td>2.93±1.95</td>
<td>3.42±0.99</td>
<td>1.44±0.43</td>
<td>1.46±0.41</td>
</tr>
<tr>
<td>Ao Peak Velocity (m/s)</td>
<td>1.22±0.40</td>
<td>1.07±0.16</td>
<td>1.10±0.18</td>
<td>1.04±0.21</td>
</tr>
<tr>
<td>Ao peak Gradient (mm Hg)</td>
<td>6.48±4.30</td>
<td>4.72±1.39</td>
<td>4.98±1.63</td>
<td>4.50±1.72</td>
</tr>
<tr>
<td>AV peak velocity (mm/s)</td>
<td>826.15±256.61</td>
<td>916.49±134.25</td>
<td>599.49±256.43</td>
<td>826.65±256.61</td>
</tr>
<tr>
<td>Av mean velocity (m/s)</td>
<td>1.35±0.42</td>
<td>1.44±0.25</td>
<td>1.22±0.27</td>
<td>1.21±0.443</td>
</tr>
</tbody>
</table>

5.4.4 PV analysis of effects of ISO on the LV haemodynamic parameters in WT and p38γ/δ KO

Haemodynamic analysis of hypertrophied hearts infused with ISO revealed a reduction in the %EF which only reached significance in the transgenic hearts (Figure 5-12 A). Other
parameters such as SW (Figure 5-12 B), HR, SV remained unchanged (Figure 5-13 A-B) in both genotypes. However, there was a significant increase (p< 0.05) in CO in the KO mice post ISO infusion compared with saline and WT-ISO treated hearts (Figure 5-13 C). This was inconsistent with the CO measurement acquired by echo previously in the same animals (Figure 5-10 C). The inconsistencies between the two techniques are discussed in more details in the section below. Both genotypes demonstrated a preserved EDV and EDP following treatment with ISO (Figure 5-14 A & B). The ESV and ESP also remained unchanged (Figure 5-14 C & D). We observed a lower baseline ESV (not statistically different) in the KO compared with the WT hearts (Figure 5-14 C). This is unlikely to be due to the genetic deletion of the γ and δ isoforms as the other baseline haemodynamic parameters are comparable for both genotypes.

In this study, we were able to observe a comparable baseline values using echo and PV with the exception of ESV (5-14 C). The observed non-significant reduction in the ESV of the transgenic hearts at baseline compared with the WT hearts (Figure 5-14 C) was absent when using echo (Table 5-2). Overall, the data was more variable between the two techniques after treatment with ISO. There was a significant increase in LV diastolic and systolic volumes only in the WT as measured by echo (Table 5-2). PV analysis however, revealed no marked differences in the EDV (Figure 5-14 A) or ESV (Figure 5-14 C) of either genotypes post ISO infusion. These inconsistencies in the volume related parameters between the PV and echo are possibly associated with the way the two techniques are used to record measurements. The correct volume measurement by PV relies on accurate positioning of both the proximal and distal volume transducers within the LV without any contact with the surrounding walls. These requirements make PV more prone to user error if the technique is not mastered properly. As established earlier, we used an apical approach to insert the catheter through the thick hypertrophied walls into the LV. It is, therefore, likely that the proximal volume transducer became misplaced during the experiment and resulted in erroneous readings. Unlike PV, we were able to obtain a more consistent range of data when using echo. Interestingly, by using echo, we were also able to distinguish subtle statistical differences in the functional responses of WT compared to the null mice such as the %EF and %FS in hypertrophied hearts (Figure 5-10 A and B). Using PV, we were only able to detect a significant reduction in the %EF of the null mice post ISO treatment (Figure 5-12 A). Unlike echo which demonstrated an unchanged CO post ISO infusion (Figure 5-10 C), PV
suggested a significant increase in the CO the transgenic mice after treatment with ISO, despite the preserved HR and SV (Figure 5-13 A-C). At this point we were not certain whether this was associated with the way the two techniques recorded the parameters or it was associated with user error when utilising the catheter. It was possible that the incorrect positioning of the catheter (particularly the proximal volume transducers) within the LV, had resulted in untrue readings (see discussion, section 5.5.3).

From the discussion above it can be concluded that the two techniques are most useful when used in conjunctions with each other in order to obtain a broad range of reliable data. Echo is more suitable for measuring the morphological changes of the LV and assessing functional parameters such as %EF and %FS. Although invasive, PV is a reliable approach for measuring functional parameter of the LV particularly the pressure and volume related measurements. This is, of course, subjected to reliable catheterisation of the animals to ensure correct catheter positioning. It is also possible that PV analysis is not suitable for relatively small n numbers and is more reliable when used on larger group sizes.

Figure 5-12 Haemodynamic analysis of WT and p38γδ KO mice subjected to chronic infusion of ISO using admittance catheter.

WT and p38γδ KO were treated with ISO mice and saline. After two weeks, the hearts were catheterised through an apical route and the following haemodynamic parameters were measured using the admittance catheter inside the LV: (A) Ejection fraction (EF) and (B) Stroke work (SW). Values are mean ± SEM, n=6.
Figure 5-13 Analysis of LV function of WT and p38γ/δ KO mice subjected to chronic infusion of ISO using pressure volume admittance catheter.

WT and p38γ/δ KO were hearts treated with ISO and saline. After two weeks, the hearts were catheterised through an apical route and the following haemodynamic parameters were measured using the admittance catheter inside the LV: (A) Heart rate (HR); (B), stroke volume (SV) and; (C) cardiac output (CO). Values are mean± SEM, n=6.
Figure 5-14 Pressure volume analysis of LV function of WT and p38γδ KO mice subjected to chronic infusion of ISO.

WT and p38γδ KO mice were treated with ISO and saline. After two weeks hearts were catheterised though an apical route and the following haemodynamic parameters were measured using the admittance catheter inside the LV: (A) end diastolic volume (EDV); (B) end diastolic pressure (EDP); (C) end systolic volume (ESV) and; (D) end systolic pressure (ESP). Values are mean ± SEM, n=6.
5.5 Discussion

5.5.1 Validation and characterisation of hypertrophy model

Norepinephrine, through stimulation of β-AR, is a well known trigger of cardiac hypertrophy. Extent of left ventricular dysfunction in human pathology has been shown to correlate to plasma norepinephrine concentration independently of arterial blood pressure (Kelm et al., 1996). In an in vivo cardiac hypertrophy model, hypertrophy is induced by stimulation of β1-AR by chronic infusion of agonist, ISO (Limbird and Vaughan, 1999). ISO also stimulate β2-AR. Chronic β2-AR stimulation has also been shown to induce fibrosis by binding directly to CF and also by increasing CF proliferation leading to cardiac remodelling and diastolic dysfunction (Jalil et al., 1989; Stacy et al., 2007).

Diastolic dysfunction often occurs initially in hypertrophy when the ventricular filling (preload) is impaired. The term diastolic dysfunction refers to changes in ventricular diastolic properties that have an adverse effect on SV. About 50% of heart failure patients have diastolic dysfunction, with or without normal systolic function as determined by normal %EF. Ventricular filling (i.e. EDV and hence sarcomere length) depends upon the venous return and the compliance of the ventricle during diastole. Ventricular compliance is determined by the physical properties of the cardiac muscle and the state of ventricular contraction and relaxation. For example, in ventricular hypertrophy the compliance is reduced as the ventricles are stiffer which leads to a higher EDP at any given EDV. Reduced ventricular compliance is due to increased collagen and changes in the ratio of collagen fibre types in the ECM that occurs during hypertrophy (Galderisi, 2005; Spinale, 2002). It has been demonstrated that ISO increases plasma rennin, Ang I and Ang II three to four fold (Leenen et al., 2001). In addition β2-AR stimulation by ISO results in the expression of IL-18 and inflammatory cytokines linked to fibrosis and cardiac dysfunction (Chandrasekar et al., 2004). Increased ventricular stiffness results in a reduction in SV, EDV with a small decrease (in some cases) in %EF. In addition, SW will also decrease. The diastolic dysfunction can be better explained by the Frank-starling relationship (Figure 5-15).
A second mechanism that results in diastolic dysfunction by impaired ventricular relaxation, is due to reduced rate of Ca\(^{2+}\) uptake by the SR. Under normal conditions, towards the end of excitation-contraction coupling, the SR actively sequester Ca\(^{2+}\) so that the concentration of Ca\(^{2+}\) in the vicinity of troponin-C is reduced allowing the Ca\(^{2+}\) to leave its binding sites on the troponin-C and thereby permits disengagement of actin from myosin. This is a necessary step to achieve rapid and complete relaxation. Consequently mechanisms which impair relaxation will reduce the rate of ventricular filling, particularly during rapid filling.

In this project, the validation of the model was first examined in WT hearts by measuring heart weight and tibia length to body weight ratio in response to ISO given at a dose of 30mg/Kg/day also used by previous investigators to induce hypertrophy in mice (Webb et al., 2010; Zhang et al., 2007a). As the mice have reached adulthood (25-30g, age 8-12 weeks), any increase in the heart weight compared to the whole body weight and/or tibia length indicates hypertrophy. These measurements are often used as indices of hypertrophy.
The data in the present study showed that β-AR activation induces a geometric remodelling of the LV as assessed by dilatation and increased free and septal wall thickness in addition to increased heart to body weight and tibia length ratio. The functional analysis of the left ventricle in response to ISO revealed a significant reduction in %EF, %FS of both genotypes (measured by echo) with preserved SV and EDP (afterload) (measured by PV). These findings are in contrary to the diastolic dysfunction (often characterised by high EDP and maintained %EF (Diwan and Dorn, 2007) expected in response to ISO. In fact, our data (reduced contraction/systolic function accompanied with LV dilatation) is typical of “hypertrophy de-compensation” also known as cardiomyopathy (Figure 5-16). This condition is best described by the Laplace relationship which establishes that wall stress (afterload) of a thin-walled sphere is determined by intraluminal pressure, chamber size and wall thickness;

\[ \sigma = \frac{pr}{2h} \]

Where \( \sigma \) is wall stress, \( p \) is intracavity pressure, \( r \) is the internal radius of the chamber, and \( h \) is the thickness of the chamber wall. Thus, in a situation such as hypertrophy (induced by ISO), where there is increased stress on the heart, a reactive increase in wall thickness can normalise wall stress and preserve systolic function (compensatory hypertrophy) (Grossman et al., 1975). However, long term maladaptive remodelling of reactive hypertrophy is associated with progressive ventricular dilatation (Figure 5-16). This occurs due to increased ratio of \( r/h \) despite constant intracavity pressure. This geometrical increase in wall stress generates a haemodynamic stress on the heart, further stimulating the already overloaded signalling pathways and tipping the balance from a cell growth to one of cell death (Diwan and Dorn, 2007).

It is not clear why using ISO lead to the development of compensated hypertrophy (poor contractility and LV dilatation) in the current study. These observations may suggest possible activation of other signalling pathways implicated in the development of cardiomyopathy in response to ISO. This is further discussed in the following section.
Chapter 5  

Involvement of p38-MAPK isoforms in Cardiac Hypertrophy  

5.5.2 Does ISO induce compensated cardiac hypertrophy or de-compensated cardiomyopathy?

Role of p38α MAPK in hypertrophy has been investigated in vivo by using various methods including aortic banding and ISO infusion (Braz et al., 2003; Nishida et al., 2004; Zhang et al., 2003a). As described previously, ISO induces cardiac hypertrophy and fibrosis by activating the β-AR signalling pathway, resulting in diastolic dysfunction. The inconsistency between our data (with ISO) and previous published data could be associated with the activation of other downstream signalling effectors. Impaired calcium homeostasis is a prominent feature in the transition from compensatory hypertrophy to heart failure and causes contractile dysfunction and developed arrhythmias. It is now well appreciated that downregulation of the SERCA is a major cause of calcium deregulation and impaired potentiation of contractile force in heart failure (Frank et al., 2003; Meyer et al., 1995). Several studies have demonstrated that SERCA expression and hence Ca^{2+} SR uptake is reduced in overloaded
hypertrophy and heart failure (Frank et al., 2003; Schultz et al., 2004). As described in the introduction section of this chapter, PKA is one of the downstream effectors activated by cAMP upon β-AR stimulation. PKA activity affects various aspects of hypertrophy signalling including phosphorylation and inactivation of the SERCA inhibitor. This has the effect of increasing Ca\textsuperscript{2+} influx into cytoplasm and increasing Ca\textsuperscript{2+} uptake into the SR, causing myocyte contraction and vasodilatation. Indeed, one of the potential mechanisms mediating the effects of β-blockers may involve reversing PKA hyper-phosphorylation of the calcium release channel/cardiac ryanodine receptor (Reiken et al., 2003; Wehrens and Marks, 2004). Therefore, it is possible that the de-compensatory hypertrophic response we observed in the current study be associated with the activation of cAMP/PKA/Ca\textsuperscript{2+} pathway.

Another possible molecular signalling pathway by which ISO can induce cardiomyopathy was recently shown to involve the activation of calcineurin /NFAT and ERK (Yeh et al., 2010b). In this study, it was demonstrated that ISO induces hypertrophy by down-regulation of cGMP and PKG and upregulating calcineurin A /NFAT signalling. The cardiac hypertrophic effects of ISO were blunted in presence of a novel xanthine based derivative, KMUP-1. Pre-treatment with this inhibitor attenuated cardiac hypertrophy and fibrosis and improved the survival of ISO treated rats (Yeh et al., 2010b). In addition, recently, it was reported that ISO induces cardiac hypertrophy by increasing the activation of AT 1 (angiotensin I ) receptor which play a crucial part in the development of cardiac hypertrophy and oxidative stress under β-AR stimulation (Zhang et al., 2007a). In this study AT1 receptor blocker, olmesartan (OLM) treatment suppressed cardiac mass enlargement as well as oxidative stress in chronically-infused mice without any reduction in heart rates. In this study it was demonstrated that the cardiac AT1 receptor mRNA expression in ISO-infused mice was two-third the level in control mice whereas the levels in Ang II-infused or OLM-treated mice were not affected. These data may suggest that ISO infusion may down regulate cardiac AT1a receptor by a cAMP-dependent mechanism via β-AR, rather than by mechanisms involving the AT1 receptor themselves. Although the cardiac AT2 receptor m-RNA expression was not affected by ISO infusion, OLM treatment or gene deletion of AT1a receptors AT2s activated under these conditions might contribute to a reduction of cardiac mass and fibrotic alteration in OLM-ISO and ISO-infused AT1 (Jones et al., 2004).
5.5.3 Role of p38γ and δ MAPK isoforms in ISO-induced cardiac hypertrophy

Investigating the potential roles of p38γ and δ MAPK in cardiac hypertrophy revealed subtle phenotypic differences in the responses of the WT and p38γδ KO to ISO induced cardiac hypertrophy. This was demonstrated by a significant increase in LV volume during diastole and systole and a marked increase in diastolic LVID of WT compared with the transgenic hearts (by echo), suggesting that the null hearts are to some degree protected against LV dilatation. In addition, the significant reduction in cardiac performance (% EF and %FS) appeared to be less prominent in the null mice post ISO treatment compared with the WTs (p<0.01 in WT compared to p<0.05 in p38γδ KO). Infusion of ISO resulted in a comparable thickening of the anterior, posterior and septal wall in WT and p38γδ KO as assessed by echocardiography. Overall, these findings may suggest that p38γ and δ MAPK have a role in protecting against LV dilatation in geometric remodelling with β-AR modulated hypertrophy. However, this needs to be confirmed by further molecular investigation to identify the cell type expressing these isoforms and their exact contributions to cardiac remodelling with larger n numbers in each group. In addition, the following points should also be taken into consideration when interpreting the current data; 1) the complexity of the signalling cascades involved in hypertrophy; and 2) technical limitation of the study.

5.5.3.1 Complexity of hypertrophic response and substrate sharing of MAPKs

Hypertrophy engages a series of complex signalling pathways, involving many important MAP kinases such as JNK, ERK and p38α MAPK. These key kinases are likely to be the prime targets activated in response to hypertrophy. Therefore any possible effects of p38γ and δ MAPK may become masked by the overriding activation of these kinases through competing for the same downstream substrates. One of the downstream substrates of p38γ MAPK recently identified in cardiac fibroblasts is Sab25 (Court NW et al., 2004). It was shown that p38γ MAPK localises in the mitochondria of cardiac myocytes and HEK293 cells where it phosphorylates Sab25 on serine 321 (Court NW et al., 2004). This protein, first described as a binding partner of Bruton’s tyrosine kinase (Btk) (Matsushita et al., 1998; Yamadori et al., 1999) is also an in vitro substrate of the JNKs (Wiltshire et al., 2002).
The sharing of substrates by protein kinases can provide important levels of control but can also lead to production of the same endpoint, even in the absence of one of the upstream regulators.

Indeed cross talk between the parallel signalling pathways is always a drawback when investigating cardiac hypertrophy in vivo. This can be to some extent rectified by using the most appropriate hypertrophic agent/model. In this study we used ISO to induce hypertrophy. However, from the above discussion, it is clear that the exact mechanism by which ISO induces cardiac hypertrophy is not fully understood and new molecular pathways activated by ISO are emerging. Perhaps this is a criticism to the model we adopted to examine the complicated role of p38 MAPK isoforms in vivo and a non-pharmacological approach would have proved more informative. Analysing the phosphorylation of upstream activator (M KK3/6), downstream substrate (MAPKAPK2) of p38 MAPK, in addition to other MAPK family members such as ERK and JNK by immunoblotting would have been also beneficial to accurately delineate the activation of p38 MAPK when characterising the model. In addition, dilated cardiomyopathy and heart failure are often associated with cardiac myocytes apoptosis. The role of p38α MAPK in hypertrophy has mainly been associated with increased apoptosis and fibrosis. Therefore using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay and histological analysis in this model would have been useful to evaluate apoptosis and fibrosis respectively in response to ISO in the present study. This would have also provided a better insight in the mechanisms implicated in preserved LV dilatation we observed in the null mice compared to the WT.

5.5.3.2 Technical limitations of the study

Although, there was no difference in hypertrophic responses of either genotype, we encountered some difficulties interpreting the volume related haemodynamic data such as ESV, EDV and CO obtained from PV analysis. In this study, we used an apical approach to advance the admittance catheter inside the LV. Incorrect positioning of the catheter, due to user error, could have led to misalignment of the conductance electrode within the LV (Figure 5-17). Consequently, although pressure readings from these animals were within the normal range, there was a large degree of variability in the volume related data. To minimise incorrect catheter positioning carotid catheterisation should be used as opposed to apical
approach in future studies. This will ensure that both the pressure and volume transducers are correctly positioned within the LV. In the following chapter, we adapted the carotid approach for PV studies.

Figure 5-17 A schematic diagram showing catheters positioning through an apical puncture

(A) The admittance catheter is perfectly positioned in a normal heart, so that both pressure transducers and volume transducer lie within the LV. (B) Incorrect catheter positioning due to user error can lead to misalignment of the volume transducer within the left ventricle and embedding of the conductance electrode in the septal myocardium to incorrect readings.

We can conclude that p38γ and δ MAPK may to some extend protect against LV dilation in both eccentric and concentric hypertrophy. However due to the inconsistency of the echo and PV data we cannot be certain that these subtle phenotypic differences are exclusively associated with the deletion of the γ and δ isoforms. As discussed previously, p38γ and δ
MAPK have shown to phosphorylate various cytoskeletal proteins. It may, therefore, be possible that involvement of these two isoforms are implicated at a later, more chronic time course in response such as remodelling post-MI and progression towards heart failure. We tested this hypothesis in the following chapter.
6 INVESTIGATING THE ROLE OF P38γ AND δ MAPK IN POST-INFARCTION LEFT VENTRICULAR REMODELLING

6.1 Introduction

Left ventricular (LV) remodelling is a dynamic process characterised by necrosis and thinning of the infarcted myocardium (early phase) and LV dilation. Fibrosis occurs both at the site of the infarct and in non infarcted myocardium with hypertrophy of viable myocytes (late phase). LV remodelling may persist and contributes to functional de-compensation and eventually the development of the clinical syndrome of heart failure (Pfeffer and Braunwald, 1990). Identifying the cellular and molecular substrates underlying LV remodelling post-MI is an important strategy in the development of novel therapies to impede heart failure.

Activation of MAPKs have been implicated in several mechanisms of LV remodelling in the progression towards heart failure. These include apoptosis, induction of the inflammatory response and cell proliferation. With respect to p38 MAPK, a few studies have investigated the role of the p38α isoform in cardiac apoptosis and remodelling. Myocardial p38 MAPK is rapidly activated in rats after MI induced by coronary artery ligation (Shimizu et al., 1998). Sustained p38 MAPK activation in the heart has been associated with LV remodelling and dysfunction both in human (Cook et al., 1999) and in animal models (Liao et al., 2001). Recently it was reported that mice with cardiomyocyte-specific expression of a dominant negative mutant form of p38α MAPK subjected to MI display a marked reduction in infarct size and increased ventricular systolic function after 7 days compared with WT littermates (Ren et al., 2005). In addition, these mice showed a reduction in cardiac myocyte apoptosis which was accompanied by a reduction in Bcl-XL deamination that sensitises cells to apoptosis (Ren et al., 2005).

LV remodelling also involves the rearrangement of the cytoskeleton and extracellular matrix (Wilson et al., 2003). So far, the role of p38α MAPK in cardiac remodelling has been investigated in the context of apoptosis and inflammatory response. To date, no studies have investigated the potential role of other p38 MAPK isoforms in cardiac remodelling post-MI.
Of all of p38 MAPK isoforms, p38$\gamma$ and $\delta$ have shown to be involved in the regulation of cytoskeletal and microtubules rearrangement in response to stress (Feijoo et al., 2005; Sabio et al., 2005). In the previous chapter, we were able to observe a few subtle phenotypic differences between the WT and the p38$\gamma$$\delta$ KO in response to $\beta$-AR induced cardiac hypertrophy. These observations made us question the possible contributions of p38$\gamma$ and $\delta$ MAPK in chronic phase of remodelling. Therefore, the current study was designed to investigate the involvements of p38$\gamma$ and $\delta$ MAPK in response to remodelling post-MI and progression towards heart failure.

The most popular method used to induce myocardial ischemia is ligation of one or more coronary arteries, in particular the left anterior descending coronary artery (LAD). Occlusion of the LAD produces a pathphysiologically relevant wave of ischemic injury and a transition zone separating the infarcted and non-infarcted area. Also, the injury can lead to trans-mural infarction as has been shown to occur in man. In this model, the ligation can be permanent (without reperfusion) or temporary followed by a period of reperfusion. After 4 weeks, the permanent ligation model leads to large infarct volumes and is ideal for studying LV remodelling and heart failure.

6.2 Aim

In this study we used an in vivo infarction model with permanent LAD occlusion to investigate the possible contributions of p38$\gamma$ and $\delta$ MAPK in post-infarction cardiac remodelling.
6.3 Methods and Materials

The experiments in this chapter are divided into two sections. The first section explains the optimisation of the model (see Figure 6-1 A-C) through three different protocols and the second part has been allocated to the investigation of p38γ and δ isoforms in LV remodelling using the optimised model (see Figure 6-1 D).

6.3.1 Surgery and post-operative care

Mice were anaesthetised, shaved under the lower jaw and the chest and intubated as described in (Chapter 2, section 2.11.2). Animals hydrated by 1 mL sterile saline (0.9% w/v NaCl) injected IP to prevent dehydration during the operation. A small incision was made to the overlying skin and the intercostal muscles between the second and the third ribs to gain access to the heart. Ligation of the LAD was performed using an 8/0 silk. Sham-operated animals were also subjected to the entire surgical procedure but without the ligation. For recovery, the chest was closed by suturing the two ribs and then the overlying skin by interrupted stitches of 5/0 silk suture (Johnson & Johnson). Mice were injected with 100 µL of analgesic Buprenorphine (0.3 mg/mL diluted 1:20 in sterile water) before recovery. The pressure of the lungs (controlled by the ventilator) was increased to 6 cm H₂O to reduce the risk of post-surgical pneumothorax. The animals were left at 28°C for 24 h until active in the cage, before being placed back in room temperature in the holding areas.

6.3.2 Echocardiography

6.3.2.1 Characterisation of the model

In the characterisation study, WT mice were subjected to echocardiography before, and 3 days, post-MI. Anterior and posterior wall movement were measured by tissue Doppler (TD) in the short axis view, parasternal long and short axes were used to assess LV mass and dimensions (septal, anterior and posterior wall (LVS, LVAW and LVPW) and LV interior dimensions (LVID). Cardiac output (CO), fractional shortening (FS), ejection fraction (EF), stroke volume (SV), blood velocity and flow were measured to determine LV function.
6.3.2.2 Remodelling Study

In the remodelling study, WT and transgenic mice were subjected to echocardiography before and 4 weeks post-MI. All the above measurements (apart from the anterior and posterior wall motions) were determined.

Figure 6-1 The characterisation (A-C) and optimisation of the myocardial infarction (MI) model (D) by permanent Left Anterior Descending Artery (LAD).

Characterisation study: The reproducibility of ligation was assessed in an open chest model by measuring area at risk (AAR) by apical injection of Evans blue 10 min post ligation in WT mice (A). WT hearts were catheterised with a pressure volume (PV) conductance catheter via the apex before stabilisation for 40 min and haemodynamic parameters measured at baseline and 10 min post ligation. Ischaemic volume was measured by Evans blue as before (B). The mice were subjected to echo before being subjected to LAD ligation were recovered and kept for three days and echoed. Infarct size was measured at the end of experiments by apical injection of Evans blue (C).

(D) Established protocol for the remodelling study: In the final protocol, mice were echoed, subjected to remodelling for 4 weeks. Animals were then echoed again and haemodynamics measured by PV analysis. At the end of the experiments hearts were removed and fixed in paraformaldehyde.
6.3.3 Pressure- volume analysis

Haemodynamic measurements were recorded at baseline and 10 min post LAD ligation. In the remodelling study, pressure volume analysis was performed one day after echocardiography and WT and p38γδ KO mice were catheterised through the left carotid artery. In both cases, haemodynamic parameters; heart rate (HR), End systolic and diastolic volume (ESV, EDV) and pressure (ESP, EDP), stroke work (SW), cardiac output (CO) and stroke volume (SV) were recorded as described in the Materials and Methods (Chapter 2, section 2.11.3). At the end of the experiment, mice were injected with 200 µL of KCl (0.9% w/v in saline) to relax the hearts in diastole.

6.3.4 Infarct size measurements

At the end of the experiment, a bolus of 0.5 mL Evans Blue dye (2% v/w in sterile filtered saline) was slowly infused into the LV cavity via the apex (Figure 2-13 A in Chapter 2). The needle was then gently withdrawn and the heart carefully excised and fixed in a solution of 10% paraformaldehyde (w/v in PBS) for 24 h at 4°C. After fixation, the heart was mounted in 5% agarose (w/v in PBS) with the apex uppermost. The agarose-embedded hearts were then sectioned from apex to base into 700 µm thick slices using a vibratome (Agar Scientific) and analysed by Sigma Scan v5.0, (SPSS) as described in Chapter 2, section 2.11.4.

6.3.5 Statistical Analysis

In characterisation study using PV and echocardiography, haemodynamic parameters were pooled and linear regression analyses were carried out to determine the relationship between the changes in each parameter and the ischaemic zone (% LV muscle volume) measured. Data are presented as scatter plots where each point is an individual experiment. Linear regression and 95% confidence intervals are represented by a single solid and a pair of dashed lines respectively. All analysis was performed using GraphPad Prism. R² (goodness of fit) and linear regression equation are shown in the figure only where the slope of the regression were considered by the analysis to be significantly non-zero. A value of p<0.05 was considered statistically significant. Data obtained from PV and echo analysis in the remodelling study are expressed as means ± SEM. Statistical comparisons were made by one
way ANOVA followed by Tukey post test with the GraphPad Prism. A value of p<0.05 was considered significant.
6.4 Results

6.4.1 Optimisation of LAD ligation in a non-recovery model

Creation of a moderately sized and small transmural MI with precision is paramount to long term survival in this model, enabling mechanistic studies of long term LV remodelling and contractile dysfunction. However, a relatively uniform size has proven to be a formidable task in mice due to the following reasons; 1) the branches of LAD penetrate the myocardium close to their origin and are not accessible to view, even with the assistance of an operating microscope; therefore ligation has to be attempted “blindly”. 2) various anatomical variation of LAD exists in mice with branches following a different course. These factors affect the reproducibility of the model which is linked with a wide range of ischaemic volume and consequently infarct size. In addition, large transmural infarcts due to ligation of the LAD at its most proximal region are often associated with short-term survival of less than 4 weeks required for LV remodelling.

It is, therefore, necessary to optimise the surgical techniques to permit a reliable creation of infarct size and long-term survival rate. To achieve these objectives, we first optimised the reproducibility of the model by obtaining a consistent area at risk size as determined by Evans blue (3% w/v) infusion 10 min following ligation. We also used PV and echocardiography analysis to test whether these techniques can be used to predict infarct size post-MI in vivo.

6.4.2 Reproducibility study

A total of 11 WT mice were subjected to a permanent LAD ligation (Figure 6-1 A). In this study, we were able to achieve an area at risk size (AAR, expressed as percentage LV, AAR) of 45.67 ± 3.83% following the ligation (Figure 6-2 A). The data was spread over a range of 28.90 % to 68.30% with a median of 46.00 % (Figure 6-2 A & B). A positive correlation was observed when AAR was plotted against a given LV muscle volume (Figure 6-2 C). The graph demonstrated a tight spread of data between 95% confidence interval which indicated a reproducible ligation (Figure 6-2 C).
Figure 6.2 Characterisation of the reproducibility of ligation in an open chest model by measuring area at risk. 

A representative area at risk (AAR) zone in heart slices subjected to a non-recovery left anterior descending artery (LAD) ligation stained with Evans blue (A) and the range of AAR achieved (B). Correlation ($P<0.01$) of AAR to left ventricle (LV) 10 min following LAD ligation (C). Each data point represents a single animal ($n=11$). Dotted lines show 95% confidence intervals.
6.4.3 Pressure volume analysis of LV function post MI in vivo

6.4.3.1 Baseline haemodynamic measurements

Haemodynamic parameters were determined in mice (n=11) using the micro conductance catheter before they were subjected to LAD ligation (Figure 6-1 B). These data are described in detail in Table 6-1.

Table 6-1 Haemodynamics of mouse hearts at baseline based on in vivo analysis of pressure-volume relationships.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Baseline, Mean ± SEM, (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (beats/min)</td>
<td>605.2 ± 11.8</td>
</tr>
<tr>
<td>ESP (mmHg)</td>
<td>82.3 ± 2.3</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>ESV (µL)</td>
<td>5.5 ± 1.3</td>
</tr>
<tr>
<td>EDV (µL)</td>
<td>9.6 ± 1.3</td>
</tr>
<tr>
<td>SV (µL)</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>CO (µL/min)</td>
<td>2621.7 ± 206.3</td>
</tr>
<tr>
<td>EF %</td>
<td>75.2 ± 6.0</td>
</tr>
<tr>
<td>dP/dt max</td>
<td>6444.6 ± 341.9</td>
</tr>
<tr>
<td>dP/dt min</td>
<td>-5784.7 ± 332.1</td>
</tr>
</tbody>
</table>

6.4.3.2 Effects of ligation of the LAD on maximum pressure, end systolic and diastolic pressure

Linear regression analyses of the relationship between the changes in maximum pressure, end systolic and diastolic pressure and the ischaemic zone (% LV muscle volume), showed a significant (P<0.01) correlation of the decline in maximum pressure compared to an increase
in ischaemic risk zone (Figure 6-3 A). There was no correlation in ESP and EDP, with increasing percentage of LV ischaemia (Figure 6-3 B and C).

**Figure 6-3 Pressure volume analysis of relationship between area at risk (%AAR) size and change in left ventricular function.**

Correlation (P<0.01) of %AAR to the % change in maximum pressure (A), the end systolic pressure (P>0.05) (B) and end diastolic pressure (P>0.05) (C) in relation to %AAR 10 min following LAD ligation. Each data point represents a single animal (n=9). Dotted lines show 95% confidence intervals.
6.4.3.3 Effects of LAD ligation on stroke volume (SV), cardiac output (CO) and dP/dt_max

There was no correlation between a drop in SV (Figure 6-4 A) and CO (Figure 6-4 B), with increasing ischemic zone size (P>0.05 for both). A significant negative relationship (P<0.05), however, was observed in dP/dt_max as ischaemic size increased (Figure 6-4 C).

Figure 6-4 The relationship between area at risk (%AAR) size and change in left ventricular function using pressure volume analysis.

Correlation in the % change in stroke volume (P>0.05) (A), cardiac output (P>0.05) (B), and dP/dt_max (P<0.05) (C), with %AAR, 10 min following ligation of the LAD. Each data point represents a single animal (n=9). Dotted lines show 95% confidence intervals.
6.4.3.4 Effects of LAD ligation on stroke work and maximum power

A significant drop (P<0.05) was observed in LV maximum power (Figure 6-5 A) performed by the left ventricle 10 min following occlusion of the LAD. In this case there was a 35% decline in hearts with 60% AAR compared with hearts with no detectable AAR. No correlation was observed between stroke work (Figure 6-5 B) with increase in size of AAR (Figure 6-5 B).

![Graph A](image)

**Figure 6-5** Analysis of relationship between area at risk (%AAR) size and change in maximum power and stroke work using pressure volume catheter.

Correlation in the % change in maximum power (P<0.05) (A), and the change in stroke work (P>0.05) (B), in relation to %AAR 10 min following ligation of the LAD. Each data point represents a single animal (n=9). Dotted lines show 95% confidence intervals.

6.4.3.5 Echocardiographic analysis of LV function post- MI in vivo

This part of the study aims to investigate the potential use of imaging techniques, namely echocardiography, for early detection of successful infarct induction (Figure 1-6 C). The disadvantage with our previous study was that although a drop in pressure is a good marker of a reasonably sized area at risk, it is only an acute effect and will eventually normalize after a few days. Therefore, it cannot be used as a reliable indicator of infarction in chronic protocols (e.g. investigating remodelling 4 weeks post infarction). This prompted us to use
echocardiography to assess LV function post- MI in a recovery model three days after the operation.

High-resolution murine echocardiography offers a rapid, reproducible and non-invasive method of imaging the heart and measuring a range of cardiac performance parameters in the early post-operative period (at 1-3 days). The measurement of LV wall and cavity dimensions, using M-mode parasternal long- and short-axis views, offers an initial group of parameters to test for correlation with AAR; along with calculations of EF, FS and CO from the long-axis view. Evaluation of LV anterior wall (LVAW) and posterior wall (LVPW) movement by Tissue Doppler (TD) in the parasternal short-axis view and assessment of aortic peak flow and velocity time integral (VTI) by pulse-wave Doppler (PW) give further cardiac measurements to compare to AAR and infarct size. This is important as it has been reported that infarct sizes of less than 30% are not large enough to induce remodelling (Pfeffer and Braunwald, 1990).

The aim of present study in the early post-operative period after LAD ligation is to ascertain whether any cardiac parameter can be used to separate successfully ligated hearts from non-ligated, sham-operated hearts. In addition to reducing the expense of keeping excess animals with no infarct or small infarct (of less than 30%), this study addresses two of the ‘3 R’s’: Reduction, by early detection of infarcts and re-arrangement of study and control groups with minimal additional animals; and Refinement, by removing unneeded animals soon after surgery to minimise suffering. Ultimately, this project aims to provide an objective method of determining the presence of an infarct in LAD ligated mice, and possibly predicting the size of the induced infarction.

### 6.4.4 Direct Measures

First, various baseline LV parameters were measured in non-operated animals (Table 6-2). These measurements included LV wall dimensions such as LVAW, LVPW, LVID and IVS during systole and diastole. LV function was monitored by measuring %EF, %FS, CO as well as anterior and posterior wall movements using TD during LV diastole/relaxation (TDAW\text{rel} and TDPW\text{rel}) and systole/contraction (TDAW\text{cont} TDPW\text{cont}) respectively and blood flow by measuring VTI, aortic mean gradient and velocity. Since small infarct (less than 30%) are unlikely to remodel, we pooled the two groups (shams and <30% (small)
AAR) together (Figure 6-6). For statistical analysis we compared the sham (merged with <30% AAR) with hearts with >30 (large) % AAR (Table 6-2).

Echocardiographic analysis of LVAW during diastole and systole revealed a significant reduction in hearts with large %AAR compared with sham operated animals (Figure 6-6 A-B and Table 6-2). These hearts also displayed a marked LV dilatation which was manifested by a significant increase in the LVID compared with the shams (Figure 6-6 A and C) (Table 6-2). The IVS and LVPW remained unchanged during systole and diastole agreeing with previous reports (Bayat et al., 2002; Michael et al., 1995) (Figure 6-6 A and B). There was no significant difference in the LV function measured by %EF, %FS and CO three days post coronary ligation (Table 6-2). LAD ligation, however, did result in a significant reduction in TDPWcont and TDAWrel in hearts with large %AAR compared with sham operated group (6-7 A and B and Table 6-2). The TDAWcont and TDPWrel remained unchanged (Figure 6-7 A and B and Table 6-6). Interestingly, we were able to observe a marked reduction in the VTI, mean and peak gradient and mean and peak velocity of blood three days post ligation in the hearts with large %AAR compared with the shams (Figure 6-8 and Table 6-2).

![Image](image_url)

**Figure 6-6 M-mode imaging of hearts in the parasternal views 3 days following MI.**

Traces are taken at the level of posterior papillary muscle at end-systole in parasternal long (A) and short axis (B) views from control mice and after ligation in animals with small AAR and large AAR, see results for full description.
Table 6-2 Echocardiography measurements 3 days post-MI

Baseline and small AAR groups are shown as the combined group. (‡) indicates significance between small AAR and large AAR groups. Values are mean ±SEM.

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n = 29)</th>
<th>Small AAR (&lt;30%) (n = 6)</th>
<th>Large AAR (&gt;30%) (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>27.3 ± 0.27</td>
<td>26.9 ± 1.11</td>
<td>23.8 ± 2.09</td>
</tr>
<tr>
<td><strong>Heart Rate (bpm)</strong></td>
<td>531.4 ± 6.20</td>
<td>528.1 ± 10.95</td>
<td>529.4 ± 10.66</td>
</tr>
<tr>
<td>%AAR (of LV)</td>
<td>N/A</td>
<td>5.6 ± 2.87</td>
<td>45.9 ± 3.93</td>
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<tr>
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<tbody>
<tr>
<td><strong>DIASTOLIC FUNCTION</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVAW (mm)</td>
<td>0.9 ± 0.02</td>
<td>0.8 ± 0.02</td>
<td>0.7 ± 0.07 ‡</td>
</tr>
<tr>
<td>LV PW (mm)</td>
<td>0.8 ± 0.01</td>
<td>0.9 ± 0.03</td>
<td>0.9 ± 0.07</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.9 ± 0.02</td>
<td>0.9 ± 0.05</td>
<td>1.0 ± 0.11</td>
</tr>
<tr>
<td>LVID (mm)</td>
<td>4.1 ± 0.06</td>
<td>3.8 ± 0.11</td>
<td>4.7 ± 0.12 ‡</td>
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<tbody>
<tr>
<td><strong>SYSTOLIC FUNCTION</strong></td>
<td></td>
<td></td>
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<tr>
<td>LVAW (mm)</td>
<td>1.4± 0.03</td>
<td>1.1 ± 0.08</td>
<td>0.8 ± 0.09 ‡</td>
</tr>
<tr>
<td>LV PW (mm)</td>
<td>1.2 ± 0.02</td>
<td>1.3 ± 0.04</td>
<td>1.4 ± 0.19</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>1.4± 0.03</td>
<td>1.2 ± 0.05</td>
<td>1.4 ± 0.26</td>
</tr>
<tr>
<td>LVID (mm)</td>
<td>2.6 ± 0.07</td>
<td>2.6 ± 0.18</td>
<td>3.6 ± 0.14 ‡</td>
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<tr>
<td><strong>CONTRACTILE FUNCTION</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>EF (%)</td>
<td>66.3 ± 1.37</td>
<td>63.2 ± 4.15</td>
<td>53.0 ± 3.17</td>
</tr>
<tr>
<td>FS (%)</td>
<td>36.4 ± 1.04</td>
<td>34.2 ± 3.18</td>
<td>27.2 ± 2.17</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>24.2 ± 0.75</td>
<td>20.4 ± 1.33</td>
<td>26.5 ± 4.30</td>
</tr>
<tr>
<td>TD AW (rel) (mm/s)</td>
<td>15.0 ± 0.59</td>
<td>13.3 ± 1.98</td>
<td>8.3 ± 0.78</td>
</tr>
<tr>
<td>TD PW (cont) (mm/s)</td>
<td>21.8 ± 0.59</td>
<td>18.7 ± 1.31</td>
<td>12.6 ± 0.85 ‡</td>
</tr>
<tr>
<td>TD AW (rel) (mm/s)</td>
<td>24.8 ± 0.86</td>
<td>18.2 ± 2.37</td>
<td>8.0 ± 1.19 ‡</td>
</tr>
<tr>
<td>TD PW (cont) (mm/s)</td>
<td>27.8 ± 0.88</td>
<td>19.5 ± 2.46</td>
<td>18.4 ± 1.22</td>
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<tr>
<td><strong>AORTIC FLOW</strong></td>
<td></td>
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</tr>
<tr>
<td>VTI (cm)</td>
<td>4.3 ± 0.10</td>
<td>3.5 ± 0.17</td>
<td>2.5 ± 0.07 ‡</td>
</tr>
<tr>
<td>Mean gradient (mmHg)</td>
<td>2.7 ± 0.11</td>
<td>2.2 ± 0.25</td>
<td>1.3 ± 0.11 ‡</td>
</tr>
<tr>
<td>Peak gradient (mmHg)</td>
<td>7.2 ± 0.23</td>
<td>5.3 ± 0.62</td>
<td>3.3 ± 0.32 ‡</td>
</tr>
<tr>
<td>Mean velocity (mm/s)</td>
<td>822.0 ± 17.1</td>
<td>727.4 ± 42.27</td>
<td>558.3 ± 25.56 ‡</td>
</tr>
<tr>
<td>Peak velocity (mm/s)</td>
<td>1332.6 ± 26.87</td>
<td>1144.4 ± 68.10</td>
<td>908.5 ± 45.16 ‡</td>
</tr>
</tbody>
</table>
Figure 6-7 Screen capture of the echocardiography display produced by tissue Doppler (TD) 3 days post-MI.

(TD) imaging of the LV anterior wall (LVAW) (A), and posterior wall (LVPW) (B), are taken at the level of posterior papillary muscle at end-systole in the parasternal short-axis view. Traces are taken from control mice and 3 days post ligation in animals with small AAR and large AAR.
Figure 6-8 Pulse-wave Doppler (PW) of the aortic arch immediately before the brachiocephalic artery junction 3 days post-MI.
Traces showing 4 consecutive cardiac cycles from control and 3 days after ligation in control and animals with small and large AAR. All AAR traces are at the same scale.

6.4.5 Direct Measurement: Correlations with AAR

Using the information in Table 6-2, parameters with significant differences between sham (merged with small AAR) and large AAR were plotted against %AAR as before for the PV study as single data points for each animal. These parameters were LVAW and LVID during systole and diastole, TDPW<sub>cont</sub>, TDAW<sub>rel</sub>, VTI, aortic mean gradient and velocity.

Diastolic and systolic LVAW thickness showed no correlation with %AAR (data not shown). Similarly, there was no significant correlation in LVID diastole (Figure 6-9 A) and systole (Figure 6-9 B) with increased %AAR.

The wall motion of both PW<sub>cont</sub> (Figure 6-10 A) and AW<sub>rel</sub> (Figure 6-10 B) revealed no correlation with %AAR. The 95% confidence intervals were too wide to discern between the effects of the procedure in sham-operated and small AAR hearts and the effects of the ischemia present in hearts with larger infarctions.

Larger %AAR also reduced aortic flow parameters from small AAR to large AAR. Aortic mean gradient (Figure 6-11 A) and mean velocity (Figure 6-11 B) calculations showed a significant (p<0.05) negative correlation with %AAR. Aortic VTI (Figure 6-11 C) also diminished with increasing %AAR (p<0.05), all of which links with the LV dilation and wall motion impairments observed in these hearts.
Figure 6-9 Echocardiographic analysis of relationship between area at risk (%AAR) size and LV internal dimension.

Internal dimension (LVID) at diastole (A) and systole (B) taken three days after LAD ligation plotted against percent area at risk (%AAR) of the LV with linear regression. Each data point represents a single animal (n=10). Dotted lines show 95% confidence intervals.
Figure 6-10 Echocardiographic analysis of relationship between area at risk (%AAR) size and LV anterior and posterior walls movement.

Posterior wall contraction (PW_{cont}, A) and anterior wall relaxation (AW_{rel}, B) taken three days after LAD ligation plotted against percent area at risk (%AAR) of the LV, with linear regression. Each data point represents a single animal (n=10). Dotted lines show 95% confidence intervals.
Figure 6-11 Echocardiographic analysis of relationship between area at risk (%AAR) size and measurements of aortic functional parameters.

Mean gradient (A); mean velocity (B); and aortic velocity time integral (Ao VTI, C) taken three days after LAD ligation plotted against percent area at risk (%AAR) of the LV, with linear regression. Each data point represents a single animal (n=10). Dotted lines show 95% confidence intervals.

6.4.6 Morphological analysis of LV remodelling in WT and p38γδ KO 4 weeks post-MI

In this part of the study we used the protocol summarised in Figure 1-6 D. Analysis of hearts subjected to MI revealed a marked decline in LV free wall thickness with no apparent
change in interventricular septal wall thickness (Figure 6-12 A and B). The LV free wall thickness decreased from 1.60 ± 0.15 mm to 0.64 ± 0.26 mm in WT and from 1.6 ± 0.33 mm to 0.88 ± 0.26 mm in p38γδ KO hearts (Figure 6-12 A). There was no significant difference in the morphological analysis of LV remodelling in WT hearts compared with the KO animals 4 weeks post-MI (Figure 6-12 C).

Figure 6-12 Morphological analysis of hearts 4 weeks post-MI.
Hearts from age and weight-matched WT and p38γδ KO mice and sham operated mice subjected to MI. Animals were sacrificed 4 weeks post infarction and hearts were excised and sliced. LV free wall (A) and septal wall dimensions (B) were measured. (C) Representative slices of hearts from WT and p38γδ KO mice that were harvested 28 days following true and sham ligation of the LAD. Data represent mean ± SEM, n=6.

6.4.7 Echocardiographic analysis of LV remodelling in WT and p38γδ KO hearts post-MI

Echocardiography revealed a significant increase in LV volume during systole accompanied by a reduction in LV mass in WT and transgenic mice four weeks post-MI in both
genotypes (Table 6-3). There was also a significant reduction in LV anterior wall (AW) mass only in the KO mice (Table 6-3). The data demonstrated a significant reduction in the thickness of LV anterior wall (LVAW) during systole and diastole compared with sham operated mice in both WT and p38γ/δ KO hearts four weeks post-MI (Figure 6-13 A and B). During systole, LVPW significantly reduced in the WT only (Figure 6-13 D), but remained unchanged in both genotypes during diastole (Figure 6-13 C). Interestingly, LV dilatation was more prominent in the WT remodelled hearts, which was manifested by a significant decrease in LVS (Figure 6-14 A) and a marked increase in LVID (Figure 6-14 C) during diastole. During systole, both genotypes demonstrated LV dilatation evident by a marked reduction in LVS (Figure 6-14 B) and a significant rise in LVID (Figure 6-14 D). Intriguingly, although there were no significant differences in the responses of the two genotypes, the changes reported during systole were statistically more significant in WT remodelled hearts compared with the transgenic hearts (i.e. p<0.01 in WT vs. p<0.05 in the null mice (Figure 6-14 B and D).

Table 6-3 Echocardiographic measurements from M-mode readings four weeks post-MI.

LV volume diastole, left ventricular volume during diastole; LV volume (systole), left ventricular volume during diastole; LV mass, left ventricular mass; LV Mass (AW), left ventricular mass (anterior wall). * p<0.01 compared with control group in WT and # p< 0.05 compared with control group in γδ KO. Values are mean ± SED.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT LAD (n=6)</th>
<th>γδ KO LAD (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV Volume, Diastole (µL)</td>
<td>107.48 ± 29.91</td>
<td>71.04 ± 8.43</td>
</tr>
<tr>
<td>LV Volume, Systole (µL)</td>
<td>89.12 ± 39.02*</td>
<td>36.60 ± 14.92#</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>82.44 ± 28.37*</td>
<td>52.02 ± 4.18#</td>
</tr>
<tr>
<td>LV Mass, AW (mg)</td>
<td>70.33 ± 14.88</td>
<td>49.40 ± 2.99#</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Sham LAD (n=4)</th>
<th>Sham (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV Volume, Diastole (µL)</td>
<td>67.05 ± 13.68</td>
</tr>
<tr>
<td>LV Volume, Systole (µL)</td>
<td>19.98 ± 8.78</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>136.89 ± 41.90</td>
</tr>
<tr>
<td>LV Mass, AW (mg)</td>
<td>116.17 ± 16.32</td>
</tr>
</tbody>
</table>
Chapter 6

p38-MAPK isoforms and myocardial remodelling

Figure 6-13 Echocardiographic analysis of LV anterior and posterior walls thickness 4 weeks post-MI.

Hearts from age and weight matched WT and p38γδ KO mice and sham operated mice subjected to MI. After 4 weeks, mice were subjected to echo and parameters measured; Left ventricular anterior wall (LVAW) thickness during diastole and systole (A) and (B) respectively Left ventricular posterior wall (LVPW) thickness during diastole and systole (C) and (D) respectively. Data represent mean ± SEM, n=6.
Figure 6-14  Echocardiographic analysis of LV septal and interior dimensions 4 weeks post-MI.

Hearts from age and weight matched WT and p38γδ KO mice and sham operated mice subjected to MI. After 4 weeks mice were subjected to echo and parameters measured; Left ventricular septal wall (LVS) thickness during diastole and systole (A) and (B) respectively. Left ventricle interior dimension (LVID) during diastole and systole (C) and (D) respectively. Data represents mean ± SEM, n=6.

There was a significant reduction in contractility of remodelled hearts apparent by a large reduction in the %EF and %FS compared with sham-operated group (Figure 6-15 A and B). However, CO remained unchanged in all groups (Figure 6-15 C).
Hearts from age and weight matched WT and p38γδ KO mice and sham operated mice subjected to MI. Following 4 weeks mice were subjected to echo to measure ejection fraction (EF) (A), fractional shortening (FS) (B) and cardiac output (CO) (C). Data represents mean ± SEM, n=6.
All the echocardiographic measurements were performed in m-mode imaging (Figure 6-16 A-D).

**Figure 6-16** Representative echocardiographic images from parasternal long axis view 4 weeks post-MI.

Hearts from age-weight matched WT and p38γδ KO mice (A-B) and sham operated (C-D) mice subjected to MI. 28 days later the mice were subjected to ultrasound analysis. LV remodelling can be detected by a reduction in wall motion and LV dilatation.
6.4.8 Pressure-volume analysis of LV remodelling in WT and p38γδ KO mice post-MI

Pressure-volume analysis of hearts subjected to permanent ligation of the LAD showed a significant increase in EDV and ESV in WT but not p38γδ KO compared with sham-operated animals (Figure 6-17 A and C). The EDP and ESP in remodelled hearts of both remained unchanged (Figure 6-17 B and D).

The contractile dysfunction was manifested in the significant reduction in the %EF and SW with preserved dP/dt_max compared with the shams (Figure 6-18 A-C). There was no significant change in HR, SV and CO post-MI in either genotype (Figure 6-19 A-C).

Overall, there was no significant difference in haemodynamic parameters of WT and p38γδ KO mice four weeks post-MI.
Figure 6-17 In vivo haemodynamic measurements using pressure volume analysis 4 weeks post-MI.

Hearts from age and weight matched WT and p38γδ KO mice and sham operated mice subjected to MI. After 4 weeks, mice were catheterised through the internal carotid artery and haemodynamic parameters were measured using the admittance catheter inside the LV. (A) End diastolic volume (EDV); (B) End diastolic pressure (EDP); (C) End systolic volume (ESV); (D) End systolic pressure (ESP). Values are mean ± SEM, n=6.
Hearts from age and weight matched WT and p38γδ KO mice and sham operated mice subjected to MI. After 4 weeks mice were catheterised through the internal carotid artery and haemodynamic parameters were measured using the admittance catheter inside the LV. (A), Ejection fraction (EF); (B), Maximum first derivative of pressure (dP/dtmax) and (C) Stroke work (SW). Data are presented as mean ± SEM, n=6.
Figure 6-19 Haemodynamic analysis of LV function using pressure volume analysis 4 weeks post-MI.

Hearts from age and weight matched WT and p38γδ KO mice and sham operated mice subjected to MI. After 4 weeks, mice were catheterised through the internal carotid artery and haemodynamic parameters were measured using the admittance catheter inside the LV. (A), Heart rate (HR); (B), Stroke volume (SV) and (C), Cardiac output (CO). Values are mean ± SEM, n=6.
6.5 Discussion

6.5.1 Validation and characterisation of LV remodelling model

Permanent occlusion of the LAD is the most commonly used method to study the changes that occur during LV remodelling. However, the anatomy of the coronary arteries varies in mice. This has caused problems with reproducibility of infarct volumes following ligation of the LAD (Pfeffer and Braunwald, 1990). It has been reported that the LAD occasionally branches early and occlusion of other branches is necessary to produce a large infarct (Fisher and Marber, 2002). Small infarct volume may be insufficient to trigger LV remodelling in mice 4 weeks after ligation of the LAD (Pfeffer and Braunwald, 1990).

In order to improve the reproducibility of AAR, we used a non recovery model to ensure that the LAD was ligated in the same place (Figure 6-2 B). Care was taken to ensure that the LAD was always ligated efficiently (1-2 mm from the bottom edge of the left atrium) in order to generate reproducible data. In this study a relatively consistent ischaemic size was achieved within the 95% confidence intervals (Figure 6-2 A-C).

Using pressure-volume analysis, we next aimed to investigate acute changes in cardiac function during regional ischemia following occlusion of the LAD and to investigate whether a single haemodynamic parameter or group of parameters can be used as a determinant of ischaemic size and, ultimately, the extent of infarct.

Our findings show that there is a strong negative correlation between LV AAR and maximum LV pressure, rate of change of contraction, \( \frac{dP}{dt_{\text{max}}} \) and maximum power. Upon closer inspection, from our data we can predict (P<0.001) that an AAR of at least 60% would result in drop in maximal LV pressure of approximately 55% after occlusion. Whilst a small or non measurable AAR was only associated with a drop in pressure of about 12%. A similar, although less robust (P<0.05), correlation is observed between \( \frac{dP}{dt_{\text{max}}} \) and Maximal Power and AAR following tying of the LAD. AAR of 60% or more can be achieved when a 25% and 30% reduction in \( \frac{dP}{dt_{\text{max}}} \) and maximum work respectively is observed.

Decreases in maximum pressure, \( \frac{dP}{dt_{\text{max}}} \) and maximum power appear to be the most immediate, and reproducible, myocardial adaptations to a sudden decrease in blood flow following LAD ligation. A reduction in maximum pressure and systolic pressure can be best
explained by poor systolic ejection. This could be due to impaired muscle function and poor contractility caused by poor muscle perfusion following occlusion of a major coronary artery.

In this study, we were hoping for a haemodynamic parameter or group of parameters to hold a close, linear relationship with AAR following ligation of the LAD. Thus, when subjected to linear regression analysis and plotted, a graph with a steep gradient and tight confidence intervals would result (Figure 6-20 A). Such a relationship would enable us to accurately determine AAR and therefore predict infarct progression, with high confidence. For instance, from the hypothetical graph shown in Figure 6-20 A, if the parameter dropped to 80% of baseline (shown in green), we could predict an AAR of between 10 and 18%. Similarly, observing a decrease of 50% 10 min after the onset of ischaemia we would expect AAR of 40 to 45% (shown in red).

However, from the data generated in this study, having recorded a decrease in maximum pressure to 80% of baseline following LAD ligation, we can be 95% confident that an AAR up to 30% will be achieved (Figure 6-20 B) although it could conceivably be as little as 0%. This graph also suggests that a drop in maximum pressure to 60% would indicate an AAR of at least 40% of the LV within the 95% confidence levels (Figure 6-20 B). Although a similar relationship can be observed between Maximal power, dP/dt_max and ischaemic size, we cannot determine with any statistical certainty the degree of ischemia when the parameter lies within a certain range. Because of the variation in the data and the confidence of the correlation, if the measured dP/dt_max or Maximal Power drops to ~70% and ~45% of baseline respectively (shown by the red dotted line in Figure 6-20 C and D) we are unable to calculate the risk zone. Therefore in this study, dP/dt_max and Maximal Power could not be used to predict the degree of ischaemia following LAD ligation.

The drawback with using the maximum pressure as an end-point in determining infarct size is associated with its acute effects. Drop in pressure tends to normalise in a few days in vivo and therefore, it is not a useful parameter to use in long term studies such as remodelling. Our data prompted us to use echocardiography to analyse LV function three days post- MI.
This study was designed to investigate whether a single haemodynamic parameter or group of parameters can be used as a determinant of ischaemic size and, ultimately, the extent of infarct. Using tissue Doppler as expected, echocardiography illustrated that LAD ligation leads to changes in cardiac dimensions, as well reducing CO, function (Bayat et al., 2002; Pfeffer and Braunwald, 1990) and wall motion (Stypmann et al., 2009; Suematsu et al., 1995) and aortic flow measurements. However, discriminating between the effects of the surgical procedure and the changes caused by large AAR was more complex.
Specifically, large infarcts induced increased LV dilation compared to small infarcts, which appeared to correlate with %AAR (Figure 6-10). However owing to the variability in the data, the 95% confidence intervals would lead to the prediction of achieving an AAR of >25% with the smallest LVID size seen in the large AAR group in this study (4.6 mm diastolic, 3.4 mm systolic), which is not specific enough for reliable %AAR prediction. Similar predictions were seen for LV wall motion measurements, with PW_{cont} (14.1 mm/s; Figure 6-9 A) and AW_{rel} (9.4 mm/s; Figure 6-10 B) giving >20% and >22% AAR, respectively, for the maximum velocities seen in animals with large AAR. AW_{rel} was further compromised as a viable measure as an animal in the small AAR group showed a lower velocity than two mice with large AAR. Aortic flow measurements also gave broad predictions, with aortic VTI presenting the narrowest 95% confidence values and a prediction of >28% AAR for the highest velocity in the large AAR group (2.6 cm; Figure 6-11 C). Aortic mean gradient (1.3 mmHg; Figure 6-11 A) and mean velocity (586.3 mm/s; Figure 6-11 B) both indicated AAR of >20% for the maximum large AAR value; however mean velocity showed overlap between the large and small infarct groups.

From some of the obtained correlations in this part of the study (Figure 6-9 A and B), there appear to be a strong association between certain haemodynamic parameters (i.e. r^2), whereas the calculated P values are not statistically significant. In other words, despite an apparent strong correlation, in reality the correlation may be derived by chance. One other explanation for this could be associated with r^2 and P relationship; r^2 is the goodness of fit (linearity) whereas P is the probability that the gradients are significantly non-zero. Therefore, a high r^2 does not necessarily indicate a significant correlation, unless it is concomitant with significantly non-zero gradient (P value).

Considering all of the above, perhaps one can argue that examining the relationship between the %AAR and change in haemodynamic parameters using correlation coefficient is not the most appropriate method. The implicated factors could be the variability of the data and possibly the relatively small sample size used in these experiments. Although we were able to make interesting observations, we cannot use the data to predict infarct size confidently.

In our final protocol to study the role of p38γ and δ MAPK in LV remodelling, we performed a follow-up echocardiography three days post-MI to confirm infarction. However, we found that some of the mice with large infarcts died shortly after the follow-up echo, possibly due
to the anaesthetic effect. In addition, using pressure volume and echo we were not able to identify a reliable parameter to precisely predict infarct size. Therefore, the most useful, out of three methods to characterise the model and generate consistent ischaemic size was the first reproducibility experiment (Figure 6-2). In our experience, the best way to produce a reproducible model was by practice and surgical training. In our hands, consistency in ligating the LAD (from 1-2 mm from the edge of the left atrium to the middle of the heart) was the key to generating a reasonable size infarct to cause remodelling. Therefore the final protocol (Figure 6-1 D) was designed by taking the above into consideration. In this protocol, ultrasound echo was performed before and only after 4 weeks post surgery in order to examine LV remodelling.

6.5.2 Are the data consistent with LV remodelling?

The syndrome of cardiac remodelling has traditionally viewed as functional disorders and is classified into systolic and diastolic dysfunction. As described in the previous chapter the compensatory hypertrophy occurs at the acute phase of cardiac remodelling and is mainly associated with diastolic dysfunction (Chapter 5 section 5.5.1). However, progressive remodelling of the LV, as a consequence of structural increase in ventricular chamber volume, results in the manifestation of systolic dysfunction (impaired ventricular contraction). Chamber dilatation occurs as an early response that results in the reduced wall motion that is mandated to generate a normal SV from a large ventricular EDV. In chronic heart failure, this is most likely due to changes in the signal transduction mechanisms implicated in cardiac apoptosis, scar formation, CF proliferation and ECM rearrangement as well as mechanisms involved in the regulation of cardiac excitation-contraction coupling (Cohn, 1995). In addition to the underlying etiological cardiac pathology, it has also been suggested that structural alterations can represents as intrinsic morphological change that progress over time in response to an initiating event (Cohn, 1995).

The mechanical properties of cardiac muscle are the basis for an inherent mechanism for altering SV: the ventricles contract during systole more forcefully when filled to greater degree during diastole. This relationship between SV and EDV (preload) can be explained by the Frank Starling relationship (Figure 6-21 A). This allows the cardiac output to be synchronized with the venous return, arterial blood supply and without depending upon external regulation to make alterations. Following an MI, the loss of cardiac inotropy causes
a downward shift in the Frank-Starling curve (Figure 6-21 A). This results in a decreased SV and a compensatory rise in preload. The rise in preload is considered compensatory as it activates the Frank-Starling mechanism to help maintain SV despite the loss of inotropy. The reduction in loss of contractility can be explained by the force-velocity relationship. Briefly, at any given preload and afterload, a loss in inotropy results in a decrease in the shortening velocity of cardiac fibres. As there is only a limited period of time available for EF, a reduced velocity of %EF results in less blood ejected per SV. The residual volume of blood within the ventricle is increased (increased ESV) because less blood is ejected. This can further be depicted using pressure-volume loops (Figure 6-22 B). Loss of intrinsic inotropy decreased the slope of the end-systolic pressure volume relationship (ESPVR). This leads to an increase in ESV. There is also an increase in EDV (compensatory increase in preload), but this increase is not as great as the increase in ESV. Therefore, the net effect is a decrease in SV (which narrows the pressure volume loop). Because SV decreases and EDV increases, there is a substantial reduction in %EF. SW (area within loop) is also decreased (Figure 6-21 B).

![Figure 6-21 (A) Operation of Frank-Starling law under normal conditions and in heart failure. (B) Systolic and diastolic dysfunction presented by pressure-volume loops.](image)

(A) At constant heart rate and ejection pressure, stroke volume rises with increasing end-diastolic pressure (black line). In heart failure, the inotropy is impaired which results in a rise in end systolic pressure. As the contractility is impaired, more blood is left in the ventricles which lead to a reduction in stroke volume and a compensatory increase in preload (end diastolic pressure). This will shift the Frank-starling cure down and to the right (red line). (B) Systolic dysfunction (decreased inotropy) and diastolic dysfunction (decreased compliance) result in a depressed end systolic pressure volume relationship and a raised slope of passing filling respectively. This will lead to an increased end systolic volume and a reduced end diastolic volume which causes a dramatic reduction on stoke
volume and ejection fraction. Adapted from www.cvphysiology.com

Using PV analysis in this chapter, systolic dysfunction was apparent by a significant reduction in %EF in both genotypes (Figure 6-18 A) and a compensatory diastolic function by elevated preload (EDV) only in the WT hearts (Figure 6-17 A). Using echocardiography, we were able to observe a significant reduction in the %FS of both genotypes. Echo also revealed a significant increase in LVID (systole in both genotypes and diastole only in WT) (Figure 6-14 A-D). The LV dilatation together with increased end diastolic volume indicates increased ventricular compliance, suggests impaired ventricular relaxation and consequently LV filling. However, despite a marked reduction in the %EF and an increase in EDV, SV remained unchanged, which is not consistent with systolic dysfunction described above. CO and HR (determinant of SV) did not change post MI compared with the sham operated animals in either genotypes. This partly explains why the SV remained unchanged. Using PV, a marked increase was observed in EDV and ESV (Figure 6-17 A and B) post-MI only in the WT. In addition, we were not able to detect any changes in ESP, EDP (Figure 6-17 B and D) and $dP/dt_{\text{max}}$ (Figure 6-18 B). Therefore, it is possible that the reduction in %EF (shown by both echo and PV Figure 6-15 A and Figure 6-18 A) and %FS (Figure 6-15 B) in this study is in part a reflection of increased chamber volume that will obligatory result in a low %EF in the absence of peripheral demand for a high SV. Perhaps in this case, remodelling, not contractile dysfunction is the key to the severity of depression of %EF.

6.5.3 Pathophysiology of LV remodelling is partially dependent on p38γ and δ isoforms

The two signalling cascades have been particularly well studied both in cultured myocytes and in experimental models of LV remodelling; the PI3K and MAPK pathways. In MAPK family, p38 MAPK and JNK have been more commonly associated with pathologic dilatation and cardiomyocyte apoptosis whereas ERK has been found to play an anti-apoptotic role in promoting physiological hypertrophy (Braz et al., 2002; Bueno et al., 2000b; Ferrandi et al., 2004; Ren et al., 2005). p38 MAPK is activated by ischaemia, haemodynamic stresses, neurohormonal factors such as Ang II (van et al., 1999) endothelin and phenylephrine (Clerk et al., 1998). These p38 MAPK dependent processes (i.e. pathological remodelling and cardiac myocyte apoptosis) characterise the cellular sequel
post-MI, suggesting p38 MAPK activation may contribute to progressive LV remodelling and the transition to heart failure.

Various experiments have implicated a role for p38 MAPK in cardiac remodelling in vivo (Behr et al., 2001; Cain et al., 1999; Cook et al., 1999; Liao et al., 2001; Ren et al., 2005). Sustained p38 MAPK activation in the heart has been associated with LV remodelling and dysfunction arising from various aetiologies both in humans (Cook et al., 1999) and in animal models (Behr et al., 2001; Liao et al., 2001). In the failing human heart owing to ischaemic injury, inhibition of p38 MAPK has been shown to improve ex vivo function of ischaemic myocardium (Cain et al., 1999). Using a gene-switch transgenic strategy with activated mutants of upstream kinases MKK3bE and MKK6bE, Liao and co-workers showed a significant induction of p38 MAPK activity and premature death at 7-9 weeks in the transgenic mice (Liao et al., 2001). Both groups exhibited a significant interstitial fibrosis and expression of foetal marker genes (α and β MHC). Functional analysis (by both echo and PV) revealed a similar systolic dysfunction and restrictive diastolic abnormalities related to markedly increased chamber stiffness. In this study, end systolic dilatation, wall thinning and myocyte atrophy were only observed in MKK3bK, indicating MKK3 as the main activator p38 MAPK in cardiac remodelling (Liao et al., 2001). Involvement of p38 MAPK in cardiac remodelling post MI was also demonstrated in rats subjected to permanent coronary ligation (See et al., 2004). In these animals, treatment with RWJ-67657 (RWJ) (inhibitor of α and β isoforms) 7 days post MI impeded the development of chronic heart failure (manifested by reduced lung weight to body weight ratio), preserved LV function and dimension and inhibited infarct expansion. Treatment with RWJ also lead to a preserved %FS and increased myocyte hypertrophy and reduced myocardial collagen and α-smooth muscle actin (SMA) compared with not-treated animals (See et al., 2004). Interestingly, RWJ had suppressive effects on TGF-β-stimulated collagen synthesis and gene expression on neonatal CF. These observations point to a potential mechanism by which p38 MAPK regulate collagen expression by CF (See et al., 2004).

In this chapter, were able to detect subtle differences in the responses of WT compared to the p38γδ KO in chronic LV remodelling. Our data suggested that the WT are more susceptible to LV dilatation. This was demonstrated by a significant decrease in LVS and a marked increase in LVID only in WT during diastole. Although both genotypes exhibited LV dilatation during systole, the data was more significant in the WT remodelled hearts.
Furthermore, only WT hearts demonstrated a significant increase in EDV and ESV (measured by PV). This response was absent in the null mice. The data in this chapter is in keeping with the previous chapter (Chapter 5) which also suggested that p38γδ null mice are to some extent protected against dilatation and also demonstrate a better preserved function following β-AR stimulation with ISO. Interestingly, in a recent study of in vivo infarction, a marked increase was observed in the phosphorylation of p38γ in the infarct and remote area of insult in an in vivo mouse model of MI (Yeh et al., 2010a).

The findings in this chapter together with recent reports, can therefore, to some extent indicate that the γ and δ isoforms are important and somehow contribute to the process of LV remodelling. However, the exact mechanisms by which they regulate this process remains unclear and warrants further investigation. In order to fully investigate the role of the γ and δ isoforms perhaps, it would have been more useful to monitor the remodelling process in both genotypes over a time course (3, 7, 21 and 24 days) after the coronary artery ligation. Investigating the extend of fibrosis, apoptosis and analysis of isoform expression/activation as well as functional examinations, would have perhaps enabled us to gain a better insight of the possible involvement of p38 isoforms in LV remodelling post MI.

As with cardiac hypertrophy, the process of remodelling involves activation of many complex signalling pathways. A large degree of cross talk between these signalling pathways and substrates sharing could explain the negative results in this study. In a recent study, our group demonstrated that even knocking out MKK3, one of the major upstream activators of p38 MAPK, has no significant consequence in the progression of pathological remodelling post infarction (Clark et al., 2007). In addition, the heterogeneity of cellular responses and different pattern of MAPK signalling in cardiac myocytes and non-myocytes (such as CF) should also be considered when interpreting the data. These implications were recently examined in a study investigating the distinct activation of p38 MAPK and ERK signalling in remote and infarcted myocardium in an in vivo post-MI remodelling in mice (Yeh et al., 2010a). In this study, it was demonstrated that phosphorylation of p38 MAPK increased steadily in the remote myocardium, while remaining constant or declining in the infarct/border zone. Phosphorylation of ERK remained higher in the infarct than in the remote myocardium during the process. JNK phosphorylation decreased in the remote myocardium, however, its activation increased significantly after 2 weeks in the infarct region and remained stable. A 30% decreased was observed in the phosphorylation of AKT.
(a key PI3K intermediate) only early after MI in the remote myocardium and remained unchanged during the remodelling process (Yeh et al., 2010a). Interestingly, a progressive increase of p38α and p38γ MAPK isoforms was reported from week 1-12 post-MI. It was also shown that, phosphorylated ERK localised to fibrotic areas populated primarily by CF, whereas staining p38 MAPK phosphorylation was stronger in areas of progressive cardiomyocytes apoptosis. These observations highlight the fact that strategies for therapeutic manipulation of signalling patterns, particularly in the complex setting of post-MI remodelling and cardiomyopathy, may require cell-type specific intervention. For example, ERK activation in cardiac myocytes might inhibit apoptosis and loss of functional myocardium, but may result in increased fibrosis in CF. On the contrary, inhibition of p38 MAPK may attenuate cardiac myocyte apoptosis and fibrosis by regulating collagen turn over by CF (See et al., 2004; Yeh et al., 2010a).

It should be noted that the remodelling process is a complex process involving the activation of many intracellular processes in the heart which is beyond the scope of this Thesis. (Figure 6-22). The distinct activation of these signalling pathways may in part account for the heterogeneity of clinical presentation of heart dysfunction in patients. For instance, about half of the patients have contractile failure and a dilated heart (that is systolic heart failure) whereas other half seem to have a normal contraction and a non-dilated, but often hypertrophied heart (Mudd and Kass, 2008). Indeed, untangling the molecular mechanisms of remodelling has formed one of the avenues in the development of new treatments for tackling heart failure. In addition to the activation of the compensatory signalling pathways, the chronic phase is associated with fibrosis and scar formation. It has been though that the persistence of myoFb can facilitate scaring and fibrosis which leads to pathological remodelling and eventually heart failure (Sun and Weber, 2000). It has also become apparent that resident myocardial CF are not the sole source of myoFb in the remodelling heart and a role for hematopoietic cell derived fibroblast has also been reported in various in vivo model of remodelling (Fujita et al., 2007; Haudek et al., 2006; Haudek et al., 2008).

These implications are clearly very important to consider when developing new pharmacological treatments. For instance, treatment with β-blockers halts left ventricular remodelling, improves left ventricular ejection fraction and has a positive effect on mortality outcomes (Sabbah, 2004). Although β- blockers are currently the most efficacious class of drugs in the treatment of heart failure, there is some debate as to the exact nature of their
action. This is in part reflected in the activation of β-AR subtypes. It has been shown that in both adult rat CF and human myoFb (expressing predominately β2-AR) adrenaline (a non-selective agonist) was a more potent inducer of cAMP than noradrenaline (a β1-AR selective inhibitor) (Meszaros et al., 2000; Turner et al., 2003). However, recent treatment for patient with heart failure has been in favour of β1-selective-blockers (such as Metoprolol). Such treatments tends to ignore the potential benefits of inhibiting the β2-AR on CF. Perhaps this is the overlooked explanation for the greater benefit observed with Carvedilol (non-selective α-β-antagonist) compared with the β1-AR selective inhibitor Metoprolol in COMET (Carvedilol or Metoprolol European Trial) clinical trial (Kveiborg et al., 2007).
Figure 6-22 Cardiomyocyte signalling pathways in pathophysiology of heart failure.

Stress stimuli include nitric oxide, neurohormones (such as natriuretic peptides and angiotensin II, the latter of which binds to G\textsubscript{S} or G\textsubscript{11}-protein-coupled receptors), neurotransmitters (such as catecholamines, which bind to \(\beta\)-adrenergic receptors (\(\beta\)-ARs)), cytokines and growth factors. After cell-surface receptors bind to these ligands, the signal is transmitted to protein kinases, which in turn activate signalling nodes (where many pathways converge). These nodes include calcium (Ca\textsuperscript{2+})/calmodulin-dependent kinase II (CAMKII), Akt, glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)) and cyclic GMP (cGMP)-dependent protein kinase (PKG). These pathways are involved in physiological responses; however, in the failing heart, there are more stress stimuli, thereby amplifying these pathways and generating imbalances among them. AC, adenylyl cyclase; AKAP1, PKA anchor protein 1; ANP, atrial natriuretic peptide; cAMP, cyclic AMP; DAG, diacylglycerol; FOXO, forkhead-box O proteins; HDAC, histone deacetylase; INPP5F, inositol polyphosphate-5-phosphatase F; InsP\(_3\), inositol-1,4,5-trisphosphate; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor 2; mRNA, messenger RNA; miRNA, microRNA; NFAT, nuclear factor of activated T cells; NKX2-5, NK2 transcription factor related, locus 5; NO, nitric oxide; pGC, particulate guanylyl cyclase; PICOT, PKC-interacting cousin of thioredoxin; PI(3)K-Y, phosphatidylinositol 3-OH-kinase-\(\gamma\); PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PDE5, phosphodiesterase 5; PKD, protein kinase D; PLC, phospholipase C; PtdIns(4,5)P\(_2\), phosphatidylinositol-4,5-bisphosphate; PTEN, phosphatase and tensin homologue; RCAN1, regulator of calcineurin 1; ROCK, Rho-associated, coiled-coil-containing protein kinase; sGC, soluble guanylyl cyclase; SRF, serum response factor; STAT, signal transducer and activator of transcription. Adapted from (Mudd and Kass, 2008)
7 SUMMARY AND CONCLUSIONS

7.1 General Summary of data

The isoforms-specific roles of p38 MAPK in the adult heart are largely unknown, partly due to the historical lack of specific tools to either manipulate or measure selective p38 MAPK isoform activity. Improved understanding of p38 MAPK isoform regulation and determination of their contributions in myocardium will, undoubtedly benefit research into designing of pharmacological inhibitors with greater selectivity and move away from the drawbacks of chronic systemic inhibition. In this Thesis we showed p38α and γ to be the most abundantly expressed isoforms in the mouse heart. Our data confirmed that p38α MAPK is the dominant isoform in the murine myocardium and is activated in response to ischaemia, ischaemia reperfusion and a number of pro-inflammatory cytokines. We propose that p38β MAPK is implicated in pharmacological preconditioning whereas p38γ and δ isoforms appear to be important in the myocardial response to osmotic stress. In addition, the γ and δ isoforms may, somehow, be involved in LV remodelling and functional changes during cardiac hypertrophy and following MI.

7.1.1 Endogenous expression and localization of p38 MAPK isoforms in murine heart

Using commercially available isoform-specific antibodies, in Chapter 3, we were able to demonstrate that all p38 MAPK isoforms are expressed in the murine heart with p38α and γ being the most abundant. The other two isoforms, p38β and δ seemed to be expressed at lower abundance and were only detected by confocal microscopy of isolated cardiac myocytes. In addition, the transcript for all the p38 MAPK isoforms were detected in murine hearts. Experiments in isolated cardiac myocytes using immunofluorescence demonstrated a diverse localization of p38 MAPK isoforms which suggest distinct substrate selectivity and consequently different functions. For instance, expression of p38α and β MAPK in the cytoplasm as well as the nucleus suggests that these isoforms control multiple cellular functions such as, cytoskeletal proteins and translational machinery components. Punctate distribution of p38γ MAPK throughout the cytoplasm will allow its interaction with various cytoplasmic proteins. Our data also demonstrated localization of p38δ MAPK at the intercalated discs which serve to anchor actin filaments between cells. These findings
suggested that p38γ and δ MAPK may be part of a signalling pathway activated to enhance survival in response to stress.

### 7.1.2 Involvement of p38β MAPK in preconditioning

Although we were not able to delineate a significant role for p38β MAPK isoform in response to ischaemia-reperfusion injury, we made a very interesting observation when investigating the role of this isoform in CORM-3 mediated cardioprotection.

Using Langendorff perfusion in Chapter 4, we have shown that mice lacking the β isoform are refractory to CORM-3 (CO releasing molecule) mediated cardioprotection. The data demonstrate that CORM-3 pre-treatment followed by a 5 min washout of hearts prior to an *in vitro* ischaemia-reperfusion insult result in decreased infarct size and increased LV function. This effect was reversed in WT hearts in presence of the p38 MAPK inhibitor SB203580. These observations may indicate that p38β MAPK isoform is an important mediator of pharmacological cardioprotection in our model. However, the exact mechanism by which the cardioprotection is achieved remains unclear and requires further experiments. These are discussed in section 7.7-2 of this Chapter.

### 7.1.3 Involvement of p38γ and δ MAPK in osmotic stress in an *ex vivo* model

Using an isolated mouse heart perfusion model in Chapter 4, we explored the activation of p38γ and δ isoforms in myocardium in response to cytokines (TNF-α and IL-1), ischaemia-reperfusion and osmotic stress (induced by sorbitol). Our data suggested that neither of these isoforms is likely to play a significant role in response to ischaemia and inflammatory cytokines such as TNF-α and IL-1. Both ischaemia and the inflammatory response are prime activators of p38α MAPK. As this isoform is the most dominant of the other three and is therefore likely that the stimuli used in our studies will activate the signalling pathway involving p38α MAPK isoforms. Interestingly, the functional analysis of hearts treated with sorbitol revealed a significant reduction in left ventricular developed pressure in response to sorbitol in WT which was significantly ameliorated in p38γδ KO hearts. These data were consistent with western blot data showing a reduction in the level of p38 MAPK phosphorylation in KO mice compared with WT after sorbitol treatment. Although these observations are interesting, the exact signalling pathway involving p38γ and δ MAPK in response to sorbitol remains unclear and warrants further investigation.
7.1.4 LV remodelling in response to cardiac hypertrophy and 4 weeks post- MI is independent of p38γ and δ MAPK

Investigating the contribution of p38γ and δ MAPK isoforms in cardiac hypertrophy and infarction revealed that both genotypes develop LV hypertrophy and remodelling. However, interestingly, it appears that the null mice are to some extent protected against LV dilatation and also demonstrate a better preserved function following β-AR stimulation by ISO. These findings may suggest that p38γ and δ MAPK are involved and somehow contribute to the process of LV remodelling. The exact mechanisms by which these isoforms regulate these processes remain unclear.

7.2 Critique of Methods

7.2.1 Nature of models used

In this Thesis we were able to detect all four isoforms using RT-PCR but not by western blotting. We discussed the implication of different techniques in detecting different isoforms in Chapter 3 (section 3.9.3). However, it should also be noted that we did not include a negative control without the RT-enzyme to ensure our data is from the amplification of mRNA not genomic DNA. This issue could have been resolved by using an intron spanning primers prior to PCR to ensure absence of genomic contaminations in the samples.

Due to the lack of isoform-specific phospho-specific antibody, we were forced to use a phospho p38 MAPK pan-isoform antibody to investigate the activation of different isotypes. Although the antibody has shown to be able to detect all four isoforms by resolving different molecular weights corresponding to α, β, γ and δ, we were not able to demonstrate this in the present study. In addition, we were unable to use the ELISA kit specific for measuring the activity of p38γ MAPK, due to a high background, possibly generated by non-specific binding to the non-target proteins. Perhaps, using the ELISA would have been more useful if performed on cultured isolated mouse cardiac myocytes as opposed to whole heart homogenate. To examine this option, we attempted to isolate and culture mouse myocytes from the WT and p38γδ KO. However, despite our efforts, we were unable to culture the cells and they died after 1 h in the culture medium (data not shown).
In this Thesis we set out to examine the contribution of p38\(\gamma\) and \(\delta\) MAPK isoforms in complex settings such as hypertrophy and LV remodelling when little was known about the role of these isoforms in the heart. Using transgenic mice is a powerful tool for studying the functional roles of these proteins. Despite this, possibly using a cardiac derived \textit{in vitro} model (such as isolated mouse cardiac myocytes from the null mice) would have been more appropriate to investigate the implicated signalling and identifying downstream targets. However, our attempt to culture mouse cardiac myocytes was unsuccessful and we were not able to perform any experiments on these cells (data not shown). Another implication in the current study, is of course, the imperative role of CFs. As discussed in the first chapter (General Introduction, section 1.3.1), CFs (and their trans-differentiation to myoCFs) are important in fibrosis and scar formation during hypertrophy and remodelling. However, by using whole heart tissue in our experiments we were unable to distinguish the contributions of CFs (expressing different isoforms) from cardiac myocytes. In addition, although murine models are often used to investigate the underlying molecular and physiological implications of various diseases, the findings from such studies need to be evaluated in a human derived model. This is important, as the expression of p38 MAPK isoforms in cardiac myocytes and non myocytes (CF) may vary between human and mouse heart and may consequently contribute distinctively in response to different myocardial stresses (Yeh et al., 2010a).

Another potential concern with our study was using p38\(\gamma\)\(\delta\) double KO. Although these mice were viable, exhibited a healthy life span and displayed a normal global cardiac structure and function, they might have compensated in other ways which we were not able to detect. It is fully appreciated that genetic manipulation (change in the level of protein expression by overexpression or elimination of a protein) in the heart might induce a non-specific biological (or toxicity) effect. This could have been determined by using microarray analysis to examine any changes in the protein expression profile of these mice which could have contributed indirectly to the responses of these mice to the stimuli used in this Thesis. Perhaps, a more useful model would have been to generate a cardiomyocyte- specific single \(\gamma\) and \(\delta\) conditional KO to accurately delineate between the functions of the two isoforms. This would have particularly been beneficial in our experiment with sorbitol (Chapter 4, Figure 4-16 and 4-17). In addition, treatment of the p38\(\gamma\) and \(\delta\) KO mice with pharmacological inhibitor of \(\alpha\) and \(\beta\) such as SB203580 and non-competitive inhibitors such
as BIRB compound (which inhibits all four isoforms) would have helped us to identify the responsible isoforms more clearly.

7.2.2 Technical challenges of pressure-volume catheter

The pressure-volume catheter can provide a valuable understanding of the pathophysiology in various *in vivo* models such as hypertrophy and remodelling. As shown in this Thesis, a range of objective parameters can be obtained by using this system which cannot be derived by less expensive methods such as cardiac ultrasound.

We were, however, faced with some challenges in the hypertrophy and remodelling models when using the pressure volume catheter in Chapter 5 and 6. As discussed in chapter 5, we used an apical puncture to advance the catheter inside the LV. However, we encountered some difficulties in the position of the catheter which, lead to some of the variation in volume related parameters. When we catheterised the mice through the carotid artery and entered the heart via the aortic route, however, a more consistent set of data was achieved. Despite this, the positing of the catheter in the LV was not very secure in the remodelled hearts due to number of factors:

1) The orientation of the remodelled hearts was no longer in line with the chest which, was caused by adhesion between the pericardium/myocardium and the rib cage. In some cases it required partial dissection of the diaphragm to tease out the scar tissue to free the heart for subsequent morphological analysis.

2) The anatomy of the remodelled hearts had changed dramatically post-MI with a significant increase in size and dimensions. The above factors made the catheter positioning very insecure and limited our ability to perform an inferior vena cava (IVC) occlusion. Although, we were able to obtain a range of valuable measurements (such as dP/dt\text{max} and dP/dt\text{min}) from this study, certain powerful parameters such as end diastolic pressure volume relationship (EDPVR) were problematic to acquire. In addition, the new technologies used (ADVantage system) has not been fully characterised using infarcted hearts. The parameters obtained to calibrate and quantify the volume signal may vary significantly in the infarcted, remodelled or hypertrophied heart.

Despite these obstacles, the advantages of the pressure volume analysis over its disadvantages to better understand the pathophysiology associated with various *in vivo*
models must be emphasised. This technique is most beneficial when used in conjunction with other in vivo techniques such as echocardiography.

7.3 Novel aspects of the Thesis in relation to human disease and pharmacological treatment

Our data confirmed that p38α MAPK is the dominant isoform in murine myocardium and is activated in response to ischaemia, ischaemia reperfusion and a number of pro-inflammatory cytokines. Compelling evidence supports both protective and promoting roles of p38 MAPK in the regulation of cell death in various cell (Ivanov and Ronai, 2000; Nemoto et al., 1998; Xia et al., 1995), including cardiomyocytes (Communal et al., 2000; Hoover et al., 2000; Mackay and Mochly-Rosen, 1999). Therefore, it was thought that the different effects of pharmacological p38 MAPK inhibition on apoptosis in various cell types may reflect heterogeneity in expression and/ activation of p38 MAPK isoforms. Another possibility was that p38α MAPK has a dual role in promoting/protecting cell death. This hypothesis is supported by Molkentin’s group, in which dnp38α transgenic mice showed a reduction in cardiac injury and cell death following ischaemia-reperfusion (Kaiser et al., 2004). Consistent with the findings in this Thesis and also considering current literature with regards to the role of p38α MAPK in apoptosis, it appears that, in the heart at least, p38α MAPK is the key isoform activated in response to various myocardial stresses such as ischemia-reperfusion, hypertrophy and LV remodelling post infarction. The inhibition of the beneficial isoform/s in the heart has always been a concern in the design and development of novel p38 MAPK inhibitors and has accounted for the inconsistency of data by investigators.

We made interesting observations with regards to the role of p38β (in pharmacological preconditioning) and p38 γ and δ MAPK isoforms in osmotic stress in response to sorbitol. In addition, we observed a few subtle differences in the responses of the p38γδ KO mice with β-AR (induced by ISO infusion) cardiac hypertrophy and also 4 weeks post-MI compared with the WT mice. These observations may suggest these isoforms are involved and contribute to the process of LV remodelling. However, the exact mechanisms by which these isoforms regulate remodelling remain uncertain. These observations need to be further investigated in a more in depth molecular approaches to obtain a better understanding of the exact mechanisms and downstream targets of these isoforms.
7.4 Future work

7.4.1 Elucidating the role of p38α and p38β MAPK in cardiac protection

Currently, the interpretation of the data concerning the role of p38β MAPK in cardioprotection is limited by the lack of knowledge on the role of p38α MAPK isoform in CORM-3 mediated cardioprotection. An approach to elucidate the function of p38β MAPK in CORM-3 mediated cardioprotection, would be to use mouse hearts expressing an inhibitor resistant form of p38β MAPK. These mice have p38β MAPK which is insensitive to SB203580 but have maintained p38α MAPK isoform sensitive to SB203580. By comparing responses of WT and inhibition-resistance (IR) forms of p38β MAPK, it would be possible to determine which effects are isoform dependent and which are the result of documented off-target action (Bain et al., 2007; Godl et al., 2003). These experiments are currently underway, and form the basis of another research project.

7.4.2 Investigating the role of p38γ MAPK and δ MAPK in channel interactions and trafficking

As described previously, SAP97 is a member of membrane associated guanylate kinase (MAGUK) proteins which are important for the organisation of ion channels and receptors at neurones. MAGUK and in particular SAP97 is found at the level of intercalated disc in the atrial (Godreau et al., 2002) and ventricular myocardium (Murata et al., 2001), where it co-localizes with voltage-gated Kv1.5 channels (Godreau et al., 2002; Murata et al., 2001). Kv1.5 shaker channels are concentrated at the level of intercalated discs in atrial myocardium. Like nodes of Ranvier or neural synapses, this may be crucial for the normal transmission of action potentials. Moreover, cardiac SAP97 and Kv1.5 subunits have been co-precipitated, suggesting a direct interaction between the two proteins. A recent study examining the effect of SAP97 on the function and localization of Kv1.5 subunit in cardiac myocytes, showed that SAP97 regulates the K⁺ current in cardiac myocytes by retaining and immobilising kv1.5 subunits in the plasma membrane (Abi-Char et al., 2008).

In addition, immunoaffinity purification and affinity chromatography from skeletal muscle and cardiac muscles has shown that dystrophin associated protein (substrates for p38γ and δ MAPK) and SAP97 bind strong inward rectifier potassium channels (Kir2.1, kir 2.2, kir 2.3). As with p38γ MAPK, these channels also contain a PDZ domain binding motif, which
allows interactions with scaffolding protein/trafficking proteins (Leonoudakis et al., 2004). It is suggested that this association may be important to target and traffic channels to specific subcellular locations, as well as anchor and stabilize channels in the plasma membrane (Leonoudakis et al., 2004).

Considering the unique structure of the p38γ isoform with its PDZ binding domain and distinct localization of p38δ MAPK at the intercalated discs, it would therefore, be interesting to explore the role of this isoform in the context of channel interaction and trafficking between different binding partners.
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Chapter 8

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