The role of PI3Kα in cardioprotection

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DECLARATION

I, Francisco Javier Rosselló Lozano, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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PUBLICATIONS

List of the publications to date resulting from this 3-year work period.

First author publications related to this thesis:

Yellon DM, **Rossello X**. Xenon A Noble Member of the Cardioprotection Club. J Am Coll Cardiol. 2017;70(21): 2661-2.

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Rossello X, Riquelme JA, He Z, Taferner S, Vanhaesebroeck B, Davidson S, Yellon DM. The role of PI3Kα in cardioprotection. Basic Res Cardiol. 2017;112(6):66.

Rossello X, Riquelme J, Davidson S, Yellon DM. Role of PI3K in myocardial ischemic preconditioning: mapping prosurvival cascades at the trigger phase and at reperfusion. Journal of Cellular and Molecular Medicine (2017, in press).

Rossello X, Yellon DM. Apoptosis, A Double-Edge Sword! JACC: Basic to Translational Science. 2017, 2 (4): 498.

Rossello X, Yellon DM. A new era in the management of type 2 diabetes: is cardioprotection at long last a reality! Int J Cardiol. 2016;228:198-200.

Rossello X, Yellon DM. Cardioprotection: The Disconnect Between Bench and Bedside. Circulation. 2016;134:574-575.

Rossello X, Yellon DM. A critical review on the translational journey of cardioprotective therapies. Int J Cardiol. 2016;220:176-84.

Rossello X, Burke N, Stoppe C, Bernhagen J,Davidson SM, Yellon DM. Exogenous administration of recombinant MIF at physiological concentrations failed to attenuate infarct size in a Langendorff perfused isolated mouse heart model. Cardiovasc Drugs Ther. 2016;30:445-45

Rossello X, Hall AR, Bell RM, Yellon DM. Characterization of the Langendorff Perfused Isolated Mouse Heart Model of Global Ischemia–Reperfusion Injury: Impact of Ischemia and

Reperfusion Length on Infarct Size and LDH Release J Cardiovasc Pharmacol Ther. 2016;21: 286-295

Co-author research publications related to this thesis

Bromage DI, Pickard JM, **Rossello X**, Ziff OJ, Burke N, Yellon DM, Davidson SM. Remote ischaemic conditioning reduces infarct size in animal in vivo models of ischaemia-reperfusion injury: a systematic review and meta-analysis. Cardiovasc Res. 2017;113: 288-297.

Burke N, Hall AR, **Rossello X,** Beikoghli Kalkhoran S, Hausenloy DJ. Phenanthroline protects the murine heart against ischaemia-reperfusion injury via mitochondrial mechanisms. *Manuscript in preparation.*

ABSTRACT

Background. Ischaemic preconditioning (IPC) is an endogenous cardioprotective phenomenon, which can limit infarct size following ischaemia-reperfusion injury (IRI) through the activation of intracellular signalling pathways, such as the PI3K kinase cascade. Little is known about the individual roles of PI3K isoforms in IRI. This thesis aimed to elucidate the role of PI3Kα in cardioprotection.

Methods and Results. Initial studies focussed on establishing the Langendorff-perfused isolated heart model of mouse IRI, and the relative contribution of different lengths of ischemia and reperfusion to myocardial infarction was rigorously determined. The PI3K signalling cascade and its interaction with other pathways were also dissected before evaluating the role of PI3Kα.

PI3Kα was shown to be critical in mediating IPC-induced heart protection against IRI. Using the *ex vivo* model of myocardial infarction, we showed that PI3Kα is required during the IPC reperfusion phase to reduce myocardial infarct size, whilst not mediating the effect during the trigger phase. These findings were confirmed in an *in vivo* setting. Using insulin as a canonical activator of PI3Kα, we also demonstrated that this isoform is not only necessary for IPC to confer cardioprotection, but sufficient for its specific activator to promote myocardial salvage against IRI. PI3Kα activation mediates its effect through the end-effector namely the mitochondrial permeability transition pore, as demonstrated by delaying its opening in primary isolated cardiomyocytes. Importantly, the PI3Kα protein levels are comparable between mouse and human heart tissue, and its activation can be modulated in both tissues. These last observations highlights the potential ability of PI3Kα to be translated into the clinical setting.

Conclusions. These studies have clearly demonstrated that PI3Kα plays a crucial role at reperfusion. This suggests that strategies specifically enhancing the α isoform of PI3K, at reperfusion, could provide a direct target for clinical treatment of IRI.

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ABBREVIATIONS

Standard units of measurement are used throughout this report according to accepted conventions. A comprehensive list of non-standard abbreviations is provided below.

ACE	Angiotensin converting enzyme
Akt/PKB	Akt/protein kinase B
AMI	Acute myocardial infarction
AUC	Area under the curve
BAD	Bcl-2 associated death promoter
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CsA	Cyclosporine A
DMEM	Dulbecco's Modified Eagle Medium
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal–regulated kinase
GIK	Glucose-insulin-potassium
GPCR	G-protein-coupled receptor
GSK-3β	Glycogen synthase kinase-3β
HUVEC	Human umbilical vein endothelial cells
IGF-1	Insulin-like growth factor-1
IPC	Ischaemic preconditioning
IRI	Ischaemia reperfusion injury
IS	Infarct size
JAK	Janus kinase

LAD	Left anterior descending (coronary artery)
LDH	Lactate dehydrogenase
MCEC	Mouse cardiac endothelial cell
mPTP	Mitochondrial permeability transition pore
mTOR	mammalian target of rapamycin
p70s6k	Ribosomal protein S6 kinase beta-1
PDK1	Phosphoinositide-dependent protein kinase-1
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKG	Protein kinase G
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PPCI	Primary percutaneous coronary intervention
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
RIC	Remote ischaemic conditioning
RIPC	Remote ischaemic preconditioning
RISK	Reperfusion Injury Salvage Kinase
ROS	Reactive oxygen species
SAFE	Survivor Activator Factor Enhancement
STAT3	Signal transducer and activator of transcription 3
STEMI	ST-segment elevation myocardial infarction
RTK	Receptor tyrosine kinase
TMRM	Tetra-methyl rhodamine methyl ester
TNFα	Tumor Necrosis Factor-α
TTC	Triphenyl tetrazolium chloride
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UCL	University College London

Chapter 1 GENERAL INTRODUCTION

1.1 Epidemiology of coronary artery disease

The Global Burden of Disease study has recently estimated 422.7 million cases of cardiovascular disease and 17.92 million cardiovascular deaths worldwide in 2015 (1). Coronary artery disease (CAD) is the leading cause of cardiovascular health lost globally and also one of the major causes of mortality and morbidity when compared to non-cardiovascular diseases (2,3). By way of illustration, about 16 million people have CAD and around 8 million have had an acute myocardial infarction (AMI) in the United States. Furthermore there are about 735,000 AMIs every year, which translates into someone having an AMI every 43 seconds in the USA (4).

ST-segment elevation acute myocardial infarction (STEMI) is a major manifestation of CAD, usually precipitated by an abrupt occlusion of an epicardial coronary artery due to a sudden rupture of an atherosclerotic plaque (5). As a result, the myocardium distal to the occlusion site becomes ischaemic and its outcome depends on subsequent spontaneous or interventional coronary reperfusion (6). Early reperfusion by primary percutaneous coronary intervention (PPCI) limits myocardial infarct size (IS) and changes the fate of the infarcted area (7,8). The process of restoring blood flow to the ischaemic myocardium induces additional myocardial damage, known as "myocardial ischaemia-reperfusion injury (IRI)"(9,10), that negatively impacts on IS as well as on death and disability rates. The concept of myocardial IRI is further explained in sections 1.2 and 1.3.

The implementation of PPCI and chronic evidence-based medications in the last 20 years have resulted in a widespread improvement in prognosis in Western countries (11–14). Overall, in-hospital death rates for STEMI have dropped over the last few decades (12). However, morbidity and mortality still remain unacceptably high in STEMI patients, with 1-year death rate reported in 7-11% and heart failure in 22% (15,16). Moreover, the increased cardiovascular risk burden and the incidence of AMI is growing disproportionately in some geographic regions, turning STEMI into a major health problem in developing countries (17,18).

To improve outcomes in patients with STEMI, further efforts are needed in two main non-mutually exclusive directions. On one hand, there is still room to improve both the implementation and the timings of PPCI (19), though this might not apply to all regions worldwide – in countries where the time from ambulance arrival to re-opening the occluded coronary artery in a catheterization laboratory has shortened to about 90 minutes, further shortening of this time has not demonstrated to improve mortality (20).

On the other hand, novel therapies targeting myocardial IRI are needed to be administered as adjuncts to PPCI in order to improve patient survival and prevent the onset of heart failure. This last point describes the work as outlined in this thesis - i.e. to investigate a molecular target that when activated at reperfusion ameliorates the impact of myocardial IRI, reducing the resultant infarct size, with an improvement in clinical outcome.

1.2   Brief historical perspective on cardioprotective therapies and reperfusion injury concepts

The major determinants of myocardial IS following an AMI were historically considered only as a function of the duration of ischaemia, area at risk and collateral blood flow (21,22). In 1971, Braunwald *et al* proposed that the extent and severity of myocardial tissue damage after coronary occlusion could be modified by therapeutic manipulations applied during ischaemia (23). This work was the starting signal for studies examining therapies designed to limit myocardial IS, known generally as cardioprotective therapies. Therapies able to reduce IS have been tested under the hypothesis that smaller infarctions will result in fewer adverse clinical events in the long-term (24–26).

Many attempts to limit myocardial IS by pharmacotherapy in the absence of reperfusion (pre thrombolytic era) dominated research in the 1970s (27), although the concept of cardioprotection eventually evolved to those therapies aimed at the attenuation of injurious results of myocardial IRI (28).

Myocardial reperfusion injury was first postulated in 1960 by Jennings *et al.*, describing landmark histologic features of reperfused ischaemic canine myocardium, such

cell swelling, contracture of myofibrils, disruption of the sarcolemma and the appearance of intra-mitochondrial calcium phosphate particles (29,30). However, it was in 1985 when Braunwald and Kloner wrote that myocardial reperfusion may be viewed as a double-edged sword(31), putting this subject on the research agenda.

Table 1-1 presents an overview of landmark studies in IRI and cardioprotection.

Year	Author	Achievement
1972	Maroko *et al.* (32)	First evidence that reperfusion limits extent of necrosis
1977	Reimer *et al.* (22)	Description of the wavefront progression of necrosis (from endocardium to epicardium)
1986	Murry *et al.* (33)	First evidence that ischaemic preconditioning reduces IS (first window of protection)
1993	Marber *et al.* (34)	Description of the second window of protection
1993	Przyklenk *et al.* (35)	First evidence that remote ischaemic preconditioning reduces IS
2002	Schulman *et al.* (36)	Description of the Reperfusion Injury Salvage Kinase (RISK) pathway
2003	Zhao *et al.* (37)	First evidence that ischaemic postconditioning reduces IS

Table 1-1: Landmark studies in IRI and conditioning-related cardioprotective therapies

Some of the most relevant studies in the field of myocardial IRI and cardioprotective therapies based on the conditioning phenomena.

Abbreviations: IS, infarct size.

1.3 Pathophysiology of myocardial ischaemia/reperfusion injury

Time is muscle in patients undergoing an STEMI: the less time the coronary artery is occluded, the smaller the IS and the better the outcome for the patient (38,39). Although myocardial reperfusion is essential to salvage viable myocardium, it comes at a price. Hence, myocardial reperfusion paradoxically damages the vulnerable post-ischaemic myocardium. Studies in animal models of AMI suggest that reperfusion injury may account for up to 50% of the final myocardial IS (10), as Figure 1-1 illustrates. Hence, the prognosis after an STEMI is greatly dependent on the IS resulting from both ischaemia and reperfusion induced injury (25,26).
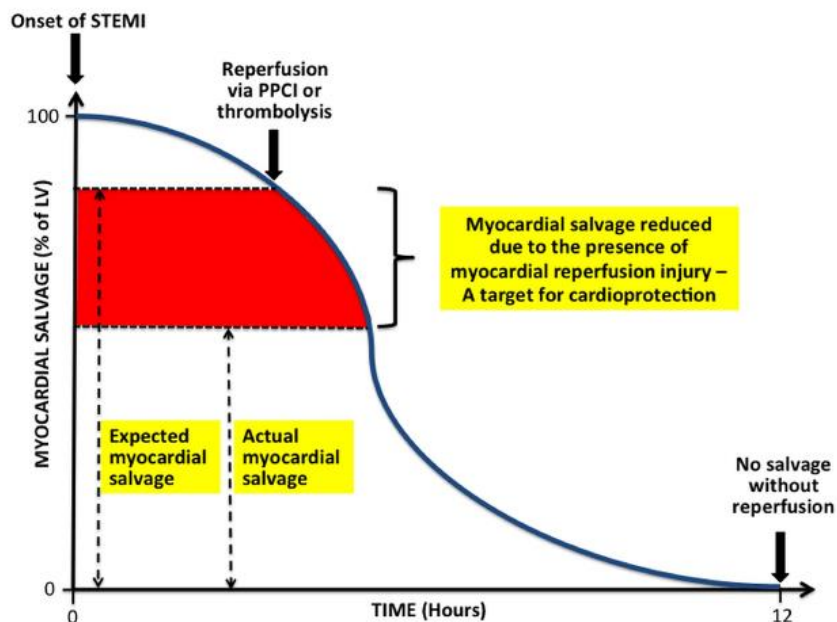
Figure 1-1: Reperfusion injury contributes to myocardial infarct size after therapeutic restoration of blood flow

Rough representation of the contribution of reperfusion injury to overall IS in STEMI patients. Reproduced with permission from authors (40).

Myocardial reperfusion injury can manifest in several forms (10,40):

A) Myocardial stunning. This is a reversible mechanical dysfunction that usually lasts for a few days after restoration of normal coronary flow. It is usually self-terminating, and is believed to result from oxidative stress and intracellular calcium (41).

B) The no-reflow phenomenon, also known as microvascular obstruction. It refers to the impedance of microvascular blood flow encountered after opening the infarct-related coronary artery, and its aetiology is multifactorial, including capillary damage, external capillary compression by endothelial cells, cardiomyocyte swelling, micro-embolization and neutrophil plugging (42).

C) Reperfusion arrhythmias. Usually idioventricular rhythm and ventricular arrhythmias, most of them self-terminating or easily managed from a clinical perspective.

D) Lethal reperfusion injury. This is the main cause of reperfusion-induced death of cardiomyocytes that have been reversibly injured during the ischaemia. Lethal reperfusion injury is the death of cardiomyocytes either as a consequence of reperfusion-triggered cell death and/or as a consequence of some event occurring during ischaemia that manifests during the reperfusion phase. Those phenomena triggering lethal reperfusion injury have been described elsewhere (10,41) and might be summarized as a cytosolic and mitochondrial calcium overload, oxidative stress and rapid restoration of intracellular pH which result in the opening of the mitochondrial permeability transition pore (mPTP) (described below).

Timely reperfusion is the most effective intervention to treat acute myocardial infarction - it has demonstrated to improve the prognosis of STEMI patients and its use is currently indicated in this context. However, reperfusion itself is a double-edged sword that represents an unmet clinical need. Further development of interventions targeting reperfusion injury are needed to reduce both mortality and morbidity following an acute myocardial infarction.

1.4 Cardioprotection via ischaemic conditioning

In 1986, Murry *et al.* published a seminal study demonstrating that several short (5 min) cycles of non-injurious ischaemia and reperfusion render the myocardium significantly protection from a subsequent sustained ischaemic insult (33). This phenomenon whereby the myocardium can endogenously be protected from lethal IRI was defined as "ischaemic preconditioning" (IPC). This finding, firstly described in dogs, has been subsequently replicated in numerous pre-clinical studies (43), as well as in other organs (44). The mechanism of protection conferred by IPC is described in next sections 1.5 and 1.6.

The concept of IPC has evolved into "ischaemic conditioning", a broader term that encompasses a number of related endogenous cardioprotective strategies, applied either to the heart (ischaemic preconditioning or postconditioning) or from afar (remote ischaemic pre-, per- or postconditioning). Table 1-1 provides a chronological summary of the evolution of the concept "ischaemic conditioning", which is further elaborated in the subsequent paragraphs.

In 1993, Przyklenk *et al.* applied four episodes of 5 min circumflex coronary artery occlusion separated by 5 min of reperfusion, before subjecting the left anterior descending (LAD) artery to 1 h sustained occlusion and subsequent 4.5 h reperfusion in a canine model (35), finding a 10% reduction myocardial IS in those circumflex preconditioned dogs. This phenomenon was termed "protection at a distance" later to be called remote ischaemic conditioning (RIC) and has been shown similar cardioprotective effects when applied to other remote organs and tissues, such as kidneys and skeletal muscle (41,45,46). In 1997, Birnahum *et al.* demonstrated that the application of short cycles of ischaemia and reperfusion to a rabbit limb (a RIC manoeuvre) remotely conferred protection to the heart (45). This finding boosted both pre-clinical and clinical research, as it meant that cardioprotection could be easily translated to the clinical arena by inflating a blood pressure cuff on the arm (47).

In 2003, Zhao *et al.* described a similar reduction in myocardial IS following the application of brief cycles of ischaemia/reperfusion in the same coronary artery either before or after the index ischaemia (37). The novelty of this intervention applied in a canine

model was the use of repetitive ischaemia/reperfusion stimulus in early reperfusion of the LAD territory. This intervention was named ischaemic postconditioning (37).

The translational potential of IPC is inevitably limited by the necessity to apply the intervention before the index ischaemia, which is unpredictable in many clinical scenarios such as the STEMI. Ischaemic postconditioning has been already tested in the clinical setting with mixed results (48–51). However, ischaemic postconditioning involves an invasive approach associated with peri-procedural risks, including coronary artery dissection or perforation, access site complications or stroke (13). On the contrary, RIC has emerged as a non-invasive alternative that can be applied either before (52–54), during (55–57) or after (58,59) index ischaemia (named remote pre-, per- or postconditioning, respectively).

Overall, large clinical trials assessing the impact of RIC on hard endpoints in the context of cardiac surgery have been disappointing (52,53). One of the main reasons that can potentially explain the disconnect between pre-clinical and clinical findings is the amount of myocardium at risk. Whilst STEMI patients (and particularly those with an anterior infarct) have a large territory at risk and therefore may potentially benefit from cardioprotective therapies, patients undergoing cardiac surgery have shorter ischaemic times, and their myocardium at risk is already subjected to cardioprotective procedures such as cardioplegia and hypothermia, therefore leaving little room to further protect the tissue. In fact, the term "infarct size" is usually referred to the consequence of having an STEMI, whilst "peri-procedural myocardial injury" is the concept used in the context of cardiac surgery (60). A deeper analysis on why some cardioprotective interventions have failed to be translated into the clinics can be found in some of our reviews and editorials (61,62), as well as elsewhere (13,63,64). With regard to the ultimate application of RIC in STEMI patients, there is great expectation for the outcome of the combination of two large ongoing clinical trials, namely the CONDI2/ERIC-PPCI study (57), which is a European (Denmark, Serbia, Spain, and UK respectively) prospective, randomized, controlled, clinical trial involving 5300 STEMI patients undergoing PPCI with the aim of evaluating the improvement of long-term clinical outcomes following the application RIC.

Figure 1-2 depicts the wide spectrum of ischaemic conditioning interventions and summarizes all the potential clinical scenarios where these therapies might be applied

(10,65). STEMI patients undergoing PPCI represent the most promising population that could benefit from cardioprotection, however other situations, where the heart is subjected to acute global IRI, such in cardiac surgery, cardiac transplantation and cardiac arrest, are also targetable.

In this thesis, the focus is on "classic" IPC, whereby brief cycles of coronary occlusion and reperfusion elicits protection from prolonged IRI. Despite acknowledging that this form of conditioning is non-translatable itself, the elucidation of signalling pathways underlying classic ischaemic conditioning should help to identify molecular targets amenable to pharmacological manipulation.
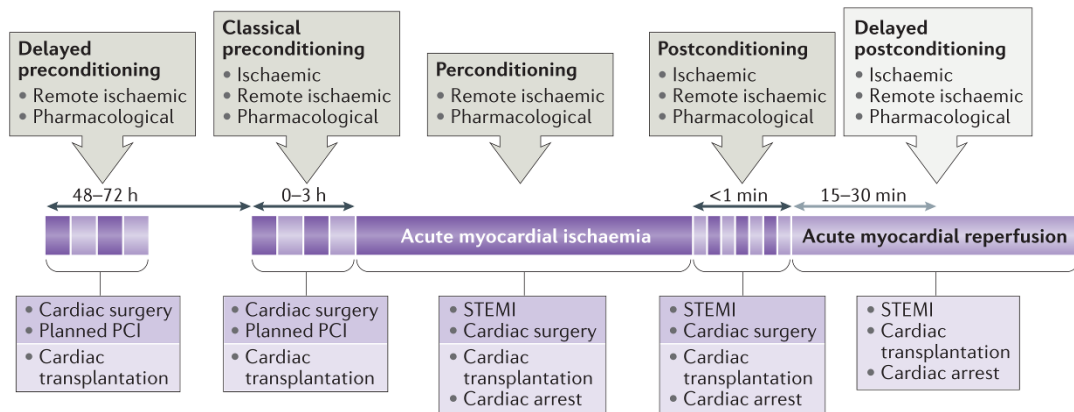
Figure 1-2: Ischaemic conditioning forms

This illustration depicts the different forms of ischaemic conditioning, and their timing with regard to the index myocardial ischaemia and reperfusion insult.

Cardioprotection can be induced by applying short cycles of ischaemia and reperfusion directly to the heart either: (1) 24–48 h prior the myocardial index ischaemia (Second window or *delayed ischaemic pre-conditioning*); (2) within 3 h of the index myocardial ischaemia (*IPC*); (3) within 1 min of reperfusion following the index myocardial ischaemia (*postconditioning*); and (4) 15–30 min after the onset of myocardial reperfusion following the index myocardial ischaemia (*delayed ischaemic post-conditioning*).

Cardioprotection can also be elicited by applying brief cycles of ischaemia and reperfusion to a remote organ or tissue (such as the arm or leg) either: (1) 24–48 h prior the index myocardial ischaemia (*delayed remote ischaemic pre-conditioning*); (2) within 3 h of the index myocardial ischaemia (*remote IPC*); (3) during the index myocardial ischaemia (*remote ischaemic perconditioning*); (4) within 1 min of reperfusion following the index myocardial ischaemia (*remote postconditioning*); (5) 15–30 min after the onset of myocardial reperfusion following the index myocardial ischaemia (*delayed remote postconditioning*).

Two other forms of conditioning have been also described: (1); based on drugs mimicking the activation of pro-survival pathways (pharmacological conditioning); and (2) based on a daily application of the stimulus for a long period – i.e. 1 month (chronic conditioning).

Below, in purple, the clinical settings in which they have been tested (dark shading) or those in which there is potential for application (light shading). Reproduced with permission from authors(41).

Abbreviations: PCI, percutaneous coronary intervention; STEMI, ST-segment elevation myocardial infarction

1.5   Ischaemic preconditioning

Besides reperfusion itself, IPC is considered the most powerful intervention available to protect the heart against myocardial IRI and has become the paradigm for cardioprotection (66). This intervention whereby brief cycles of coronary occlusion and reperfusion elicits protection has also been proved to be present in humans (67). After more than three decades of research on preconditioning, significant advances have been made in our understanding of both the mechanisms underlying IRI and the cardiac endogenous protection conferred by IPC.

1.5.1   Mechanisms of signal transduction in IPC

There is a consensus to recognize three hierarchical levels of signal transduction(28,68): triggers (usually sarcolemmal membrane receptors), intracellular mediators (the signalling cascades that help initiate and propagate the signal) and end-effectors (mechanisms that actually cause the attenuation of cellular injury and death during the lethal ischaemic insult). Figure 1-3 presents few examples of this signal transduction pattern.

This classification of IPC signalling is based on a causal/temporal sequence of events. The IPC stimulus initially results in production of <u>triggers</u> from the cardiomyocyte (such as acetylcholine, adenosine, bradykinin and opioids) (69). These stimuli, also known as "autocoids", bind to their respective sarcolemmal surface receptors to initiate the preconditioning signalling.   These receptors are on the plasma membrane of cardiomyocytes and are usually G-protein-coupled receptors (GPCRs), such as adenosine A1 receptors(70), bradykinin B2 receptors (71) and δ-opioid receptor (72,73). In addition to GPCRs, some receptor tyrosine kinase (RTKs) can also mediate this autocoid-induced signalling, as some of these receptor agonists have been demonstrated to trigger the preconditioning response. This is evident in the case of insulin (74) or Fibroblast Growth Factor-2 (75). Of note, therapeutic approaches pharmacologically targeting these receptors would be expected to yield a meaningful cardioprotective effect.

The activation of the receptors on the plasma membrane of cardiomyocytes results in the recruitment of the <u>mediators</u>, consisting of two main protein kinases cascades that

help to propagate the precondioning signal and eventually converge on the mitochondria (76,77). These two signalling pathways underlying cardioprotection are the Reperfusion Injury Salvage Kinase (RISK) pathway (comprising PI3K–Akt and MEK1/2–ERK1/2) and the Survivor Activator Factor Enhancement (SAFE) pathway (comprising TNFα and JAK-STAT3) (78–81). Some have advocated a third signalling cascade based on the protein kinase G (PKG) and involving nitric oxide (82). Figure 1-4 depicts by colours the spatiotemporal pattern of these two main signalling cascades mediating cardioprotection in an archetypal cardiomyocyte. These prosurvival pathways have been shown to inhibit downstream mediators such as glycogen synthase kinase (GSK-3β), and activate endothelial nitric oxide synthase (eNOS) and protein kinase C ε (PKCε), which then mediate an inhibitory effect on mitochondrial permeability transition pore (mPTP) opening.

The last link of the chain of this sequential-step process to promote cellular salvage against lethal reperfusion injury are the <u>end-effectors</u>. The most important known end-effector is the mPTP, which seems to be a common final step for all the above mentioned cardioprotective signalling cascades (83). Thus, the inhibition of mPTP opening at the onset of reperfusion appears to underpin the IS-limiting effects of IPC and other endogenous cardioprotective therapies (84,85). This is further explained in section 1.5.4.

There have been attempts to pharmacologically manipulate these three levels of the signal transduction cascade. At the trigger level, Liu *et al*. demonstrated for the first time in 1991 that the preconditioned state can be achieved administering autocoids: infusing adenosine or adenosine A1 receptor-selective agonist into the coronary flow for 5 min prior to a long occlusion put the heart into an IPC-like protected state, whilst the adenosine receptor antagonist blocked the IPC protective effect (70). At mediator level, insulin have demonstrated to protect the heart through the activation of PI3K pro-survival kinase (86), a well-recognized mediator. At end-effector level, cyclosporine A has shown cardioprotective effect through the inhibition of mPTP opening (87). The concept of modulating the IPC-induced molecular signalling pattern is known as pharmacologic conditioning and will be discussed further in next sections.

Hierarchical levels of signal transduction	Examples
Triggers	Autocoids (Adenosine, bradykinin) Growth factors (IGF-1, insulin) Neurohormones (acetycholine, opioids) Cytokines (TNFα)
Mediators	Protein Kinase C RISK pathway (PI3K-Akt and ERK 1/2) SAFE pathway (STAT3)
Effectors	mPTP K_{ATP} channels Connexin 43

Figure 1-3: Hierarchical levels of signal transduction in cardioprotection

Abbreviations: IGF1, insulin-like growth factor-1; mPTP, mitochondrial permeability transition pore; RISK, Reperfusion Injury Salvage Kinase; TNF, Tumour necrosis factor; SAFE, Survivor Activator Factor Enhancement.

The cellular mechanisms of classical preconditioning can be classified in terms of triggers, mediators and effectors. IPC triggers a change in the myocardium, rendering it resistant to a subsequent infarction. This effect is mediated by a series of signal transduction pathways, which in turn end up on one or more end-effectors. The effectors are responsible for actually providing the protection during the subsequent prolonged ischaemia/reperfusion insult. This figure in not comprehensive and only depicts a few examples of triggers, mediators and effectors involved in cardioprotection.
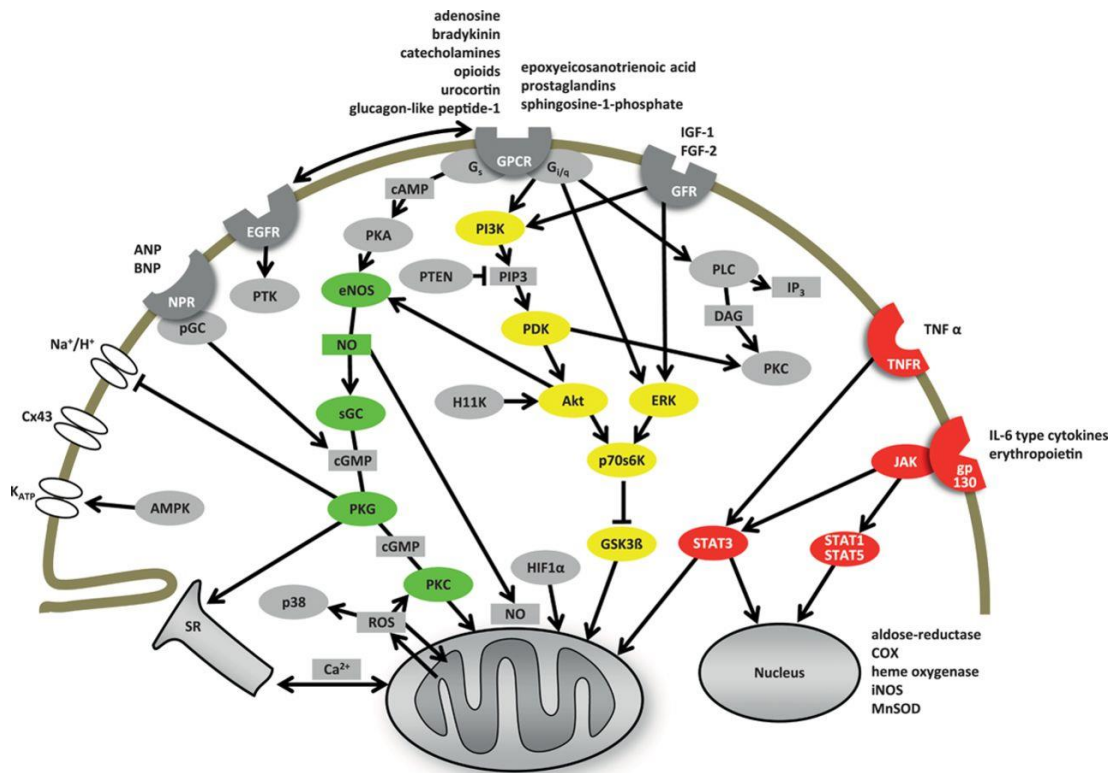
Figure 1-4: Simplified scheme of cardioprotective signal transduction.

The RISK pathway is displayed in yellow in the middle, the SAFE pathway in red on the right side, whilst the eNOS/PKG pathway is shown in green on the left side. Reproduced from Circulation Research. 2015;116:674-699 (78).

Abbreviations: Akt, protein kinase B; AMPK, cyclic adenosine monophosphate–activated kinase; BNP, brain natriuretic peptide; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; Cx 43, connexin 43; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; ERK, extracellular regulated kinase; FGF, fibroblast growth factor; Gs/Gi/q, stimulatory/inhibitory G protein; GPCR, G protein-coupled receptor; gp130, glycoprotein 130; GSK3ß, glycogen synthase kinase 3 ß; H2S, hydrogen sulfide; H11K, H11 kinase; HIF1α, hypoxia inducible factor 1α; IGF, insulin-like growth factor; iNOS, inducible NO synthase; IP3, inositoltrisphosphate; JAK, Janus kinase; KATP, ATP-dependent potassium channel; Na+/H+, sodium/proton-exchanger; NPR, natriuretic peptide receptor; pGC, particulate guanylate cyclase; p38, mitogen-activated protein kinase p38; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PKG, protein kinase G; PLC, phospholipase C; PTEN, phosphatase and tensin homolog; PTK, protein tyrosin kinase; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SR, sarcoplasmic reticulum; STAT, signal transducer and activator of transcription; and TNFα, tumor necrosis factor α.

1.5.2   Pro-survival pathways in IPC: RISK and SAFE pathways

The RISK and the SAFE pathways are the IPC mediators that have received more attention and are currently better understood, particularly the former. Following an IPC stimulus, the activation of both cascades has been demonstrated to occur at two time-points, following a biphasic pattern response (88), as exemplified in Figure 1-5. These two pathways are activated: (1) during the preconditioning cycles, prior to the index ischaemic episode, this phase known as "trigger phase"; and (2) during the onset of reperfusion, known as the early phase of reperfusion or the "mediator phase" (10,66). The last concept, that IPC mediators are actually activated at the onset of reperfusion was unknown 15 years ago and came from a seminal observation made at The Hatter Cardiovascular Institute (see section 1.6.1). Of note, the importance of this finding is its potential translational power – this molecular signalling can be mimicked by pharmacological agents to produce benefits for patients undergoing myocardial IRI, either in the ambulance or the cath lab.
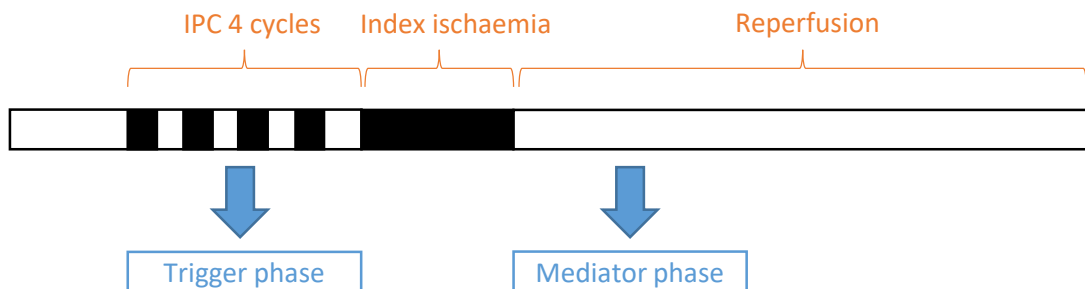
Figure 1-5: The activation of pro-survival pathways follow a biphasic pattern

In orange (top of the illustration), the names for each part of a given experiment simulating the application of 4 cycles preconditioning followed by a prolonged IRI protocol. Black boxes represent periods of ischaemia and white boxes represent periods of perfusion. In blue (bottom of the illustration), the time-periods where the pro-survival pathways are activated, namely the trigger phase (during the IPC protocol) and the "mediator" phase (at the onset of reperfusion).

In 2002, Yellon's group coined the term Reperfusion Injury Salvage Kinase (RISK) pathway to refer to a group of pro-survival protein kinases, which confer cardioprotection when activated specifically at the time of reperfusion (36,89,90), providing an amenable pharmacological target for cardioprotection. This pathway, which is actually a combination of two parallel cascades, PI3K-Akt and MEK1-ERK1/2, was thoroughly dissected through a series of elegant pharmacological studies where the protective effect of several interventions were blocked with the co-administration of both PI3K and ERK inhibitors at different time points (89). The importance of the RISK pathway lies is two main concepts: (1) this pathway must be activated at the time of early reperfusion for IPC to protect against IRI; and (2) the RISK pathway may be recruited not only by ischaemic conditioning, but also by other pharmacological agents such insulin, adenosine or statins (86,91). As the focus of this thesis is on further mapping the RISK pathway, this concept is further elaborated in section 1.6.

In 2005, Lecour *et al.* demonstrated that the administration of TNF-α before index ischaemia (used as pharmacologic IPC-mimetic) was cardioprotective without involving the RISK signalling cascade (92). Four years later, they described that the administration of TNF-α at reperfusion was recruiting a RISK-independent alternative pathway, coined as the SAFE pathway (81,93,94), and they also linked the activation of this pathway with preconditioning (95). Less is known about this signalling cascade: TNF-α binds TNF receptor 2 thus activating Janus Kinase (JAK) and Signal transducer and activator of transcription 3 (STAT3). Interestingly, STAT3 is a classical transcription factor that it also seen to have an immediate impact on mitochondrial respiration, improving complex I respiration and inhibiting mPTP opening (96). Few experimental studies have linked the activation of this pathway with the cardioprotective effect of melatonin(97) and lipoproteins(98). In humans, it seems that STAT5, instead of STAT3, may play a relevant role in cardioprotection (99,100).

In summary, multiple protective pathways seems to be involved in IPC. The RISK pathway has a major role as a mediator of the protective of IPC and its signalling architecture is currently relatively well understood. In contrast, RISK-independent pathways, such as SAFE and PKC/eNOS cascades, are yet to be fully elucidated, but also seem to have a relevant role in cardioprotection. Of note, cardioprotective signalling

cascades have been mostly simplified in the literature, partly to make its logical order more understandable, and partly because the fragmented knowledge in which it is based. However, these cascades are believed to be highly interactive. Crosstalk between the two components of the RISK pathway (101,102) and between the RISK and the SAFE pathways (95,103) have been already described in a few studies.

1.5.3 Role of mPTP in IPC

The mPTP is considered the most important end-effector, as it has been suggested to be a point of convergence of most cardioprotective pathways (78). It was first described in the late 1970's by Hayworth and Hunter as a calcium-sensitive mitochondrial pore able to modify mitochondrial membrane permeability (104–106). Several structural components have been proposed to be part of the mPTP, such as the inner membrane transport protein adenine nucleotide translocase, the outer membrane voltage-dependent anion channel, and the mitochondrial phosphate carrier. However, most of them have been discredited as essential components of the pore and the mPTP structure still remains largely unknown.

What is known about the mPTP is that it is a nonselective channel of the inner mitochondrial membrane (107) which is formed under conditions of cellular stress or injury, such as the circumstances surrounding IRI. During myocardial ischaemia, the mPTP remains closed, only to open within the first few minutes after myocardial reperfusion in response to mitochondrial Ca^{2+} overload, oxidative stress (108), restoration of a physiologic pH and ATP depletion (83,109). When opened for a long term, results in the mitochondrial membrane potential collapse, oxidative phosphorylation uncoupling, the rupture of the outer mitochondrial membrane and the release of cytochrome c from the intermembrane space into the cytosol, where it activates proteolytic processes (110) resulting in cell death (83).

The mPTP is regulated by cyclophilin D, which decreases the threshold for mPTP opening in response to calcium and inorganic phosphate (111). Using cyclosporine A (CsA), a lipophilic cyclopeptide drug which inhibits cyclophilin D, Hausenloy *et al.* reported in the isolated rat heart model that IPC protect the myocardium by inhibiting mPTP opening at the onset of reperfusion (87), therefore demonstrating that mPTP inhibition underpins the IS-

sparing protective effect elicited by IPC (84,85). Using sanglifehrin-A, they also demonstrated that mPTP opening could be pharmacologically induced (112).

Overall, there is a growing body of evidence suggesting the importance of the mPTP in IRI. On one hand, the genetic ablation of cyclopholin D has been associated to the reduction of myocardial IS (107,111). On the other hand, the pharmacologic inhibition of cyclophilin D with CsA has been demonstrated to prevent mPTP opening (113). Despite the publication of a positive proof-of-concept study in patients with STEMI demonstrating for CsA to have an impact on surrogate endpoints (myocardial IS and ventricular remodelling) (114,115), the subsequent larger randomized clinical trial ("Does Cyclosporine Improve Clinical Outcome in ST-Elevation Myocardial Infarction Patients", or CIRCUS trial) assessing clinical outcomes failed to show benefits for CsA (116). In another study published later, the "CYCLosporinE A in reperfused acute myocardial infarction" or CYCLE trial, the primary endpoint of improving ST-segment resolution was also proved neutral (117). Other drugs aimed at mPTP inhibition in STEMI patients have also failed in demonstrate superiority in surrogate endpoints (118). Overall, these neutral results are more likely explained by both the ineffective mPTP inhibition provided by the drug itself and its inability to reach the jeopardized myocardium (discussed elsewhere in (119,120)) rather than the mPTP lacking importance in IRI in the clinical setting. The need for alternative drugs with increased specificity for the mPTP is still considered a viable option for developing novel cardioprotective therapies.

1.5.4   Cellular targets for conditioning

As reviewed above, a wide range of triggers, mediators and end-effectors have already been identified as part of the recruitment conditioning process. It has been largely assumed that the conditioning phenomenon applies to all cell types in the heart, or at least specifically to the cardiomyocyte (121). Cardiomyocyte death is the main cause of heart failure, arrhythmias and death in patients with STEMI. Further, cardiomyocytes have become central to recapitulate reductionist models of preconditioning against IRI through hypoxia/reoxygenation experiments. However, it remains largely unknown to what extent other cells can contribute in the conditioning phenomena on top of cardiomyocytes. There

are some evidence pointing to a role for platelets, endothelial cells and fibroblasts in cardioprotection.

By volumetric determination, cardiomyocytes represents around 75-80% of the total myocardium, whilst other cells appears to contribute little to the volume – endothelium by 3% and fibroblasts by 2% (121). This is important because the gold-standard measure of area at risk and infarct size, by Evans blue and Triphenyl Tetrazolium Chloride (TTC) respectively, is based in volumetric measures. However, when it comes to numbers, the proportions are slightly different and the adult myocardium is composed of ~56% myocytes, 27% fibroblasts, 7% endothelial cells, and 10% vascular smooth muscle cells, with a similar percentages in the left ventricle, right ventricle and septa, as demonstrated by Banerjee *et al.* (122). What has not been explored in great detail yet is whether preconditioning can elicit its effect through processes dependent of non-cardiomyocyte cell populations.

Due to the high metabolic demand, the heart has an extensive microvascular blood supply system. Some advocate that the endothelium might have a relevant role in cardioprotection due to both its optimal situation to interact with blood signals and its paracrine capacity/ability. As first point of contact between the myocardium and humoral factors, the endothelium constitutes a "blood-heart barrier" (123). There is some evidence demonstrating that the eluent collected from preconditioned endothelial cells is able to provide some protection against IRI in naïve primary cardiomyocytes (124). In the same vein, Teng *et al*. demonstrated with a transgenic mouse model, which restricted the expression of EPO receptor to hematopoietic and endothelial cells, that hearts from these mice can still be pharmacologically conditioned by the administration of EPO, therefore suggesting a major role for the endothelial cell response to EPO to achieve an acute cardioprotective effect (125). As a paracrine organ, the endothelium has been demonstrated to trigger protection in cardiomyocytes through receptor/ligand interaction and gaseotransmitter. Endothelin-1 (ET1) receptor and bradykynin B(2) receptor are both present in cardiomyocytes (126,127) and when pharmacologically activated both trigger a preconditioning-like effect (126,128). In regard to gaseous signals, nitric oxide has been long associated with ischaemic conditioning though the role of eNOS (the endothelial isoform of nitric oxide synthase), as demonstrated in eNOS knockout mice (129).

Besides being a provider of protective triggers and mediators to cardiomyocytes, there is also the possibility for the endothelium to be a target itself for cardioprotection. The signalling pathways and end-effector mechanisms described in cardioprotection are not specific to cardiomyocytes. Some publications have shown a higher vulnerability to IRI of the endothelium when compared to cardiomyocytes (130,131). Targeting endothelial receptors through adenosine agonists A1 and A3 or angiotensin II (132) preserve not only the endothelium-dependent vasodilation, but also cardiomyocyte viability. Indeed preserving microvascular function will provide further blood supply to the injured cardiomyocytes. In the clinical setting, pre-infarct angina (a preconditioning clinical manifestation) has been associated with attenuation of no-reflow in STEMI patients undergoing PPCI (133).

Other cells can also be relevant in cardioprotection, such as platelets, where recent experimental data have demonstrated that P2Y12 inhibitors are protective at the onset of reperfusion through RISK activation(134–136), or neutrophils, that can be targeted by metoprolol to inhibit neutrophil-platelet interaction (137).

1.5.5   Windows of protection

As described in section 1.4 and Figure 1-2, the ischaemic conditioning phenomenon is an endogenous form of cardioprotection that can be expressed in several ways. IPC, specifically, comes in two different temporal forms of protection, also known as "windows" (34). The first window of protection begins immediately following the stimulus and lasts for 2–3 h, after which cardioprotection declines (termed "classical IPC" or "acute IPC"). The second window follows 12-24h later, and lasts 48–72 h (termed "delayed IPC" or "second window of protection"), although its magnitude of effect appears to be less robust (88).

The first window of protection has been described in previous sections, and consists in the activation of several protein kinases through their phosphorylation. In the second window, these protein kinases activate gene transcription factors, such HIF-1α and STAT 1/3, which facilitate the synthesis of distal mediators, such as COX-2, HSP72 and iNOS, which enables the effects of the IPC stimulus to persist into the following 2-3 days.

The second window of protection was described by Marber *et al.* in 1993. Before, some publications had suggested the protective role of heat stress proteins when induced by both myocardial ischaemia and whole body stress (138,139). To avoid the deleterious extra-cardiac response inherent to whole body heart stress, they applied a standard IPC stimulus in an *in vivo* rabbit model and demonstrated myocardial IS reduction 24 h later through elevation of the myocardial heat stress proteins HSP70 and HSP60 (34). The same year, Kuzuya *et al.* reported similar results using an in vivo canine model (140). In 2003, Yellon's group further described an essential role for the activation of the PI3K-Akt-p70s6k signalling cascade during the preconditioning cycles in a rabbit model of IPC-delayed cardioprotection (141). However, it was later when they (Bell *et al.*) linked this "delayed preconditioning" to the recruitment of the RISK pathway at reperfusion (142). Using angiotensin II as preconditioning mimetic, they demonstrated RISK activation following 1, 6 and 24 h of reperfusion, although the protective effect was only observed at 1 and 24h. An extensive review of the second window of preconditioning can be found elsewhere (76).

1.5.6   Clinical manifestations of IPC

Although IPC has been demonstrated to be highly protective, its clinical application is limited by both the need for its use before the index ischaemia and the necessity of an invasive coronary approach. In the clinical arena, two main spontaneous clinical manifestations of myocardial IPC can be observed:

- "Warm-up angina" refers to the phenomenon of increased exercise tolerance following an episode of angina (143)

- "Pre-infarct angina", defined as the cardioprotective effect of antecedent angina before an AMI resulting in smaller IS and improved clinical outcomes (144,145).

1.6   The RISK pathway: a unified signalling cascade for cardioprotection

1.6.1   Origins of the finding of the RISK pathway: necrosis vs apoptosis

There is more than one mode of cellular death as a result of IRI: both apoptosis and necrosis are thought to play a relevant role (146). At the early exposure to IRI, there is a significant increase in both apoptosis and necrosis, as evidenced by TUNEL and TTC staining, although it seems that necrosis may contribute more to the final infarct size when the tissue is exposed to IRI for longer (146,147).

Necrosis is the form of cell death that occurs following severe cellular damage, includes uncontrolled disruption of organelles, membrane rupture, and does not require ATP (148,149). On the other hand, apoptosis is the ATP-dependent programmed cell death. Apoptosis involves cytochrome-c release from injured mitochondria or autocoid cell-surface receptor (Fas Ligand) activation followed by the downstream propagation of the signal via caspases and other signalling proteins, which in turn cause the formation of nonselective pores in the outer mitochondria or the opening of the mPTP, as well as the DNA cleavage and nuclear degradation(149). Unlike necrosis, apoptosis does not result in the release of cellular content into the extracellular milieu. Pro-apoptotic proteins were the object of study to develop new targets against IRI under the hypothesis that it would be possible to salvage cardiomyocytes already committed to die when the signal of programmed cell death is potentially interrupted. It was therefore demonstrated that inhibiting caspases limits infarct size in animal models (150), although the pharmacologic inhibition of the apoptotic signalling cascade has been reported to attenuate both the apoptotic and necrotic component of the cell death secondary to lethal reperfusion injury (66). Besides reducing cell death through the inhibition of pro-apoptotic caspases, the focus was also put on antagonizing the apoptotic process through the activation of pro-survival proteins (90) - PI3K and ERK 1/2 prosurvival kinases attracted the attention of researchers as a way to preserve ischaemic myocardium.

The RISK pathway was first described by Yellon's group in 2002 whilst assessing the mechanisms underlying the cardioprotective effect induced by urocortin (36): the use of this growth factor reduced myocardial infarct size and increased the phosphorylation of

ERK 1/2 when administered upon reperfusion, whilst these effects were abolished by the co-administration of PD98059 (ERK 1/2 inhibitor) also at reperfusion.

In April 2017, Weil *et al.* (151) reported in a swine model that a single brief episode of 10 min myocardial ischaemia and 24 h reperfusion (like 1 cycle of IPC) produced an increase in serum cardiac troponin I, which was justified by the observation of transient regional apoptosis of single dispersed cardiomyocytes stained by TUNEL (148,152). The activation of the apoptotic process following a preconditioning stimulus should be put alongside the well-recognized activation of pro-survival pathways following a short protocol of IR injury. Therefore, it seems that both pro-apoptotic and pro-survival pathways are activated following such a stimulus, maybe as a counter-regulatory process. Despite it seeming contradictory, one might speculate that if the stimulus is left for longer, apoptosis contributes alongside necrosis to the final infarct size (146), whilst if left only for a shorter period, the IR injury actually leaves a "footprint", a sort of memory in form of activated pro-survival pathways, to protect the heart when a prolonged injury comes later.

1.6.2   Key signalling proteins involved in the RISK pathway

IPC-induced molecular signalling is considered the paradigm for cardioprotection. The RISK pathway, which has been demonstrated to mediate the IPC protective effect, is therefore considered a universal signalling cascade, a common pathway, shared by most cardioprotective therapies (153). The importance of comprehending this complex molecular architecture lies in the unmet clinical need of developing pharmacological agents specifically targeting pro-survival kinases to promote myocardial salvage. Figure 1-6 depicts the temporal sequence of signal transduction mediating cardioprotection. As described for IPC in section 1.5.1, the RISK pathway (a universal mediator) is activated by some triggers (in the case of IPC were autocoids, whilst in the case of pharmacological conditioning are drugs with the potential to bind either GPCRs or RTKs). Once recruited, the RISK pathway, inhibits the mPTP opening, hence delaying cell death.
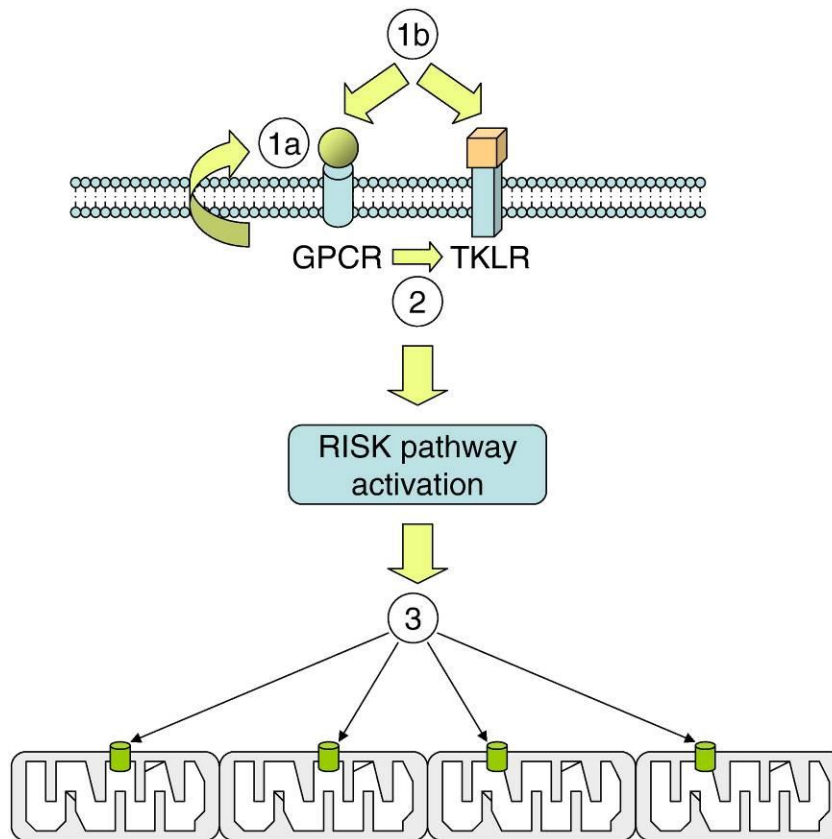
Figure 1-6: Simplified schema of the recruitment process induced by a given cardioprotective therapy

The recruitment of the RISK pathway is a three-step process. The first step is usually characterised by receptor/ligand interaction, either derived from an autocrine source (1a— for example, adenosine), or an exogenous/paracrine (1b—for example bradykinin or insulin). Once the receptor is activated, the RISK pathway is recruited (2). In the third step, the activated kinases eventually impact upon end-effector mechanisms, such as the mPTP, that increase resistance to IRI (3).

Reproduced from Bell & Yellon (121).

As a parallel cascade to PI3K-Akt, ERK 1/2 contributes to the cardioprotection elicited by all forms of ischaemic conditioning. In IPC studies, ERK1/2 appears to mediate its IPC-induced protective effect in a biphasic fashion. The activation of ERK during the trigger phase has been demonstrated to confer protection in most of the studies (89,154), although not all (155), whilst its activation at the onset of reperfusion is more clearly established (89,154). In both ischaemic postconditioning (156) and pharmacological conditioning (157), there is also a consensus on ERK 1/2 involvement at early reperfusion.

In 2000, Tong *et al.* demonstrated for the first time that the IPC-induced cardioprotective effect was mediated through the PI3K-Akt pathway in a Langendorff-perfused rat heart model (158). The prottective effect of IPC was abolished with the administration of the PI3K pharmacological inhibitor wortmannin during the preconditioning phase (trigger phase). Subsequent studies corroborated the essential role of PI3K-Akt when recruited at the trigger phase (69,155). The Hatter Cardiovascular Institute was the first laboratory to report that PI3K-Akt is also recruited at the time of reperfusion (mediator phase) (89). Later, it was also demonstrated in IPC studies, in isolated perfused rabbit hearts, that PI3K-Akt recruitment occurs for up to 1 h following reperfusion for preconditioning to protect the heart (159). Of note, both phases of PI3K-Akt activation are needed for IPC to confer protection, as inhibiting either phase of activation abolishes the IS-sparing effect of IPC (155). PI3K-Akt is also involved in other forms of ischaemic conditioning (160) .

PI3K has several downstream molecular targets, such as phosphoinositide-dependent kinase-1 (PDK1), Akt, mTOR, p70s6k and GSK3β.

PDK1 is a ubiquitously expressed 67 kDa kinase that phosphorylates and activates several kinases including Akt, protein kinase C and p70S6K.

Akt is a serine/threonine protein kinase family comprising thee different Akt isoforms, Akt1/PKBα, Akt2/PKB, Akt3/PKBβγ, each of which is encoded by a separate gene (101). Its activity is primary controlled by PI3K and Phosphatase and Tensin homologue deleted on chromosome 10 (PTEN) through the modulation of PIP3 levels. This is further explained in section 1.7.1.

Full activation of Akt happens via a sequential two-step process involving recruitment of Akt to the plasma membrane through its pleckstrin homology domain, followed by phosphorylation of a Thr-308 residue in the catalytic domain by PDK1 and a Ser-473 residue by the mTOR2. Akt is directly inactivated following dephosphorylation of the two regulatory sites by the serine-threonine phosphatase, protein phosphatase 2A (PP2A). Activated Akt phosphorylates various downstream targets including mTOR, p70s6k, GSK3β and eNOS (161).

All three known Akt isoforms are expressed in the myocardium, with the Akt1 and Akt2 being the most abundant. In fact, Akt1 is ubiquuitously expressed in all tissues and Akt2 is present mainly in insulin-responsive tissues.

mTOR is a serine/threonine kinase that is inhibited by the drug rapamycin. When activated, by Akt or other stimulants, it phosphorylates and activates p70S6K, which is a short isoform of the ribosomal S6 kinase (S6K1) and is involved in regulation of several signalling pathways such as the transcription of certain factors. Insulin administered upon reperfusion has shown ability to induce phosphorylation of both Akt and p70S6K, implicating them in the cell survival signalling cascade promoting reperfusion cardioprotection (86).

GSK-3β is a serine/threonine kinase originally identified to inactivate glycogen synthase by phosphorylation. GSK-3β is expressed in the heart and is active in unstimulated cells, where it phosphorylates targets such cyclin D, c-Jun, NFAT, in addition to glycogen synthase (162). In response to PIP3 increase, phosphorylation of serine-9 residue in the N-terminal regions of GSK-3β by Akt inhibits GSK-3β, leading to improved cell survival and hypertrophy. The importance of GSK3β in ischaemic conditioning is still contentious – i.e. two different studies using the same transgenic mouse model of a dominant negative GSK3β variant found this protein mantadory (163) or not (164) for postconditioning to confer protection.

1.6.3 Importance of isoforms in cardioprotection

A protein isoform, also known as "protein variant", is a member of a family of proteins with highly similar structure. These different forms of a protein may be produced either from different genes or from the same gene by alternative splicing, and can confer different biological effect. Some examples have been appreciated in cardioprotection.

Yellon's lab studied the role of two Akt isoforms, concluding that, Akt1, but not Akt2, was essential mediator of IPC (101). In another study, GSK-3β, but not GSK-3α, was phosphorylated after preconditioning (164). Protein kinase C, another prosurvival protein kinase, represents a paradigm for the importance of the isoforms in cardioprotection: in animal models of myocardial IRI, the activation of the protein kinase C ε isoform is protective, whilst the activation of the δ isoform is deleterious (165). Taking into account the roles of each isoform, a combined treatment with an ε-PKC activator before ischaemia and δ-PKC inhibitor at the onset of reperfusion was proved to induce greater protection against IRI than the treatment with each peptide alone (166).

1.6.4 Targeting the RISK pathway in cardioprotection

The ability to manipulate and up-regulate the RISK pathway during the early reperfusion phase may provide a potential approach to limiting reperfusion-induced cell death. Indeed, the use of pharmacological agents targeting the RISK pathway is a feasible intervention which can be applied at the onset of myocardial reperfusion for patients presenting with an STEMI, either in the ambulance or the cath lab. Therefore, strategies enhancing the RISK activity are an attractive target to develop adjuvant therapies to be used alongside cardiac catheterization.

In the laboratory setting, several drugs have been already demonstrated to protect the heart against IRI through the activation of the RISK pathway: insulin (86), statins (91), bradykinin (129), GLP-1 analogues (167), erythropoietin (168), atrial natriuretic peptide (169), metformin (170,171), dipeptidyl peptidase-IV inhibitors (172) and pioglitazone (173). The translation of most of these medications to the clinical setting has been overall disapointing - the reasons are widely discussed elsewhere (28,61,145,174). However, there

are some promising drugs which have been demonstrated to reduce myocardial IS in patients, such as exenatide (175,176), metoprolol (177–179) and atrial natriuretic peptide (180). Regarding mechanical interventions, remote ischaemic conditioning has also solidly demonstrated to be effective in the clinical setting (55,59).

Time is important when it comes to pharmacologic conditioning for three reasons:

(1) The recruitment of pro-survival kinases are protective when acutely activated, whilst their chronic activation seems to be harmful in the long-term. In the experimental setting, the chronic activation of the PI3K-Akt cascade is deleterious, inducing cardiac hypertrophy (101,181). In the clinical setting, ERK and Akt are chronically activated in the failing heart (182).

(2) Many pharmacological agents have proved effective only in those patients presenting shorter periods of ischaemia. In a subgroup analysis of the Acute Myocardial Infarction Study of Adenosine-II (AMISTAD-II) (183), patients reperfused within less than 3.2 h of symptom onset (median time to reperfusion) showed that adenosine reduced the composite endpoint of death and congestive heart failure (184). In the same vein, Exenatide was proved to be more effective in STEMI patients with shorter ischaemic time from onset of symptoms (175)

(3) The longer the cardioprotective therapy is on board, the more effective it is reducing IS. Both RIC (47) and metoprolol (185) have demonstrated that the sooner the intervention is applied in the course of the infarction, the better the surrogate outcome.

1.7 PI3K

1.7.1 PI3K is part of the RISK pathway activated by IPC

1.7.1.1 Phosphatidylinositols, PI3K and PTEN are part of the RISK pathway

Phosphatidylinositols (PtdIns) are phospholipids that comprise a phosphoglyceride esterified to the hydroxyl group of an inositol ring which can be phosphorylated and dephosphorylated at several positions by lipid kinases and phosphatases, respectively (186). Phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P3, also known as PIP3] is a short-lived second messenger, produced from phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P2, also known as PIP2]. The lipid kinase PI3K and the phosphatase PTEN are the primary regulators of these two PtdIns: PI3K produces PIP3 from PIP2, and PTEN counter-regulates the reaction, dephosphorylating PIP3 on the D3 position (187).

PI3K activity is required to protect the heart against IRI in all forms of ischaemic conditioning (188). As discussed in section 1.5, the IPC stimulus promote the release of triggers which activate either GPCR or RTKs, in turn activating signalling cascades, such as the RISK pathway. As a result of this PI3K activation, PIP3 production leads to the recruitment and activation of Akt and PDK1, and subsequently to a phosphorylation of a wide range of pro-survival downstream targets such a p70S6, eNOS and GSK3β (186).

Despite the growing interest in the importance of protein isoforms in cardioprotection (see section 1.6.3), little is known about the specific role of the PI3K variants in IRI and ischaemic conditioning. PI3K is of significant importance within the RISK pathway, and its upstream position governs the other important downstream kinases. To elucidate the role of each isoform may eventually allow us to optimize pharmacological interventions selectively thereby targeting specific PI3K isoforms.

1.7.2   Structure and function of class I PI3K

PI3K isoforms have been divided into three classes (class I, class II, class III) based on structural features and lipid substrate preferences (189). This thesis focuses specifically on class I PI3K, particularly in PI3Kα.

Class I PI3Ks are a family of lipid kinases activated by cell membrane receptors, either receptor tyrosine kinases or G protein-coupled receptors, to catalyse the production of PIP3 from PIP2 (187,190). These enzymes are heterodimers composed by a regulatory subunit (either p85 or p101/p84 family) and one of four catalytic subunit (p110α, p110β, p110γ or p110δ), forming PI3Kα, β, γ or δ, respectively (189). Structural differences at the N-terminal portion of p110 subunits allow a further subdivision in class IA and class IB. Class IA members (p110α, p110β and p110δ) form heterodimers with p85 regulatory subunit, and usually are thought to be activated downstream of receptor tyrosine kinase, although there is evidence associating PI3Kβ with GPCR (191). In contrast, the only p110γ forms heterodimers with either p84 or p101, and selectively respond to GPCR signalling, with some recent evidence suggesting a potential Ras-mediated activation.

1.7.3   PI3K isoforms: genetic disruption studies vs pharmacological studies

Two general approaches can be performed to identify the roles and mechanisms of action of PI3K isoforms in normal cardiovascular physiology and disease: genetic disruption studies and pharmacological studies targeting specific PI3K isoforms.

Mouse mutants lacking PI3Kα or PI3Kβ have shown embryonic lethal phenotype (192,193), whilst mice lacking either PI3Kγ or PI3Kδ reach adulthood (194). Transgenic mice with cardiac-specific expression of a constitutively active p110α (caPI3Kα) display increased heart and cardiomyocyte size (195). In contrast, cardiac-specific expression kinase-dead version of p110α reduces heart size. Further examples can be found in PI3K downstream effector Akt isoforms (196,197). It is not always clear whether the reported phenotypes and molecular signalling alterations of genetic modified models can be directly attributed to the the targeted gene, or if they are actually the result of "knock on" effects on other PI3K

isoforms and the signalling pathways they control. In other words, the activation of compensatory signalling pathways other than the one being under study.

Genetic approaches needs to be complemented with pharmacological studies using isoform-specific inhibitors. Cell permeable inhibitors make it possible to directly assess the phenotypic consequences of acutely inhibiting a kinase with a drug at a given time point in a physiologically relevant model. For instance, pharmacological studies with pan-specific PI3K inhibitors (LY-294002 and Wortmannin) strongly support the notion that class I PI3Ks are key mediators of cardioprotection imparted by insulin (86) and IPC (89) at reperfusion; but these inhibitors are not isoform specific (198).

A significant development over the past ten years has been the development of highly isoform-specific, small molecule, class I PI3K inhibitors. These inhibitors, which are available either commercially or through collaborations with the Cancer Institute at University College London, permit the assessment of the role of the two predominant cardiac isoforms, namely PI3Kα and PI3Kβ, in cardioprotection. This pharmacological strategy provides a physiological model which replicates the effects of small molecule kinase inhibitors more closely than classical gene knockout approaches in an acute model, such as the IRI model of myocardial infarction(199). The development of compensatory adaptions might be avoided using potent and highly selective isoform-specific PI3K inhibitors in this acute setting.

1.7.4   Comparison of features and properties between PI3K isoforms

1.7.4.1 Similar structure, different function

The triggers released as a consequence of an IPC stimulus (i.e growth factors) elicit their effect by transient alterations in the levels of PIP3, the levels of which are very low in quiescent cells but rise swiftly following growth-factor stimulation (200). This rise of PIP3 induced by the activation of PI3K mediate a broad range of cellular functions. The catalytic p110 isoforms of Class IA PI3K have a highly homologous structure, interact non-selectively with the different p85 regulatory subunits and have the same lipid substrate preference (201). Therefore, a substantial functional overlap may be expected. However, there is a growing body of evidence suggesting distinct roles for each isoform in the cell. This is

particularly highlighted by the finding that ablation of either p110α or p110β gene is developmentally lethal (192,193), indicating that the non-deleted isoform cannot fully substitute for the ablated (200). Moreover, experiments using gene-targeted mice and p110 isoform-selective inhibitors have uncovered non-redundant functions of the p110 isoforms – i.e. p110α is involved in insulin signalling (202,203), p110β in integrin signalling (204,205), and p110δ in leukocitary signalling (206). Some key parameters have been investigated to differentiate the role played by each isoform, as explained in subsequent sections.

1.7.4.2 Mechanism of activation

Class I PI3K four isoforms are highly homologous in structure, but they have distinct mechanisms of activation. Class IA PI3Ks (α, β and δ) are activated through RTKs, whilst PI3Kγ acts downstream to GPCRs (207). However, there is compelling evidence demonstrating that both RTK and GPCR can engage PI3Kβ, (191,208), placing this isoform between the two subclasses. Moreover, it has been suggested that PI3Kβ in synergistically activated by both receptors (209). Kulkarni *et al.* have demonstrated PI3Kβ to play a major role in determining the sensitivity of neutrophil activation by immune complexes in enabling a synergistic activation through both RTK and GPCRs (210). In the same way, Houslay et al. have recently reported a synergistic PIP3 formation by PI3Kβ in response to the co-activation of both GPCR and RTK in myeloid cells (211). Altogether, this need for cooperative signalling may indicate that the activation of PI3Kβ requires coincident signalling inputs to be fully activated (209).

Relevant to this thesis, RTK ligands (such as PDGF, insulin and insulin-like growth factor-1, IGF-1) have been demonstrated to activate Akt through PI3Kα, whilst several GCPR ligands has been proposed to mediate Akt activation through PI3Kβ, such as SDF-1α, sphingosine-1-phosphate and lysophosphatidic acid (191,208,210,212).

1.7.4.3 Lipid- and protein-kinase activities

Class I PI3K have two kinase functions: (1) the lipid-kinase activity mediates the phosphorylation of phosphoinositides on the D-3 position of the inositol ring (i.e. PIP2 → PIP3), therefore activating Akt and other PIP3-binding molecules, such as PDK1 and mTORC2; and (2) the protein-kinase activity causing the autophosphorylation of the

regulatory subunit p85α at Ser608, which in turn downregulates the lipid-kinase activity of PI3K (213,214).

Significant differences in the kinetic properties of p110α and p110β catalytic subunits have been described so far (215). Beeton *et al.* measured both PI3K lipid-kinase activity (using two different methods in several cell lines) and PI3K protein-kinase activity (with only one assay) (200). They concluded that: (1) p110β has a lower lipid kinase activity than p110α; (2) at low lipid concentration p110β is more active than p110α, whilst the reverse occurs at higher lipid concentration; (3) p110β also displayed a lower protein kinase activity (lower p85 phosphorylation). Altogether, p110β demonstrated less overall lipid- and protein-kinase activity compared to p110α.In that article, the authors speculate that the differences being reported in p110α and p110β kinetic properties would mean that these isoforms could be maximally effective in different cellular locations (200), based on the availability and concentrations of the substrate phosphoinositides. A few years later, the same group confirmed these differences in the regulation of PI3K by its intrinsic serine kinase activity *in vivo* – p110α possesses a higher protein kinase activity phosphorylating a serine residue in p85, whereas p110β is less effective in this phosphorylation (216).

Differences in kinase activity between PI3K isoforms have been also reported by Vanhaesebroeck's group, which evaluated lipid kinase activity in both endothelial cells and human umbilical vein endothelial cells (HUVECs), confirming that p110α is also the most active isoform in these cell types (217). With regard to PI3Kγ, Perino *et al.* have reported that p110γ lipid kinase activity is negligible in cardiomyocytes in physiological conditions, but results dramatically upregulated under adrenergic stress, such as that occurring in chronic congestive heart failure (218).

1.7.4.4 Expression and location

Some studies have quantified the amount of PI3K isoforms in several non-cardiac mouse tissues. Vanhaesebroeck's group have determined that the most abundant catalytic subunits were p110β (in liver, brain and fat) or p110δ (in spleen), with a lower abundance of 110α in most tissues tested (206). Only the muscle (the heart was not tested) showed a predominance of PI3Kα. The same group studied the expression for each class IA isoform in both mouse cardiac endothelial cells (MCECs) and HUVECs, demonstrating a similar

qualitative expression of p110α and p110β, and a very low expression of p110δ, particularly when these cells were compared with leukocytes (217). Few studies have suggested subcellular localization of PI3K isoforms as a major mechanism to determine specific functions and govern cell responses, as p110β, but not p110α, localizes to the nucleus in several cell types (219,220).

In the heart, the most highly expressed p110 catalytic subunit are p110α and p110β (221), although little is known about their absolute and relative amounts.

1.7.5   Class I PI3K isoforms

1.7.5.1 PI3Kα

PI3Kα can be exclusively activated by RTKs. Cardiomyocyte PI3Kα is mostly activated by the receptors of insulin or IGF-1, whilst other myocardial cell types such as endothelial cells or fibroblasts are engaged mostly by vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF). PI3Kα has a major role in cardiomyocyte growth and survival and this explains why systemic knockout of PI3Kα is embryonic lethal (192).

Genetic disruption in animal models have revealed a critical role for PI3Kα-Akt cascade in promoting cardiomyocyte postnatal growth and survival. In a cardiac-specific transgenic mouse model expressing a constitutively active (ca) PI3Kα mutant: PI3Kα activity was increased 6.5-fold, heart size was approximately 20% higher and cardiac function and lifespan were normal (195). On the other hand, in a cardiac-specific mouse model expressing a dominant negative (dn) PI3Kα mutant, PI3Kα activity was reduced by 77% and hearts size were reduced by approximately 20%. Under basal conditions, alterations of PI3Kα influences heart size without gross changes in cardiac contractility (222). In both animal models, ca PI3Kα and dnPI3Kα, the number of cardiomyocytes is unchanged if compared to controls, thus supporting the idea that the α isoform of PI3K controls cardiomyocyte size but not the number (222). Further, the upstream cardiac-specific IGF-1 receptor overexpression also leads to cardiac hypertrophy through the activation of PI3Kα (223). Notably, PI3Kα promotes physiological hypertrophy and sustained systolic function. In a series of articles, McMullen and colleagues have demonstrated that PI3Kα-Akt can promote physiological exercise-induced growth (224), but antagonize pathological growth

in a transgenic mouse model of dilated cardiomyopathy or a mouse model subjected to pressure overload through a constricted aorta (225,226). Altogether, these studies suggest that PI3Kα is essential to maintain cardiac function unaltered in response to a pathological cardiac insult.

With regard to myocardial infarction, little research has been undertaken to date. Lin *et al.* have demonstrated that PI3Kα is crucial for protecting the heart against pathological remodelling and failure (227). They have demonstrated that increased PI3Kα activity can protect the heart against dysfunction following a chronic myocardial infarction (227). In this myocardial infarct-induced heart failure model, designed to cause cardiac dysfunction, as a consequence of a chronic coronary occlusion (without reperfusion), caPI3Kα mice demonstrated less function impairment when compared to controls (227).

In addition to its involvement in physiological cardiac growth and protecting the heart against pathological remodelling following an AMI, PI3Kα is also critically involved in cardiac contractility. Temporally controlled overexpression of cardiac-specific PI3Kα enhances contractility in the Langendorff-perfused mouse model (228), whilst the overexpression of dnPI3Kα reduces basal contractility (229). It has been suggested that L-type voltage gated calcium channels are involved in the mechanism for PI3Kα to contribute in cardiac contractility (230).

Mutations in *PI3KCA*, the gene coding for PI3Kα, are associated with oncogenic transformation. Hence, the therapeutic potential of PI3Kα inhibitors has generated great interest in cancer patients, but also some challenges regarding their potential side-effects on the heart. Mice deficient in PI3Kα displayed an accelerated heart failure in response to dilated or hypertrophic cardiomyopathy – these results might explain the association of cardiomyopathy in cancer patients given tyrosine kinase inhibitors and raise concerns for the use of PI3Kα inhibitors in cancer patients (231).

1.7.5.2 PI3Kβ

PI3Kβ is centrally involved in Akt activation in platelets, and in thrombus formation and maintenance. This has been demonstrated in mice with targeted deletion of p110β, and by the use of a selective PI3Kβ inhibitor (204,205). It is also highly expressed in

cardiomyocytes, although no evidence has been reported on its involvement in IRI and cardioprotection.

1.7.5.3 PI3Kγ

Unlike PI3Kα, PI3Kγ is not ubiquitously expressed but enriched in specific subsets of cells, including leukocytes and cardiac cells. PI3Kγ expression is upregulated in mouse models of atherosclerosis as a result of massive leukocyte infiltration (187). Beyond atherosclerosis, PI3Kγ has emerged as a critical modulator of cardiac function (207).

PI3Kγ is known to interact with agonist-activated GPCRs. As IPC is mostly mediated by GPCR ligands, this isoform have raised the interest of some researchers. In 2004, Tong *et al.* postulated PI3Kγ as a mediator of the protection afforded by IPC (232). In transgenic mice with cardiac-specific overexpression of a catalytically inactive mutant of PI3Kγ, they observed the IPC protective effect to be lost, therefore suggesting a central role of PI3Kγ in the IPC-protective effect. Consistently, Ban *et al.* demonstrated the importance of the PI3Kγ signalling cascade in IPC, as PI3Kγ knockout mice (PI3Kγ$^{-/-}$) displayed poorer functional recovery and greater tissue injury (measured by lactate dehydrogenase release) compared to the wild-type and PI3Kγ$^{-/+}$ counterparts in a Langendorff-perfused mouse model. They also showed similar functional data on adenosine-mediated pharmacological preconditioning, suggesting PI3Kγ involvement in the protection conferred by adenosine, on the basis of GPCR activation in IPC.

PI3Kγ has also been associated with other heart diseases. In 2017, a research group from Italy demonstrated PI3Kγ to have a central role in diabetic-induced cardiomyopathy. Using both a pharmacological approach with the GE21 PI3Kγ inhibitor and a genetic approach with both knock-out and kinase-dead mice, they observed an improved cardiac function when compared to non-manipulated mice (233), therefore suggesting that PI3Kγ inhibition has the potential to be translated to patients with diabetic cardiomyopathy.

1.7.5.4 PI3Kδ

PI3Kδ is highly expressed in leukocytes, but is also present in other tissues such as neurons. Its predominant expression in the haematopoietic compartment correlates with a variety of immune functions, mainly in the adaptive immune system, with important roles

in B and T cells, as well as in mast cells and myeloid cells (190). Little is known about its potential role in the physiology and disease of the cardiovascular system.

Overall, this section lays the foundation for the investigation of specific isoforms that may play a relevant role in cardioprotection.

Chapter 2   HYPOTHESES AND AIMS

The overall aim of this PhD is to investigate the potential role of PI3Kα as mediator in cardioprotection. The principle objective and specific research aims addressed are outlined below and described with further details in subsequent chapters.

Objective (1) – To characterise the isolated perfused mouse heart model using global ischaemia-reperfusion injury (Chapter 4)

Aim 1: *Determine whether infarct size (IS) is dependent on both the duration of ischaemia and length of the reperfusion period*

Aim 2: *Describe the lactate dehydrogenase release pattern during reperfusion as an index of injury*

Aim 3: *Describe the impact of IPC on IS and lactate dehydrogenase release after long reperfusion*

Aim 4: *Determine any correlation between IS and lactate dehydrogenase release*

Objective (2) – To select the IPC protocol (Chapter 5)

Aim 1: *Compare the effect of 1-cycle vs 4-cycle of IPC to protect the heart against ischaemia/reperfusion injury*

Aim 2: *Compare the effect of 1-cycle vs 4-cycle IPC to activate the survival kinases, Akt and ERK*

Objective (3) – To characterise the role of PI3K prosurvival cascade in preconditioned mouse hearts (Chapter 6)

Aim 1: Investigate whether the pan-specific PI3K inhibitor wortmannin abrogates the cardioprotective effect of IPC when applied either during the trigger phase or the mediator phase

Aim 2: Mapping prosurvival cascades at the trigger phase and at reperfusion following PI3K inhibition in preconditioned hearts

Objective (4) – To characterise the expression of the α and β isoform of PI3K in cardiac tissue and cells (Chapter 7)

Aim 1: Investigate PI3Kα protein expression in mouse and human heart tissue, as well as in primary isolated cardiomyocytes and endothelial mouse cardiac cells

Aim 2: Investigate PI3Kβ protein expression in mouse and human heart tissue, as well as in primary isolated cardiomyocytes and endothelial mouse cardiac cells

Objective (5) - To investigate the role of PI3Kα in IPC (Chapter 8)

Aim 1: Select PI3Kα inhibitors dose

Aim 2: Investigate the role of PI3Kα in the IPC "trigger" phase in the ex vivo model

Aim 3: Investigate the role of PI3Kα in the IPC "mediator" phase in the ex vivo model

Aim 4: Investigate the role of PI3Kα in the IPC "mediator" phase in the in the in vivo model

Aim 5: Evaluate Akt activation following PI3Kα inhibition in both the IPC "trigger" phase the IPC "mediator" phase

Objective (6) - To investigate the pharmacological activation of PI3Kα at reperfusion (Chapter 9)

Aim 1: *Assess the role of PI3Kα activation to protect the heart against IRI using both its canonical activator and its specific pharmacologic inhibitor*

Aim 2: *Demonstrate Akt phosphorylation in response to PI3Kα pharmacological activation and inhibition*

Objective (7) - To investigate the pharmacological activation of PI3Kα in several cell types and tissues, as well as the mitochondrial Permeability Transition Pore (mPTP) inhibition as a potential end-effector (Chapter 10)

Aim 1: *Assess whether PI3Kα can be activated in both primary adult mouse cardiomyocyte and mouse cardiac endothelial cell line*

Aim 2: *Determine the effect of PI3Kα activation and inhibition on the susceptibility of cardiomyocytes to form the mPTP.*

Aim 3: *Assess whether the overall results can potentially be extrapolated to humans, activating and inhibiting PI3Kα in both mouse and human heart tissue.*

Objective (8) - To preliminary investigate PI3Kβ activation (Chapter 11)

Aim 1: *Demonstrate Akt phosphorylation in response to PI3Kβ activation using SDF1α in mouse isolated Langendorff-perfused hearts*

Aim 2: *Demonstrate Akt phosphorylation in response to PI3Kβ activation using SDF1α in a mouse cardiac endothelial cell line*

Chapter 3   GENERAL RESEARCH METHODS

3.1   Experimental use of animals

All experiments described within this thesis were performed at The Hatter Cardiovascular Institute, University College London, and carried out under the Home Office guidelines for the Animals (Scientific Procedure) Act 1986.

Animals used were male C57BL/6 mice (9-12 weeks, 24–28 g weight), all of them obtained pathogen-free from one supplier (Charles River, UK) and housed in 12 h light/dark cycles under identical conditions. All standard care was provided by the Biological Services Unit (BSU) at University College London (UCL). Before performing a given experiment, each animal was inspected for any adverse features, such as reduced weight gain or piloerection, and subsequently excluded before randomization if signs of not well-being.

The mouse model was chosen due to its value to elucidate the cellular mechanisms underlying those cardioprotective therapies being studied (234). Furthermore, the use of this species allows both a pharmacological and genetic approach to be undertaken. Deleting or over-expressing specific components of those signalling pathways involved in cardioprotecion, allows us also to identify specific gene products. However, interpretation of these results should be taken cautiously given that other genes may be either silenced or upregulated in compensation. In contrast, a pharmacological approach is a more translational means to study not only mechanisms, but also potential drug applications. Nevertheless, drug pharmacokinetics and pharmacodynamics should also be considered when interpreting the results. Overall, the potential use of both approaches in combination was the central reason to choose this animal model. In the end, it was not possible to obtain transgenic mice overexpressing PI3Kα from the Cancer Institute at UCL, hence this thesis is based on a pharmacological approach. To date, the colony of these transgenic mice (activated p110α colony) is being expanded and they will hopefully be available in the near future.

A variety of experimental models were used in this thesis, which are described in more detail in the following sections of this chapter. The specific experimental protocols are described in the relevant chapters.

3.2   Drugs

The Table 3-1 below summarizes the pharmacologic treatments being used across this thesis.

Drug	Company (catalog number)	Concentration
Wortmannin	Merck Millipore (681675)	100 nM
Insulin	Sigma-Aldrich (I9278)	100 nM 5 mU/L
Alpelisib (BYL719)	Selleck Chemicals (S2814-SEL)	1 and 3 µM
G326	Genentech Inc. (N/A)	1 and 3 µM
Human SDF-1α	Miltenyi Biotec Inc. (130-093-997)	25 and 100 ng/mL

Table 3-1: Overview of drugs and their relevant concentration

The Table 3-2 below summarizes the selectivity of the PI3Kα inhibitors used in this thesis. BYL719 is a potent and selective PI3Kα inhibitor, which has demonstrated to be reversible with its interruption. Its characteristics are fully described elsewhere (235,236). GDC-0326 is also a very selective PI3Kα inhibitor and its properties are fully described by Heffron et al. (237).

	BYL719 (Stratech Scientific) (236)	G-326 (Genentech) (237)
PI3Kα	4.6 nM	0.2 nM
PI3Kβ	1156 nM	133 nM
PI3Kδ	290 nM	20 nM
PI3Kγ	250 nM	51 nM

Table 3-2: Selectivity of PI3Kα inhibitors

This table illustrates the IC_{50} (concentration producing 50% inhibition of the enzymatic activity) of PI3Kα inhibitors demonstrating isoform specificity (236, 237).

Ex vivo isolated perfused mouse heart model of global IRI

This *ex vivo* model is also known as Langendorff-perfused model (238).

3.2.1 Preparation of hearts for perfusion

Mice were given terminal anaesthesia with an intra-peritoneal injection of 60 mg/kg sodium pentobarbitone (Animalcare, UK). Heparin 100 IU was co-administered to prevent thrombus formation in either coronary arteries or inside the ventricles. Once consciousness was lost, both forelimbs and the left hind limb were secured with small tape strips. Surgery was started after confirming the abolishment of the right hind limb withdrawal reflex to pain.

Upon the confirmation of deep anaesthesia, the heart was then harvested and immediately submerged in ice-cold modified Krebs-Henseleit buffer (composed of 118 mmol/L NaCl, 25 mmol/L NaHCO$_3$, 11 mmol/L glucose, 4.7 mmol/L KCl, 1.22 mmol/L MgSO$_4$.7H$_2$0, 1.21 mmol/L KH$_2$PO$_4$, and 1.84 mmol/L CaCl$_2$.2H$_2$0). A timer was started immediately to record the time taken for the heart from being removed to being perfused. The remaining lung and mediastinal tissues were dissected in the dish filled with ice-cold buffer. The heart was then swiftly cannulated with a 21-gauge cannula and fixed to the cannula with a 5/0 silk tie, to be thereafter transferred to the murine Langendorff perfusion rig and be perfused at 80 mmHg pressure. Great care was taken to avoid air bubbles in the cannula and the Langendorff apparatus, as well as to shorten as much as possible the time to cannulation to avoid ischaemic preconditioning due to perfusion delay (239).

3.2.2 Langendorff perfusion

The principles of isolated mouse perfusion were described by Langendorff in 1895 (240). The heart is perfused retrogradely through an aortic cannula, inserted above the sinuses of Valsalva. The aortic valves are forced closed as they remain in diastole by the weight of the perfusate column, therefore allowing the buffer to flow and irrigate the coronary vasculature to be eventually drained via the coronary sinus of the right atria. Perfusate pressure can be maintained either via constant pressure (gravity from a fixed

height reservoir) or via constant flow (peristaltic pump). Using this perfusion technique, a heart may be maintained with oxygenated buffer for hours (241).

Heart isolation and Langendorff perfusion were carried out with filtered modified Krebs-Henseleit buffer (see previous section for composition) aerated with a mixture of O_2 (95%) and CO_2 (5%) in order to maintain pH at 7.42 ± 0.3, as previously described (238). The pH was checked at regular intervals using a blood gas analyser (ABL90 FLEX). In the experiments described in this thesis, perfusion took place at constant hydrostatic pressure (~80 mm Hg) using a set height column of fluid above the cannulated heart.

The temperature of the circulating perfusion buffer was adjusted in order to maintain the myocardium at 37.0 ± 0.5 °C. A heated glass water jacket were used to assist in controlling any smaller temperature fluctuations of the heart. Besides, the temperature was maintained along the entire protocol by submerging the heart in an organ bath containing anoxic modified Krebs solution at 37°C, in order to avoid protect the heart through a cold-conditioning phenomenon (135,242,243).

After commencing retrograde aortic perfusion hearts were allowed to stabilise for 20 min. Pre-defined exclusion criteria were checked during this period (see Table 3-3), before starting with the experimental protocol.

Global ischaemia was achieved with the cessation of coronary flow by switching off the perfusion circuit. In a similar way, switching the tap leading to the heart changes the source of perfusate from modified Krebs-Henseleit buffer to that containing the drug of interest − i.e. PI3Kα inhibitors. Figure 3-1 provides an overview of the Langendorff apparatus and the components needed for drug administration.

Several endpoints may be used in the isolated perfused mouse heart model of global IRI, such as myocardial IS, lactate dehydrogenase (LDH) release, left ventricular pressure and electrical activity (244). In this thesis, the Langendorff was used either to collect proteins or to perform IRI experiments. In the latter case, the endpoints being studied were myocardial IS and LDH release.

MEASUREMENT	INCLUSION CRITERIA
Time from extracting the heart to perfusion	≤ 4 min
Coronary flow	1.0 – 6.0 mL/min
Temperature	37.0 ± 0.5 °C

Table 3-3: Inclusion criteria for isolated hearts undergoing Langendorff perfusion

These parameters were checked during a stabilisation – if these criteria was not meet the heart was excluded from further study.

A

A. Reservoir for modified Krebs-Henseleit buffer

B. Reservoir for drug diluted in modified Krebs-Henseleit buffer

Outlet tube from B

Outlet tube from A

Tap to switch between A and B

Organ bath

B

Cannula

Suture around aorta

Mouse heart (beating)

Modified Krebs-Henseleit buffer

Organ bath (heated)
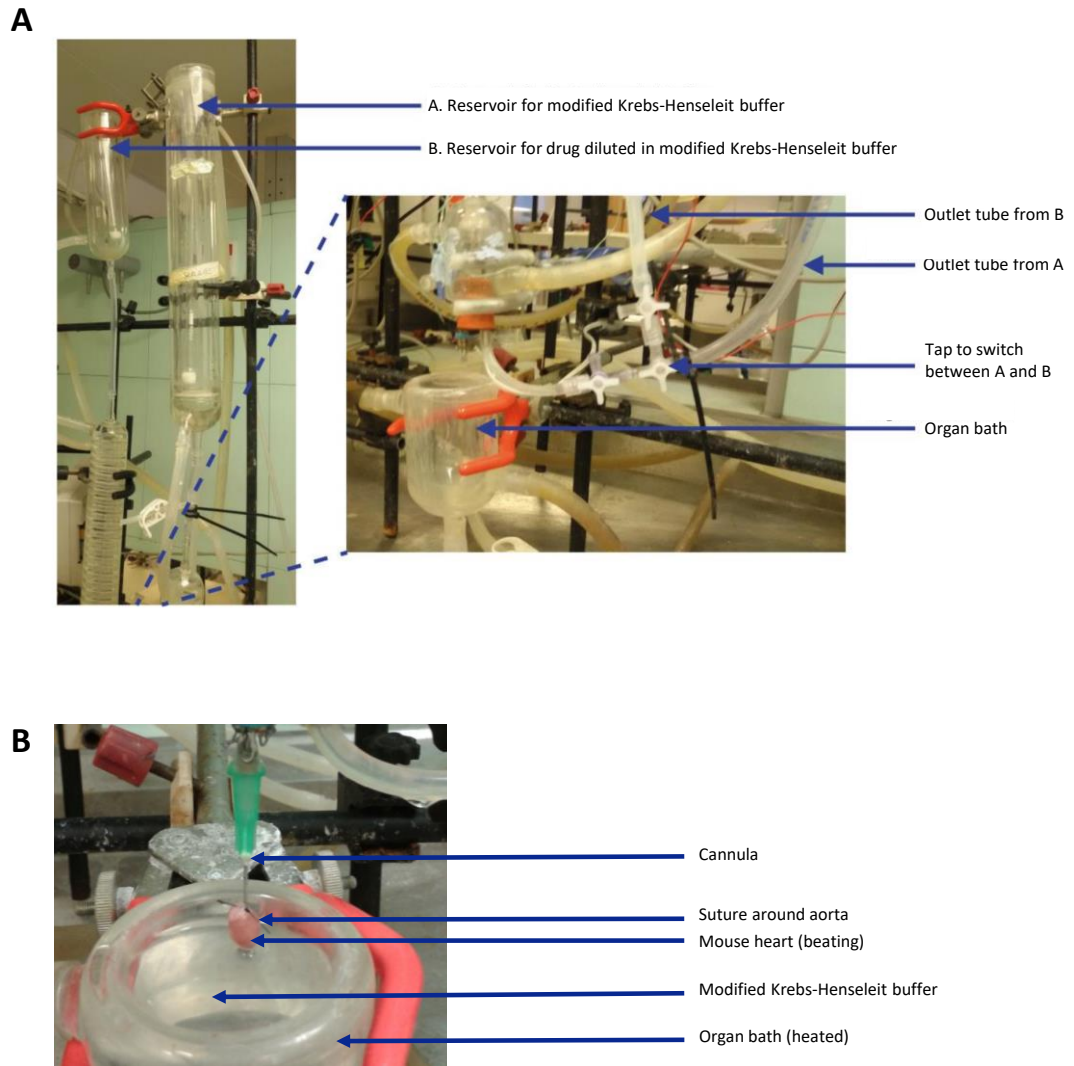
Figure 3-1: Overview of the Langendorff isolated perfused mouse heart apparatus

The top half of the illustration (panel A) depicts the Langendorff setup components needed for drug administration: a reservoir containing Krebs for normal perfusion, a reservoir used to contain drug of choice are both, the outlet tubes from both of reservoirs and the tap to switch between them (all of the indicated by arrows).

The bottom half of the illustration (panel B) shows a cannulated mouse heart through the aorta, on Langendorff apparatus. The heart is perfused with modified Krebs-Henseleit buffer and submerged in the same buffer in the organ bath at 37 ºC.

3.3 Analysis of Infarct size

After global normothermic ischaemia and reperfusion, IS was determined by injecting 5 mL of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffered saline through the aortic cannula and incubating the heart for 10 minutes at 37°C in order to demarcate the infarcted (white) versus viable (red) tissue (245). After the incubation, the heart was weighed and then frozen overnight at -20°C. Following this, it was sectioned perpendicular to the long axis into 5-7 sections > 1mm thick and the slices transferred into 10% neutral formalin buffer for 1 h. Heart slices were afterwards compressed between two Plexiglas plates and scanned using an Epson Perfection V100 Photo scanner with its corresponding Epson software. Images were taken and coded in order to blind the analyser. Planimetry analysis using Image J version 1.47 (NIH, Bethesda, MD) was carried out to accurately quantify the percentage IS in each heart as a proportion of the total heart volume.

TTC staining method has been validated against the gold-standard histological assessment of necrosis in the early phase following an AMI (246). Figure 3-2 depicts an example of heart sliced TTC staining.

A. Control heart

B. Preconditioned heart

Figure 3-2: Examples of heart 2,3,5-triphenyltetrazolium chloride (TTC) staining

Infarcted tissue is white, whilst viable tissue was stained in red.

3.4   Measurement of LDH activity

LDH is an unbound intracellular enzyme localized in the cytosol, released to the extracellular compartment by sarcolemmal membrane damage. LDH is thereafter a commonly used as a surrogate marker of cellular injury in cardiovascular research(247–249).

To study LDH activity in the isolated perfused mouse heart model, samples of coronary perfusate were collected and stored in ice until the end of the experiment, avoiding freezing at −80 °C (250). Lactate activity in the perfusate was determined by means of a commercially available assay kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, UK).

The CytoTox 96® Assay quantitatively measures LDH with a 30-minute coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of dead cells. Visible wavelength absorbance data are collected using a standard 96-well plate reader at an absorbance of 490 nm (FLUOstar Omega microplate reader, BMG Labtech).

The general chemical reactions of the CytoTox 96® Assay are as follows:

LDH

NAD^+ + lactate → pyruvate + NADH

NADH dehydrogenase

NADH + INT → NAD^+ + formazan (red)
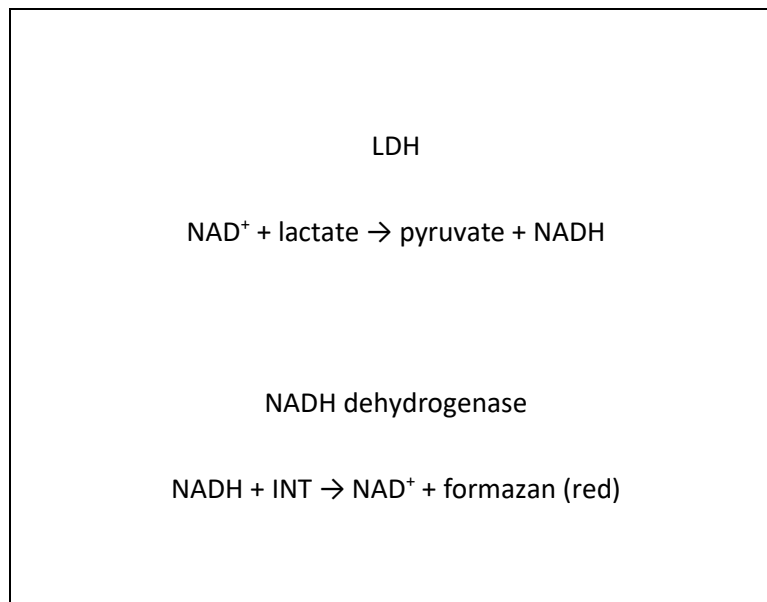
A dose-response curve with LDH positive control provided by the kit (and gradually diluted with PBS) was constructed to infer LDH concentration from the absorbance arbitrary units. The measured concentration of LDH was corrected for coronary flow and heart weight, according to previous studies (244,248,251). Thus, it was calculated as the product of effluent concentrations (pg x mL^{-1}) x coronary flow (ml x min^{-1} x g^{-1}) [LDH concentration x coronary effluent / weight] and consequently expressed as pg x g^{-1} x min^{-1}.

3.5   *In vivo* murine model of regional IRI

These experiments aimed to corroborate one specific observation found in the *ex vivo* isolated heart model (role of PI3Kα in the IPC mediator phase) and were performed by Dr David He (postdoctoral research fellow at the Hatter Cardiovascular Institute). I contributed with the experimental design, statistical analysis and critical interpretation of the data.

The primary endpoint of this *in vivo* mouse model of regional IRI was myocardial IS. Briefly, C57Bl/6 mice were anaesthetized by intraperitoneal injection of 80 mg/kg pentobarbitone at a concentration of 20 mg/ml in 0.9% (w/v) saline and maintained at 36.5 ± 0.5°C on a heating mat. Surgery was started after confirming the abolishment of pedal and tail reflexes. Mice were intubated using a 19G cannula and ventilated with room air using a MiniVent, type 845, Small Animal Ventilator (Harvard Apparatus, Kent, UK), at a flow rate of 1.0 l/min with 2 cm H$_2$O PEEP, stroke volume 200 µl at 130 strokes/min. All mice were subjected to the occlusion of the left anterior descending (LAD) for 40 min, which was verified by ST elevation in the electrocardiogram and by the presence of hypokinesia and pallor in the heart, followed by 2 h reperfusion. The animals were then killed by exsanguination via thoracic aorta. Afterwards, myocardial IS was measured by tetrazolium staining and expressed as a percentage of area at risk, determined using Evan's blue.

3.6 Isolation of adults mouse cardiomyocytes

Adult mouse ventricular cardiomyocytes were isolated using liberase heart digestion as described previously (252,253). Before proceeding with the heart removal, the perfusion buffer was prepared (consisted of NaCl 113 mmol/L, KCl 4.7 mmol/L, KH_2PO_4 0.6 mmol/L, Na_2HPO_4 0.6 mmol/L, $MgSO_4$-$7H_2O$ 1.2 mmol/L, $NaHCO_3$ 12 mmol/L, $KHCO_3$ 10 mmol/L, Hepes Na salt 0.922 mmol/L, Taurine 30 mmol/L, 2,3-butanedione-monoxime 10 mmol/L and Glucose 5.5 mmol/L) and stirred for 20 min. Its pH was corrected to 7.4 before filter sterilizing into a sterile bottle. The pre-heated isolation rig was then filled with perfusion buffer and air bubbles were carefully removed from the tubing. The solution was lightly bubbled with pure O_2 in the reservoir.

Once the Langendorff setup was ready, hearts were excised (as explained in section 3.2.1) and cannulated through the aorta before retrograde perfusion on the Langendorff apparatus at 37 ºC. Following perfusion with buffer for 5 min to clear residual blood, enzymatic digestion was performed using 30 mL perfusion buffer with 5 mg Liberase (Roche, UK) and 12.5 µmol/L $CaCl_2$ for about 20 min. The perfusion rate provided by the peristaltic pump was 3 mL/min. During this 20 min period, several parameters were used to monitor the digestion, such as the progressive paling and swelling of the heart, as well as the softness of the tissue, which was assessed by touch.

At the end of enzymatic digestion, the heart was removed and placed in a single well dish where both ventricles were isolated and mechanically disaggregated in a gentle manner. A Pasteur pipette was used to tease apart and mix the remaining non-fully digested tissue. The resulting cell suspension was filtered through a mesh and transferred for enzymatic inactivation to a tube with 10 mL of stopping buffer (perfusion buffer supplemented with fetal bovine serum 10%), and Ca^{2+} was gradually re-introduced with a three-step increasing $CaCl_2$ concentration. Cells were allowed to pellet for 10 min after each $CaCl_2$ addition until all Ca^{2+} was re-introduced. Cells were then re-suspended in M199 (Invitrogen, UK) supplemented with L-Carnitine (2 mmol/L), Creatine (5 mmol/L), Taurine (5 mmol/L), Penicillin (100 IU/ml), Streptomycin (100 IU/ml) and 25 µmol/L Blebbistatin.

3.7 Mouse cardiac endothelial cell culture

Immortalized Mouse Cardiac Endothelial Cell (MCEC) are derived from microvascular neonatal mouse cardiac endothelial cells.

MCECs were stored at -80 ºC, and after thawing were cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. To passage the cells, a PBS-wash removed all culture medium, dead cells and debris before the addition of 1-2 mL of 0.25% trypsin, incubated at 37 ºC for 2-3 minutes. Once the adhered MCECs were displaced, the cells were re-suspended in 10 mL of DMEM to inactivate the trypsin. When being seeded for experiments, MCECs were cultured at 80-90% confluence in supplemented DMEM. The cells were kept in an incubator at 37 ºC, with 95% O_2 and 5% CO_2.

MCECs were used to quantify the protein levels of PI3Kα and β as well as to study their response to the pharmacological activation and inhibition of PI3Kα.

3.8 Experiments in human right atrial tissue

Human atrial tissues were collected from Barts Heart Centre at St Bartholomew's Hospital. The study received Local Research Ethics Committee approval (REC No. 00/0275) and was carried out in accordance with the University College London Hospitals NHS Trust guidelines. All patients were provided with a Patient Information Sheet and a verbal explanation of the study, in line with Good Clinical Practice guidelines. All patients provided written informed consent and were free to participate in the Barts Cardiovascular Registry.

All patients were aged 18–80 years and their baseline characteristics were recorded upon consent. Patients with impaired renal or ventricular function, dilated left atria or a history of arrhythmias or on rhythm stabilising medications were excluded.

Right atrial appendage samples were harvested from patients undergoing cannulation for cardiopulmonary bypass either for coronary artery bypass grafting (CABG) or valve replacement. Once the cardiac surgeon provided the atrial tissue, samples were

placed in ice-cold, oxygenated modified Tyrode's buffer (NaCl 118.5 mM, KCl 4.8 mM, NaHCO$_3$ 24.8 mM, KH$_2$PO$_4$ 1.2 mM, MgSO$_4$.7H$_2$O 1.44 mM, CaCl$_2$.2H$_2$O 1.8 mM, glucose 10.0 mM, pyruvic acid 10 mM, pH 7.4) and transferred promptly to The Hatter Cardiovascular Institute.

Right atrial appendage samples were used for both assessing the basal protein expression of PI3Kα and β and undertaking a pharmacologic approach with both PI3Kα activator and inhibitor.

3.9 Analysis of protein levels

3.9.1 Obtaining protein samples from heart tissue and protein quantification

Mouse hearts and human right atrial tissues were obtained according to the relevant protocol and prepared for Western blot analysis. Tissue samples were snap-frozen in liquid nitrogen after their collection and stored at -80ºC until further processing. The tissue was homogenized in protein lysis buffer, containing Tris pH 6.8 [100 mM], NaCl [300 mM], NP40 0.5%, Halt protease inhibitor cocktail, Halt phosphatase inhibitor cocktail, 5 mM EDTA (all from Thermo Scientific, UK) and adjusted to pH 7.4. One mL of lysis buffer per mg of tissue was used to obtain an adequate protein concentration. Homogenates were incubated on ice for at least 15 min and gently vortexed before proceeding to a brief sonication to eventually centrifuge the sample at 4ºC to remove debris and DNA.

Protein content was then determined using bicinchoninic acid (BCA) protein assay reagent (Sigma, UK) and protein levels corrected accordingly to ensure equal protein loading. Briefly, this 96-well plate colorimetric assay uses bicinchoninic acid and copper sulphate at ratio of 50:1. A set of bovine serum albumin (BSA) at known concentrations was used as protein standard samples. Two µl of each sample was then added into 198 µl of BCA/copper sulphate mix per well and incubated for 15 minutes at 37°C. FLUOstar Omega microplate reader (BMG Labtech, UK) was used to measure the read out of the samples. The protein concentrations of the samples were then interpolated by plotting the absorbance results against the protein standard curve. From this, the samples were

accordingly diluted to load the same amount of protein in each gel well. If not specified in subsequent sections, 25 µg was the standard protein load.

NuPAGE LDS Sample Buffer (4X) (Thermofisher Scientific, UK) plus 5% β-mercaptothanol were added and the samples were denatured by heating to 90-100 ºC for 10 min. Prepared samples were either run immediately or stored at – 20 ºC until use.

3.9.2   Western blot analysis

NuPAGE Novex 10% Bis-Tris protein gels (Thermofisher scientific, UK) were used for electrophoresis using the Mini Protean III system (Bio-Rad, UK) filled with NuPAGE™ MOPS SDS running buffer (Thermo Scientific, UK). The first lane of each gel was loaded with 6 µL molecular weight marker (Precision Plus Protein™ Dual Color Standards, from Bio-Rad, UK) and the remaining lanes were loaded with the samples of interest under the same protein concentration. The chamber was surrounded by ice and the gel was run first at 65 V for 10 min, and thereafter at 100 V for 2-3 h.

Using wet transfer in a Bio-Rad Mini Trans-Blot, proteins were transferred either onto Immobilon-FL hydrophobic Polyvinylidene Fluoride (PVDF) transfer membrane (MerckMillipore, UK), or onto nitrocellulose blotting membrane (GE Healthcare Life Sciences, UK). Prior to use, membranes were activated for 2 min with 100% methanol or transfer buffer (containing 25 mM Tris base, 200 mM glycine and 20% methanol) respectively. The transfer was run at 100 V and 0.35 mA for 1h and confirmed visualizing protein ladder transfer.

Membranes were blocked for 1 h by incubating in 2.5% bovine serum albumin/PBS tween with gentle rocking, and subsequently incubated with appropriate primary antibodies at 4 ºC overnight. The antibodies used and their concentrations are outlined in Table 3-4. The day after, the membrane was probed with secondary antibodies at 1:10000 dilution in 50% PBS tween and 50% Odyssey® Blocking Buffer (LI-COR, UK) for 1h with gentle rocking. Usually, green fluorescent antibodies were used for loading protein and the red antibodies for the protein of interest. Levels of protein were finally quantified using the Odyssey imaging system from Li-Cor Biosciences (Image Studio Lite Ver 5.2).

Antibody	Company (reference)	Concentration
Akt	Cell Signaling Technology (#9272)	(1:1000)
Phospho-Akt (Ser473)	Cell Signaling Technology (#9271)	(1:1000)
Phospho-Akt (Thr308)	Cell Signaling Technology (#2965)	(1:1000)
p44/42 MAPK (Erk1/2)	Cell Signaling Technology (#9102)	(1:1000)
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling Technology (#9101)	(1:1000)
GSK-3β	Cell Signaling Technology (#9315)	(1:1000)
Phospho-GSK-3β (Ser9)	Cell Signaling Technology (#5558)	(1:1000)
Stat3	Cell Signaling Technology (#9139)	(1:1000)
Phospho-Stat3 (Tyr705)	Cell Signaling Technology (#9145)	(1:1000)
Phospho-PTEN (Ser380)	Cell Signaling Technology (#9551)	(1:1000)
GAPDH (Loading Control)	Abcam (#9484)	(1:20000)
PI3K p110α	Cell Signaling Technology (#4249)	(1:1000)
PI3K 110β	Santa Cruz (sc-602).	(1:500)

Table 3-4: Summary of antibodies and concentrations used for Western blot analyses

This table presents each of the antibodies used in this thesis, including the company, the catalogue number in brackets and the relevant concentration.

3.10 Assay of mPTP opening

The sensitivity of the mPTP to opening was assayed using a well-characterized cellular model of reactive oxygen species (ROS)-mediated mPTP opening(109). Briefly, tetra-methyl rhodamine methyl ester (TMRM, from S Sigma-Aldrich, UK), a lipophilic cation which is very positively charged, accumulates selectively into the negatively charged mitochondrial matrix. At high concentration (such as 12 µM), TMRM becomes quenched at the mitochondria. Constant confocal laser stimulation (at 543 nm wavelength) of TMRM generates ROS within the mitochondria thereby simulating mitochondrial ROS production during reperfusion. After few minutes of continual confocal laser scanning, ROS induces mPTP opening, producing a drop in mitochondrial membrane potential and resulting in the dequenching of TMRM, which in turn relocates to the cytoplasm (254). This leak of the dye from the mitochondria to the cytosol increases the detectable fluorescent signal, which is used as surrogate marker for mPTP opening.

Adult mouse ventricular cardiomyocytes were isolated as previously described in section 3.6. Live cardiomyocytes were incubated with the fluorescent dye TMRM at 12 µM for 15 min in Hepes based recording buffer (NaCl156 mM, KCl 3 mM, $MgSO_4.7H_2O$ 2 mM, K_2HPO_4 1.25 mM, $CaCl_2$ 2 mM, HEPES 10 mM and D-Glucose 10 mM; pH 7.4), then washed and randomly treated for 15 min with the relevant intervention (see Chapter 10). Once washed for second time, mouse cardiomyocytes were stimulated with laser illumination and imaged using confocal microscopy. The time to reach half peak signal was recorded in seconds and compared across groups.

3.11 Design and statistical analysis of experiments

All research was performed and recorded according to main guidelines and other recommendations (255,256). Briefly, the objectives and/or hypotheses to be tested were always pre-specified. If possible, positive and negative controls were added to each set of experiments (also respecting the principles of the 3Rs: Replacement, Reduction and Refinement). Sample calculations for those experiments aimed to measure myocardial IS or mPTP opening were estimated beforehand and reported accordingly in each relevant

chapter, whilst the sample size for Western blot studies were left in line with convention (n=5/group for most of the experiments). Statistical methods were pre-specified, ensuring compliance with the original design and statistical plan (257). All image assessment were performed blinded when possible. All results were reported according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, which consists of a checklist of 20 items describing the minimum information that all scientific publications reporting research using animals should include (258).

Data is presented as mean ± standard error of the mean (SEM). The statistical test used to analyse the results of a given experiment is reported in the relevant chapter. Normal distribution of the data was tested for each dataset. N values are either displayed in the figure or described in the figure legend for each experiment. Statistical significance was reported if $P<0.05$ using the following nomenclature: $*P<0.05$, $**P<0.01$ and $***P<0.001$. A $P>0.05$ was reported as non-significant (ns).

IBM SPSS Statistics software, v.20.0 (IBM Corp Armonk, New York), STATA software, version 13.1 (Stata Corp, College Station, Texas) and GraphPad Prism version 6.00 (GraphPad Software, La Jolla California) were used to perform both the analyses and the graphics.

Chapter 4   CHARACTERTERISATION OF THE ISOLATED PERFUSED MOUSE HEART MODEL OF GLOBAL ISCHAEMIA-REPERFUSION INJURY

Most of the content of this chapter has been already published as a single piece of research:

Rossello X, Hall AR, Bell RM, Yellon DM. Characterization of the Langendorff Perfused Isolated Mouse Heart Model of Global Ischaemia–Reperfusion Injury: Impact of Ischaemia and Reperfusion Length on Infarct Size and LDH Release J Cardiovasc Pharmacol Ther. 2016;21: 286-295

4.1   Introduction

The Langendorff isolated perfused mouse heart is an invaluable tool in cardiovascular research, particularly for assessing the cardioprotective efficacy of novel therapies (259). Though technically challenging, the mouse Langendorff model has become particularly popular in the last decades due to the availability of transgenic mice either overexpressing or disrupting specific gene products, and therefore providing a powerful tool to elucidate the role of signalling cascades involved in IRI.

Thorough characterization and validation of this ex-vivo model of IRI is an essential but frequently neglected step prior to important conclusions being drawn from the data they generate. Although the *ex vivo* model has excellent inter-operator reproducibility provided consistent ischaemia and reperfusion times are applied (259,260), it is worth noting that different lengths of the ischaemia and reperfusion periods vary considerably between different laboratories (261–263). Such discrepancies emphasise the need to better characterise the perfused murine model, even more considering that such methodological details have rarely been published (244,251).

Although the impact of the ischaemia duration on final IS is well-described, little is known about the optimal duration of the reperfusion period. A small number of studies have attempted to characterise the effects of ischaemia and reperfusion length as

independent variables in the isolated mouse heart model(251,264,265), concentrating on either varying the length of ischaemia or the length of reperfusion. Even fewer studies have monitored the injury wavefront by measuring the release of surrogate biomarkers, such as LDH, which compared to infarct staining, offers the advantage of tracking the injury along the time line within the same heart.

In order to study the IRI associated with acute myocardial infarction, a reproducible model of injury and measurement is required. This chapter focuses upon the characterization of the *ex vivo* Langendorff-perfused mouse heart model of global IRI to select a suitable protocol upon which cement further investigations within this thesis.

4.2   Research objectives and aims

Can myocardial infarct size be modified by both ischaemia and reperfusion length? Is LDH an adequate surrogate for myocardial infarct size?

<u>**Hypothesis**</u>

Myocardial infarct size will be larger with the increase in both the duration of the ischaemic insult and its subsequent reperfusion. LDH will be correlated with infarct size, adding the possibility to monitor the wave of myocardial injury in an isolated perfused mouse heart model of global ischaemia-reperfusion injury

<u>**Experimental aims**</u>

The overall aim was to select a protocol of ischaemia/reperfusion for subsequent experiments related to the thesis. In order to provide a step-by-step description of the Langendorff perfused isolated mouse heart model, a factorial experimental design using two endpoints was developed with the following aims:

<u>*Aim 1:*</u> *Determine whether infarct size is dependent on both the duration of ischaemia and length of the reperfusion period*

- Subject mouse Langendorff to ischaemia-reperfusion and subsequently quantify myocardial infarct size.

Aim 2: *Describe the lactate dehydrogenase release pattern during reperfusion as an index of injury*

- Collect coronary effluent samples from mouse hearts subjected to 9 protocols of IRI and measure LDH concentration using a commercially available assay kit.

Aim 3: *Describe the impact of Ischaemic preconditioning on IS and lactate dehydrogenase release after long reperfusion*

- Collect coronary effluent samples from mouse hearts subjected to IPC 4 cycles in 3 different protocols of IRI and measure LDH concentration using a commercially available assay kit.

Aim 4: *Determine any correlation between IS and lactate dehydrogenase release*

- Plot myocardial infarct size results against area under the curve (AUC) LDH release using the appropriate statistical test.

4.3   Methods and Materials

4.3.1   Study protocol

A total of 65 male C57BL/6 mice (9-12 weeks, 24–28 g weight) were used, although 8 were excluded before randomization as they were outside pre-defined exclusion criteria. Before starting the study protocol, 57 animals were allocated according to a randomized complete block design.

The study was divided into three consecutive parts, as illustrated in Figure 4-1:

1) Effect of ischaemia and reperfusion length on IS (Figure 4-1A). Thirty-six mice were divided into 9 groups in a 3x3 factorial design, including three categories of ischaemic length (25, 35 and 45 minutes) and three periods of reperfusion length (60, 120 and 180 minutes);each group containing 4 mice. LDH release during reperfusion were measured in coronary effluent aliquots sampled at selected time points in mice that underwent 180 min of reperfusion (n=12), in order to determine the release pattern, AUC and peak of LDH.

2) Effect of ischaemia length on IS and release kinetics of LDH on preconditioned hearts (Figure 4-1B). Twelve mice were subjected to ischaemic IPC at different ischaemic periods lasting for 25, 35 or 45 minutes (n=4 per group). IPC consisted of 4 cycles of 5 minutes of global ischaemia and 5 minutes of reperfusion prior to index the ischaemia.

3) IS and LDH assessment in negative and positive controls (Figure 4-1C). A negative control (n=3) was performed in order to assess the final IS and the surrogate LDH release under no ischaemic insult and maximum length of reperfusion (180 minutes). Two positive controls were allocated into two groups (60 and 180 minutes of reperfusion, n=3 per group), in order to assess the effect of reperfusion length on final IS after a maximal prolonged ischaemic insult (90 minutes), designed to mediate 100% IS and maximal LDH release.

The sample size for groups 1 and 2 was determined for IS as primary outcome (expressed as a percentage of the global myocardium at risk), based on previous results from our laboratory (266,267).
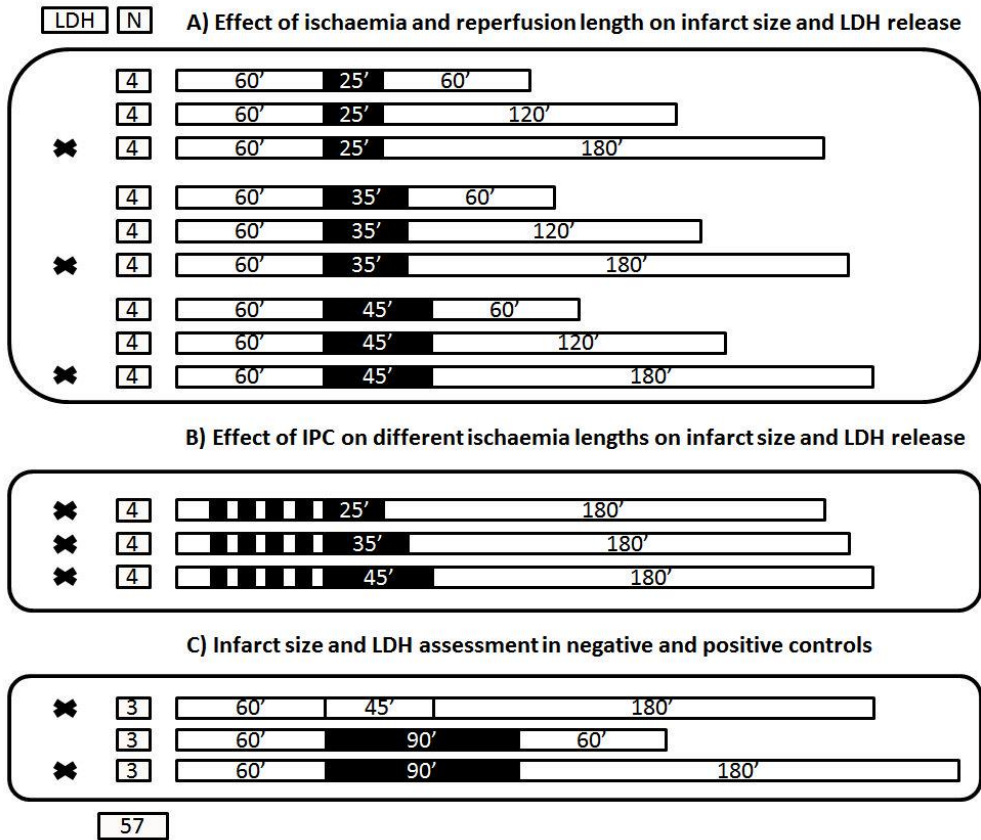
Figure 4-1: Study design

Overview of the study including subgroups and experimental protocols. The black cross indicates that LDH samples were collected. A black box represents a period of ischaemia and a white box represents a period of perfusion with Krebs-Henseleit buffer at 80 mm Hg. Abbreviations: IPC, ischaemic preconditioning; LDH, lactate dehydrogenase.

4.3.2 Langendorff-isolated perfused mouse heart

Hearts were retrogradely perfused on a murine Langendorff perfusion apparatus at 80 mmHg pressure with filtered modified Krebs-Henseleit buffer (see section 0 in Methods for further details). After observing for exclusion criteria in an initial stabilization period of 20 min, the hearts were subjected to the experimental protocols, as illustrated in Figure 4-1.

4.3.3 Myocardial infarct size

After global normothermic ischaemia, IS was determined by injecting 5 mL of TTC in phosphate buffered saline (see Methods section 3.3 for further details).

4.3.4 Release of LDH

LDH was measured for dynamic monitoring of cellular damage with the experiments lasting 180 minutes of reperfusion. Coronary effluent samples were collected at 5, 10 and 15 minutes from start of reperfusion, and then every 15 minutes until minute 180. A total of 14 samples were collected per heart. The flow rate was measured each time a LDH sample was collected. The measured concentration was corrected for coronary flow and heart weight, according to previous studies (244,248,251). The

Table 4-1 provide the raw data on the basis of which the adjusted LDH was calculated. Further details can be found in Methods, section 3.4.

Table 4-1: LDH concentration, flow rate and weight in mice that underwent 180 minutes of reperfusion

	25 minutes (I)				35 minutes (I)				45 minutes (I)				Controls			
	Control (N=4)		IPC (N=4)		Control (N=4)		IPC (N=4)		Control (N=4)		IPC (N=4)		0 minutes (N=3) (I)		90 minutes (N=3) (I)	
Weight	0.13±0.01		0.12±0.00		0.14±0.01		0.13±0.00		0.14±0.01		0.13±0.01		0.11±0.00		0.16±0.00	
Time	LDH	FR	LDH	FR	LDH	FR	LDH	FR	LDH	FR	LDH	FR	LDH	FR	LDH	FR
5	1.0±0.22	2.0±0.38	0.5±0.09	2.9±0.18	1.0±0.32	1.7±0.20	1.0±0.21	2.2±0.24	1.3±0.15	1.8±0.11	0.7±0.19	2.1±0.19	0.0±0.00	2.7±0.20	6.9±0.73	0.4±0.13
10	1.0±0.18	1.8±0.23	0.5±0.14	2.9±0.30	1.3±0.39	1.6±0.23	0.9±0.15	2.0±0.28	2.0±0.28	1.6±0.16	1.0±0.23	2.0±0.20	0.0±0.00	3.4±0.30	6.0±1.12	0.5±0.16
15	1.2±0.15	1.6±0.24	0.6±0.27	2.2±0.16	1.4±0.38	1.5±0.22	0.9±0.12	1.9±0.24	2.4±0.42	1.5±0.14	1.0±0.19	1.8±0.19	0.0±0.00	2.6±0.37	5.3±1.28	0.5±0.14
30	1.3±0.30	1.5±0.15	0.6±0.36	1.9±0.17	1.4±0.27	1.5±0.24	0.9±0.09	1.8±0.18	2.0±0.46	1.5±0.14	1.1±0.14	1.7±0.23	0.0±0.00	1.8±0.68	3.4±0.98	0.5±0.17
45	0.8±0.27	1.4±0.11	0.4±0.23	1.8±0.13	0.9±0.12	1.5±0.19	0.8±0.16	1.7±0.16	1.0±0.21	1.6±0.15	0.7±0.04	1.7±0.24	0.0±0.00	1.6±0.57	2.5±0.97	0.6±0.17
60	0.9±0.26	1.6±0.18	0.2±0.12	1.7±0.12	0.5±0.05	1.4±0.17	0.6±0.12	1.6±0.15	0.5±0.07	1.5±0.16	0.3±0.02	1.6±0.26	0.0±0.00	1.1±0.14	1.8±0.71	0.6±0.18
75	0.6±0.21	1.5±0.11	0.3±0.30	1.6±0.12	0.3±0.07	1.4±0.14	0.4±0.10	1.4±0.15	0.4±0.05	1.5±0.16	0.2±0.03	1.6±0.20	0.0±0.00	1.0±0.12	1.1±0.36	0.7±0.16
90	0.4±0.18	1.2±0.05	0.2±0.12	1.3±0.06	0.3±0.08	1.3±0.14	0.3±0.10	1.3±0.16	0.3±0.04	1.4±0.22	0.2±0.04	1.5±0.18	0.0±0.00	1.1±0.23	1.0±0.35	0.7±0.17
105	0.3±0.15	1.2±0.05	0.1±0.05	1.3±0.06	0.3±0.07	1.2±0.13	0.3±0.12	1.3±0.15	0.3±0.05	1.4±0.20	0.2±0.05	1.4±0.20	0.0±0.00	0.9±0.24	0.8±0.26	0.7±0.18

	25 minutes (I)				35 minutes (I)				45 minutes (I)				Controls			
	Control (N=4)		IPC (N=4)		Control (N=4)		IPC (N=4)		Control (N=4)		IPC (N=4)		0 minutes (N=3) (I)		90 minutes (N=3) (I)	
Weight	0.13±0.01		0.12±0.00		0.14±0.01		0.13±0.00		0.14±0.01		0.13±0.01		0.11±0.00		0.16±0.00	
Time	LDH	FR	LDH	FR	LDH	FR	LDH	FR	LDH	FR	LDH	FR	LDH	FR	LDH	FR
135	0.3±0.15	1.0±0.07	0.0±0.03	1.2±0.11	0.2±0.11	1.1±0.07	0.1±0.04	1.0±0.13	0.7±0.37	1.3±0.17	0.2±0.06	1.2±0.15	0.0±0.00	0.9±0.06	0.5±0.16	0.8±0.18
150	0.2±0.10	1.0±0.04	0.0±0.02	1.1±0.13	0.2±0.12	1.0±0.08	0.1±0.03	1.1±0.17	0.4±0.11	1.2±0.15	0.2±0.05	1.4±0.20	0.0±0.00	0.9±0.08	0.5±0.13	0.8±0.15
165	0.2±0.08	0.9±0.06	0.0±0.01	0.9±0.02	0.2±0.13	1.0±0.06	0.0±0.02	1.0±0.11	0.3±0.05	1.2±0.15	0.1±0.04	1.1±0.17	0.0±0.00	1.7±0.75	0.4±0.12	0.8±0.15
180	0.1±0.07	0.9±0.05	0.0±0.02	0.9±0.12	0.2±0.13	1.0±0.08	0.0±0.01	1.0±0.15	0.3±0.03	1.2±0.16	0.1±0.04	1.3±0.20	0.0±0.00	0.9±0.10	0.4±0.11	0.8±0.16

The LDH concentration was corrected for coronary flow and heart weight, according to previous studies(244,248,251). Thus, it was calculated as the product of effluent concentrations (pg x mL^{-1}) x coronary flow (ml x min^{-1} x g^{-1}). This table provide the crude data on the basis of which the adjusted LDH was calculated.

Units: weight (g), time (min), LDH concentration (pg x mL^{-1}) and flow rate (ml x min^{-1}). Abbreviations: I, ischaemia; IPC, ischaemia preconditioning; LDH, lactate dehydrogense; FR, flow rate.

4.3.5   Factorial study design

A complete factorial design was used in order to assess the effect of two factors (the independent variables), known as ischaemia and reperfusion length and each one represented by three equally spaced categories (25, 35 and 45 min, and 60, 120 and 180 min, respectively), on the final IS measured by TTC staining (dependant variable). The observations were summarized in a two-way table (Table 4-2), where each entry is the mean of all observations and all combination of categories are represented equally (n=4). This harmonic design is useful to estimate the following paramenters (268):

1) Estimate the separate effects on IS between different categories of one of the factors, but within the same category of the other factor, i.e. – to compare the IS between different ischaemia length within the 60 minutes of reperfusion length. These effects are represented by the pale grey boxes in the Table 4-2.

2) Estimate the main effect of each one of the factors, represented by the row and column averages shown in the margin of the Table 4-2 (highlighted in dark grey). The main effect of ischaemia (averaged over the three categories of reperfusion) was defined as the difference that can be observed with the row averages at the right of the Table 4-2, and vice versa with reperfusion (column averages at the bottom). In other words, it was estimated whether the effects of the two factors are additive and they are interacting independently of one another.

3) Calculate the interaction between both factors to be explored. The two-factor interaction *ischaemia x reperfusion* determines whether the effect of ischaemia is the same for all categories of reperfusion, that is whether the change of increasing ischaemia length is constant along the different reperfusion durations, and vice versa. Of note, the pre-specified sample was underpowered for this purpose.

4) Estimate the overall significance of a model (black box of the Table 4-2), formed by the two main effects and their interaction. The overall model is a simple equation, summarized as:

IS = (β_1) Ischaemia + (β_2) Reperfusion + (β_3) (*Ischaemia x Reperfusion)* + error

The final output of the equation is the amount of IS determined by the parameters included into the equation. Although this might seem very attractive, the extrapolation of such results should be taken cautiously, since only ischaemia duration and reperfusion length were considered as determinants of IS, whilst in the clinical setting other factors play an important role – i.e. presence of collaterals.

To summarise, although the degree of symmetry of our design offers a hierarchical model of multiple comparisons, the aim of this study was to assess the separate and main effects rather than the interaction effect and the model as a whole.

4.3.6   Statistical analyses

All values are presented at mean ± standard error of the mean.   Normal distribution was assessed by means of the Kolmogorov-Smirnov test. Linear trend test was used for comparisons on IS between groups (%), as well as to compare AUC and peak of LDH between groups. The interaction and the overall effect of the model were assessed by means of a general linear model. The Student's t test was used to compare IS (%) between control and IPC groups in the cohort of 180 minutes of reperfusion. Association between IS and peak or AUC LDH were analysed by the Pearson correlation coefficient test. A P-value <0.05 was considered significant. IBM SPSS Statistics software, v.20.0 (IBM Corp. Armonk, NY) was used for the main statistical analysis. STATA software, version 13.1 (Stata Corp, College Station, TX, USA) was used to generate the randomization sequence and to calculate and compare the AUC between groups. GraphPad Prism version 6.00 (GraphPad Software, La Jolla California, USA) was used to perform the graphics.

4.4 Results

4.4.1 Effect of ischaemia and reperfusion length on IS measured by TTC staining

All the results obtained by the 3x3 factorial study are outlined in Table 4-2.
In summary:

1) The impact of ischaemic and reperfusion duration, as illustrated by the pale grey entries in the Table 4-2, show that there is a positive trend towards an increased IS when the ischaemia and reperfusion lengths are progressively prolonged. Table 4-2 reflects the consistency of the results in each of the six comparisons, showing how IS increased from the top-left of the Table 4-2 to the bottom-right. Figure 4-2 graphically illustrates the same trend.

2) The main effect of each one of the factors is represented by the row and column averages shown in the margin of the Table 4-2 (highlighted in dark grey). The length of ischaemia has an impact on the final IS independently of the length of reperfusion. In the same way, the length of reperfusion has also a systematic impact on the final IS in each of the ischaemia duration groups. In other words, IS increases significantly with the duration of ischaemia, irrespective of reperfusion length, and vice versa.

3) No interaction was observed between ischaemia and reperfusion (p=0.773), meaning that the effect of ischaemia and reperfusion lengths have an increasing but proportional impact in all categories as the length is increased, although the observed power to assess this interaction was poor (13.8%).

4) Finally, the overall model, including ischaemia length, reperfusion duration and their interaction significantly explains the dependent variable of the equation, which is myocardial IS (p=0.001).

	Reperfusion length (min)				
	60'	120'	180'	Row average	P-value
25'	10.4 ± 4.4	18.7 ± 3.9	25.2 ± 4.9	18.1 ± 2.9	0.041
35'	18.7 ± 4.0	36.1 ± 7.2	44.9 ± 5.2	33.2 ± 4.4	0.009
45'	31.6 ± 6.0	49.7 ± 7.6	58.7 ± 3.6	46.7 ± 4.6	0.011
Column average	20.2 ± 3.7	34.8 ± 5.1	42.9 ± 4.8	32.7± 3.0	0.001
P-value	0.013	0.029	0.001	<0.001	0.001

(Row label, vertical: Ischaemia length (min))

Table 4-2: Effect of ischaemia and reperfusion length on myocardial infarct size (%)

Each of the relevant numbers in the reperfusion to ischaemia two-way table corresponds to the mean percentage IS of 4 animals. Data is expressed as mean ± standard error of the mean. The pale grey entries illustrates the separate effects, while the dark grey represents the main effects and the black colour illustrates the mean IS of all the animals and the P-value of the overall model.
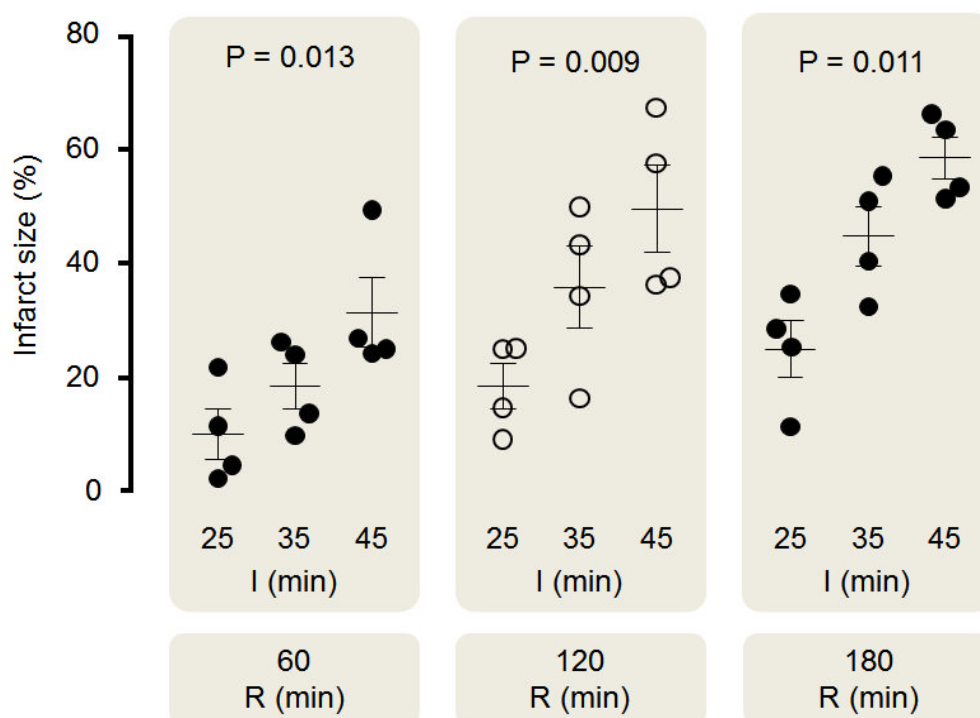
Figure 4-2: Effect of ischaemia and reperfusion length on myocardial infarct size (%)

Scatter dot blots: black lines represent mean ± SEM for infarct size (%) and circles represent individual animal data. Infarct size consistently increased with increasing durations of ischaemia and reperfusion in control hearts. Abbreviations: I, ischaemia; R, reperfusion.

4.4.2   LDH release after 180 minutes of reperfusion

LDH release during reperfusion was measured in mice that underwent 180 minutes of reperfusion (n=12). Figure 4-3 shows the LDH pattern release for each group of ischaemia length, as well as the AUC and the peak comparison between groups.

A progressive increase of LDH occurred during the first 10-15 minutes of reperfusion, followed by a progressive decline after reaching this peak. A consistent second LDH peak was observed at 60 minutes of reperfusion but only in the group of 25 minutes of ischaemia. At 90 minutes of reperfusion, a plateau release phase was observed in all groups.

Unlike IS measured by TTC staining, the AUC of the LDH did not differ significantly between groups. Nevertheless, the peak of the LDH was associated with the ischaemia length, meaning the greater the ischaemic duration, the greater the peak of LDH release.

Figure 4-3: Lactate dehydrogenase release during reperfusion in hearts under 180 minutes of reperfusion

Comparison between LDH release in hearts underwent different ischaemia lengths and 180 minutes of reperfusion. Each point represents four animals and is plotted with their mean and SEM. AUC and peak between groups were compared by mean of a linear trend test. Abbreviations: AUC, area under the curve, LDH: lactate dehydrogenase.

4.4.3 Effect of IPC on IS and release kinetics of LDH

Twelve mice were subjected to IPC before different ischaemic periods lasting for 25, 35 or 45 minutes (n=4 per group), all of them underwent 180 minutes of reperfusion. Ischaemic preconditioning (4 cycles of 5 min of ischaemia and 5 min of reperfusion) resulted in reduction of infarct size in all ischaemic duration groups: 25 minute ischaemia (25.2±4.90 vs 11.9±1.99, p=0.045); 35 minute ischaemia (44.9±5.15 vs 25.6±6.91, p=0.066) and 45 minute ischaemia (56.7±3.63 vs 38.5±3.09, p=0.006) (Figure 4-4).

Pattern of LDH release, AUC and peak of LDH were compared in control and IPC hearts that underwent 180 minutes of reperfusion, as shown in Figure 4-5. Noteworthy, IPC significantly attenuated the AUC in the 25 minutes index ischaemia group, whilst in this group the peak showed a reduction towards significance in the IPC group compared to control. AUC and peak of LDH release were not different in statistical terms after IPC in both groups of 35 and 45 minutes of ischaemia.

4.4.4 Correlation analysis between LDH assessment with myocardial IS

In all mice that underwent 180 minutes of reperfusion (n=24), a correlation was used in order to evaluate the strength of the association between the IS measured by TTC staining and the AUC and peak of LDH.

Figure 4-6 shows the correlation between the AUC of LDH release and myocardial IS. Although the association was statistically significant (R = 0.664, p<0.001), the strength of the correlation was moderate. The relation between the LDH peak and myocardial IS was even weaker (r = 0.547, p=0.006).
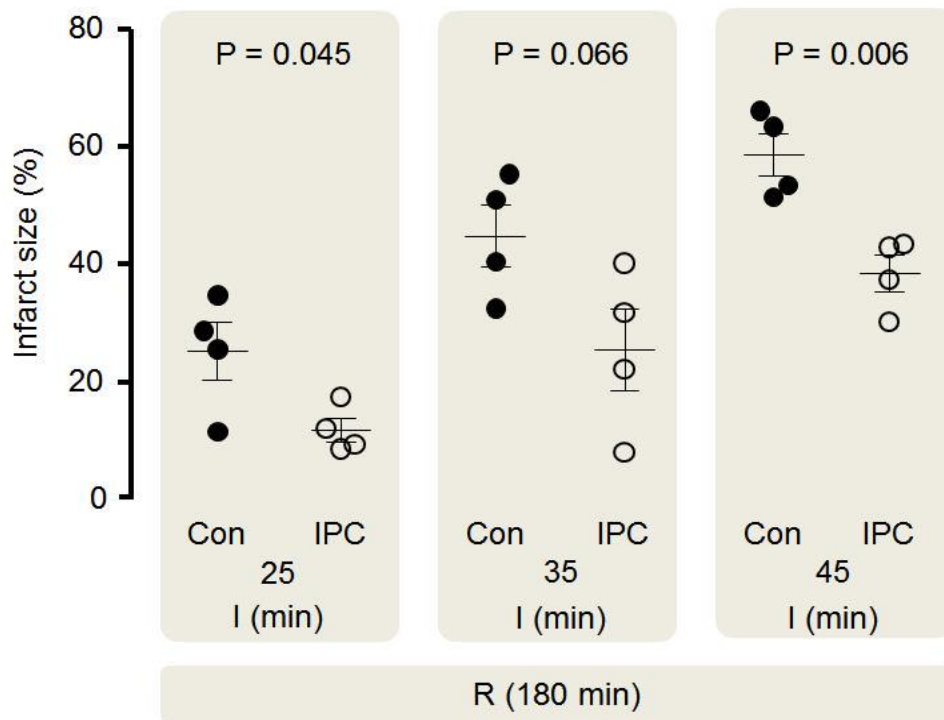
Figure 4-4: Effect of ischaemia length and ischaemic preconditioning on myocardial infarct size

IS was increased with increasing durations of ischaemia in both control and preconditioned hearts, although IPC systematically showed a lower IS at all ischaemia lengths. Abbreviations: I, ischaemia; IPC, ischaemic preconditioning, R, reperfusion.
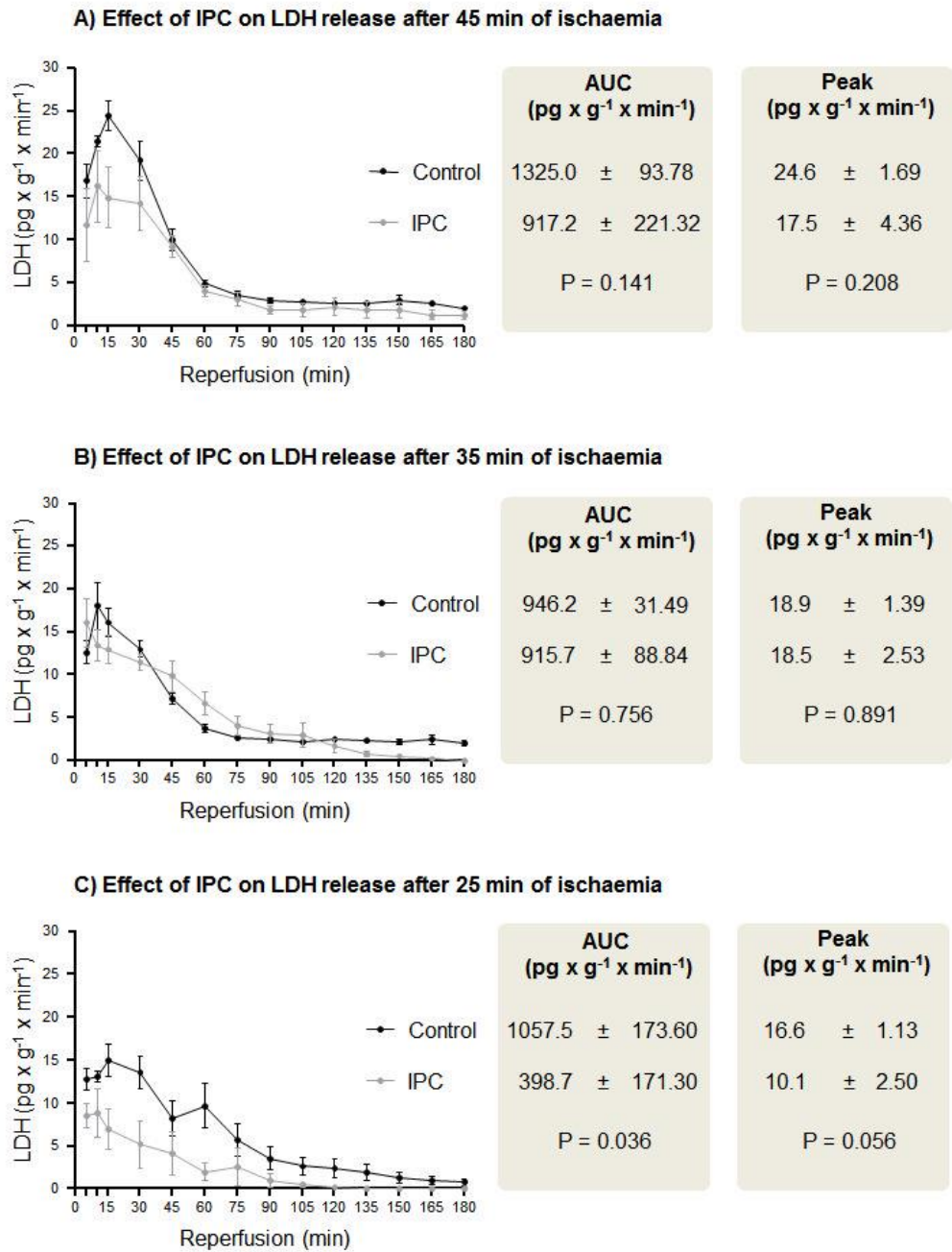
Figure 4-5: Lactate dehydrogenase release during 180 minutes of reperfusion in control and IPC hearts

Comparison between LDH release in control and preconditioned hearts, allocated in different groups of ischaemia lengths. Each point represents four animals and is plotted with their mean and SEM. AUC and peak between groups were compared by a t test in each group. Abbreviations: AUC, area under the curve; IPC, ischaemic preconditioning; LDH, lactate dehydrogenase.

A) Correlation analysis between the AUC of LDH and infarct size

R = 0.664   P < 0.001
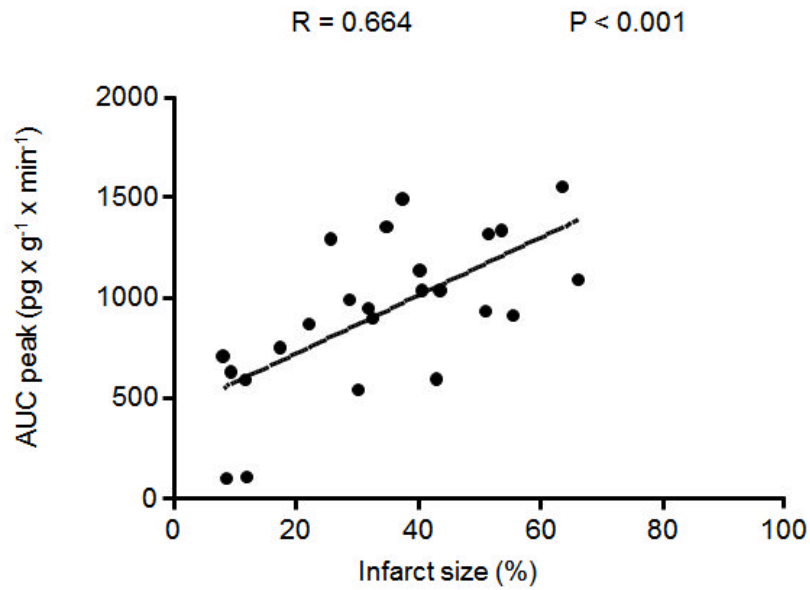
B) Correlation analysis between the peak of LDH and infarct size

R = 0.547   P = 0.006
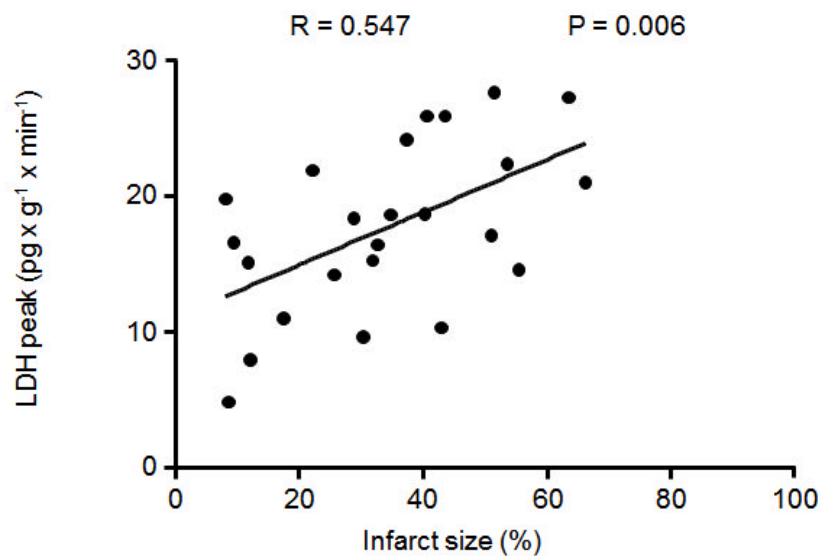
Figure 4-6: Correlation analysis between LDH AUC and peak with myocardial infarct size

The scatter plot (n=24) shows a positive correlation between the AUC of LDH release and myocardial IS while a weaker association was found between LDH peak and IS. Abbreviations: AUC, area under the curve; LDH, lactate dehydrogenase.

4.4.5 IS and LDH assessment in negative and positive controls

Negative controls without index ischaemia (n=3), but after 4 hours and 45 minutes of Langendorff perfusion, presented an IS of 3.1 ± 0.51 %, with a small and constant release of LDH (AUC of 12.67±0.67 and peak of 0.5 ±0.16 pg x g^{-1} x min^{-1}). These data are consistent with the anticipated slow deterioration of an isolated-perfused organ system, but demonstrate that perfusion alone does not represent a significant contribution to the final infarct size.

Positive controls that underwent 90 minutes of index ischaemia were allocated into two groups: mice under 60 minutes of reperfusion presented an IS of 58.9 ± 3.85 %, whilst in mice with 180 minutes of reperfusion IS was 92.6 ± 1.59%. The post-ischaemic enzyme leakage calculated in these mice was lower than any of the 25, 35 or 45 minutes of ischaemia index control groups (AUC=938.3±37.26; peak 16.7 ± 2.80 pg x g^{-1} x min^{-1}). Despite the absolute LDH release concentration being relatively high (see

Table 4-1), the coronary flow was further impaired, affecting the final result of the concentration adjusted by the coronary flow.

Perhaps somewhat paradoxically, post-ischaemic AUC enzyme leakage was not increased in the positive control, after 90 minutes of ischaemia. This might be explained as an artefact of the formula to calculate the LDH concentration: the longer the ischaemic duration, the lower the coronary flow rate during reperfusion. This was particularly prominent following 90 minutes of ischaemia, raising the possibility that LDH wash-out might be impaired following 90 minutes of infarction and the formula to adjust the LDH was highly affected by this denominator, reflecting an underestimated value when the flow rate is extremely low.

4.5   Discussion

4.5.1   Main findings

This chapter describes important methodological aspects of the mouse *ex vivo* model of IRI and provide evidence that:

1) The percentage of myocardial IS measured by TTC staining depends both on duration of ischaemia and reperfusion lengths.

2) After 180 minutes of reperfusion, there was no difference in the LDH release pattern or the AUC, although the peak of LDH was significantly different between groups.

3) After 180 minutes of ischaemia, IPC showed protection at different ischaemic lengths compared to control hearts in terms of IS, although there were no differences in the LDH release pattern. Only the 25 minute preconditioned hearts showed a reduction in the AUC and peak compared to their controls.

4) AUC and peak of LDH have a positive correlation with myocardial IS, though it was not strong enough to be used as an accurate surrogate biomarker.

4.5.2   Effect of ischaemia and reperfusion length on IS measured by TTC staining

Using a 3x3 factorial design, this systematic approach shows a mutual impact of ischaemia and reperfusion length on myocardial IS in mice *ex vivo*. Only one previous study used a similar factorial design in order to study the interdependency of ischaemia and reperfusion in an *in vivo* mouse model (269). Compared to the one factor at a time approach of previous studies (248,251,265,270), the factorial design presents the advantage of streamlining the analysis of the impact of each different periods of ischaemia and reperfusion, as the results can be interpreted from two separate perspectives.

Myocardial infarct size, measured by TTC staining, is independently associated with the durations of both ischaemia and reperfusion. The fact that increasing the duration of index ischaemia increases the IS is well known and it is considered the main determinant of the IS in the clinical setting (6,10,25,28). More controversial is the observation that

myocardial IS increases with prolonged periods of reperfusion. Some authors reported that 1 hour of reperfusion in a rat model was sufficient to demarcate infarct zones, with longer periods of reperfusion not adding further damage (270,271). Nevertheless, the majority of studies in different species published previously are in accordance with our finding (248,272). Of note, a plateau of IS was not reached, suggesting that even 180 minutes was not the maximum infarct size achievable. However, it should also be noted that other limitations are present with longer reperfusion periods in the Langendorff model, as the fate of the *ex vivo* heart is inevitable to die in the short-term.

At this point, a discussion about the optimal duration of ischaemia and reperfusion in experimental protocols is needed. Our results in Table 4-2 show consistent differences between protocols, which appear to be linear and quite equidistant, suggesting that any ischaemia/reperfusion duration protocol can be appropriate dependent upon the hypotheses one wishes to interrogate. To mimic the clinical scenario, where infarcts are typically comparatively small (around 20-30% of total left ventricular mass (178)), the use of an experimental infarction protocol that results in an equivalent injury might be appropriate to test an cardioprotective therapy that has already been experimentally demonstrated, while a protocol leading to larger infarcts, with a greater chance to reduce myocardial IS, might be useful to test a completely novel therapy in the experimental setting. Nevertheless, within 180 minutes of reperfusion, IPC was shown to be cardioprotective in both 25 and 45 minutes of ischaemia, and was shown also a trend towards significance in the 35 minutes of ischaemia, which might become significant if the sample size was increased. The demonstration of the effectiveness of IPC in the experimental groups tested means that it is possible to apply different index ischaemic protocols to the cardioprotective intervention being investigated, although Murry *et al.* demonstrated that IPC effectiveness is lost after too prolonged ischaemia in the *in vivo* canine model (33).

This systematic approach was not designed to establish the presence of an interaction between ischaemia and reperfusion and the results cannot be interpreted in this way since the observed power is too small. However, no extensive interaction is suspected since the absolute change in IS between different ischaemia and reperfusion lengths are quite parallel and constant.

4.5.3 LDH release after 180 minutes of reperfusion

The enhanced leakage of LDH following IRI was evaluated, showing the pattern, AUC and peak after different ischaemia lengths and following IPC.

The pattern of the LDH release presented an early peak at 10-15 minutes of reperfusion, followed by a progressive declining release until a plateau phase is reached at 90 minutes of reperfusion. This result contradicts a recent study by Botker's group showing a biphasic pattern of LDH release after ischaemia in isolated, perfused rat hearts, with the second phase modifiable by IPC (248). Despite the apparent disagreement between their observation (in rats) and the findings being presented in this chapter (in mice), Botker's group confirmed to us (personal communication, unpublished data) that they obtained similar results in the 35 and 45 minute index ischaemia in their studies with mice. We might speculate that this difference may purely be a species effect with the mouse having less coronary flow than the rat, therefore the increased wash-out period of LDH might blunt the second smaller peak of LDH release. In contrast to the monophasic pattern of LDH following either 35 or 45 minutes of ischaemia, a consistent bi-phasic LDH release pattern was observed following 25 minutes of global ischaemia in mouse, observing the second phase of release at 60 min reperfusion. Therefore it appears that the biphasic LDH release pattern, seen so clearly in the rat, is only observed in mouse heart following less injurious ischaemic insult durations. Why this might be the case is unclear, but may be an important observation when considering the use of AUC LDH as a surrogate for histological-based cell death assays.

In contrast to the AUC of LDH release, the peak of the LDH release curve was associated with the duration of ischaemia. The optimum sampling time point for peak LDH release in the evaluation of myocardial IRI has recently been proposed to be 60 minutes (270). In accordance, the LDH release time-course presented in this chapter revealed a major leakage occurring predominantly between 60-90 minutes in both controls and IPC treated hearts.

Curiously, despite IPC significantly attenuating IS as determined by TTC, no significant reduction of either AUC or peak of LDH following more prolonged (35 or 45 min) ischaemic lengths was found. Only following the less injurious 25 minute ischaemic insult a

significant attenuation of LDH AUC with IPC was observed, whilst the LDH peak fell just short of statistical significance. These observations are again consistent with previous reports demonstrating that enzyme efflux tends to match IS outcome following short ischaemic periods, but not following more injurious ischaemic insults (251).

4.5.4 LDH assessment and myocardial IS correlation – Is LDH a good surrogate marker of IS?

Development of models with various end-points of injury may result in a more comprehensive characterization of the myocardial insult, enabling a multi-endpoint approach. Tetrazolium staining is the most popular method to assess IS, and consequently to test the efficacy of cardioprotective therapies. Other surrogate end-points have been used – i.e. enzyme release, post ischaemic function recovery and arrhythmias assessment.

In this chapter, the LDH AUC demonstrated a better correlation than the LDH peak as a surrogate of myocardial IS, but only in experiments with a less injurious ischaemic duration. Moreover, the strength of the correlation was not enough to be comparable to an unequivocal endpoint such TTC staining, making LDH assessment unsuitable to be used as a biomarker to test cardioprotective therapies. From a technical point of view, the moderate correlation between IS and LDH AUC or peak could be explained as: 1) The considerable variability and large standard errors in absolute LDH concentration; 2) The dependence on coronary flow effluent; 3) The fact that LDH release occurs mostly within the first 90 min of reperfusion, making subsequent sample collection largely superfluous.

In my hands, IS was proportional to the duration of reperfusion. These data suggest that there is either evidence of on-going and accumulative "wave of reperfusion injury" in this model, or more likely, there is a problem with the methods being used to determine the infarct size. Given that the release of LDH appears to have largely abated after 90 minutes of reperfusion supports the latter explanation: TTC staining relies on the ability of dehydrogenase enzymes and cofactors in the tissue to react with tetrazolium salts to form a formazan pigment (245) and needs a sufficient period of wash-out. Following an ischaemic insult of 90 minutes, our results show a greater IS after 180 minutes of reperfusion length, compared to the IS after 60 minutes of reperfusion, suggesting that the

positively stained tissue is probably not healthy and may die later or that the wash-out did not take place yet or was not sufficient and as such gave a false positive result, although a "no-reflow" phenomenon cannot be excluded. In agreement with the literature (272), the longer the reperfusion period after an ischaemic insult, the more reliable the method becomes for discriminating between dead and viable tissue.

4.5.5   Study limitations

An isolated unloaded, ventricular empty mice heart model was used. Whether the same release pattern applied to other animal species and other models is unknown. The high heart rate of the mouse should be taken into account because it might have an impact on the wash-out and the subsequent TTC staining and calculation of LDH release.

No functional assessment was used in order to assess the effect of ischaemia and reperfusion length, avoiding potential manipulation injury. This might be considered as a limitation, since many groups use an intraventricular balloon in such studies. However it has been shown that an intraventricular balloon can influence pro-survival salvage pathways (273). Following the same rationale of avoiding any additional stress, no heart rhythm was recorded and consequently, no heart rate exclusion criteria was applied. Finally, the no-reflow phenomenon has been well reported in isolated heart models in which hearts are perfused only with crystalloid (42,274) and the fact TTC staining might not be reaching some no-reflow area cannot be discarded.

4.5.6   Conclusions

This chapter presents a thorough characterization of the Langendorff isolated perfused mouse heart following IRI. Myocardial IS measured by triphenyltetrazolium staining depends on both the duration of ischaemia and length of the reperfusion period. Thus, the longer the reperfusion length, the greater the infarct size, even after a prolonged ischaemic insult. Moreover, LDH assessment may not be the most reliable tool to assess infarct size and/or to examine cardioprotective effectiveness, at various times of ischaemia.

Although any protocol could be actually chosen to perform subsequent experiments throughout my PhD, the 35 min ischaemia and 120 min reperfusion protocol was the one eventually selected. First, we ruled out those protocols causing "too little" infarct, as for the aim of this PhD was crucial to demonstrate the efficacy of IPC (and the involvement of its signalling pathways) and we needed enough tissue at risk to confer a meaningful protection. Second, we discarded those protocols resulting in a too impaired tissue, particularly those protocols with long reperfusion. Third, we preferred to keep at least 120 min of reperfusion, as it is still unknown whether there is an on-going wave of reperfusion injury. Based on this criteria, we selected a standard protocol of IRI, also well reported in the literature, of 35 min ischaemia and 2 h reperfusion.

Chapter 5   SELECTION OF IPC PROTOCOL

5.1   Background

Myocardial ischaemic preconditioning whereby non-lethal episodes of coronary occlusion do not cause tissue necrosis by themselves, but render protection to a subsequent lethal ischaemia/reperfusion insult, has been the object of intense research, resulting in over 10,000 publications in the literature (88). Most of this research has focused on describing the cellular and molecular mechanisms involved in eliciting cardioprotection as well as on translating the protective effect to the clinical arena using other forms of ischaemic conditioning. In comparison, relatively little attention has been given to the assessment of the dose-response characteristics of IPC.

Dose-response characteristics of IPC have been investigated without much notice over the past 30 years. Some studies have sought to determine the "minimum" dose (translated in number and/or duration of cycles) of ischaemia/reperfusion transient episodes to confer cardioprotection. Other have studied the temporal relationship between the application of the protocol and the duration of its effect on the subsequent sustained insult – estimated in 2-3 h for the acute effect (275). Further, the consequences of overreaching the top threshold of the conditioning phenomenon when a large number of transient ischaemia/reperfusion cycles are applied, known as hyperconditioning, has also been studied (276).

Soon after the landmark publication by Murry *et al.* describing the IPC phenomenon (33), a study suggested that preconditioning was an all-or-nothing phenomenon, demonstrating protection when a single cycle was undertaken (277). Despite this first publication, most of the subsequent research papers suggest a steady graded phenomenon conferring summative protection by supplementary cycles (278), with a top limit where an excessive number of cycles can be associated with loss of protection (279). Moreover, the protection associated with IPC has been hypothesised to have a "trigger" threshold, and is therefore dependent on the combination of the number and duration of IPC cycles, which is translated in turn as whether this transient stimulus has been able to release sufficient

autocoids (or "triggers" of the three-step signalling process activated by IPC described in 1.5.1) to eventually activate downstream kinase cascades and confer protection.

Once having selected the IRI protocol as per Chapter 4, the focus was next upon the selection of the IPC protocol to be used in subsequent experiments, through the comparison between the application of 4 cycles of IPC (based on Murry's original publication) (33) with the minimum of 1 cycle algorithm.

5.2   Research objective and experimental aims

Can infarct size be reduced to the same extent when a protocol of 1 cycle IPC is compared with a 4 cycle protocol?

Hypothesis

4-cycle IPC protocol will further reduce myocardial infarct size when compared to a shorter protocol of 1-cycle IPC.

Experimental aims

The overall aim was to select a protocol of IPC for subsequent experiments related to the thesis. Below is an overview of the main aims, including the proposed models related to the research question of this chapter:

Aim 1: Compare the effect of 1-cycle vs 4-cycle IPC to protect the heart against ischaemia/reperfusion injury.

- Subject mouse Langendorff to ischaemia-reperfusion and subsequently quantify myocardial infarct size.

Aim 2: Compare the effect of 1-cycle vs 4-cycle IPC to activate the survival kinases, Akt and ERK

- Collect protein from mouse hearts subjected to IPC protocols and use Western blot analysis to measure levels of Akt and ERK activation.

5.3 Methods

5.3.1 Experimental design and study protocols

According to aim 1 and 2, two sets of experiments were performed. C57BL/6 mice were divided into 3 study groups in order to examine myocardial IS reduction. Hearts in the IPC 1 cycle group underwent 1 cycle of 5 min ischaemia and 5 min reperfusion, whilst hearts in the IPC 4 cycle group were subjected to 4 repeated cycles (total duration of the protocol was 40 min). Hearts in the control group received no intervention, being perfused in time matched fashion (60 min). Figure 5-1 illustrates the study design and experimental protocols. Secondly, Akt and ERK activation were assessed using Western blot analysis in perfused mouse hearts subjected to the IPC protocols being investigated. Figure 5-2 summarizes this second study design.

Details on the protocol of both the ex vivo Langendoff-perfused mouse model and the Western blot technique being developed can be found in section 0 and section 3.9, respectively. Regarding the latter, proteins were transferred onto Immobilon-FL hydrophobic Polyvinylidene Fluoride (PVDF) transfer membrane (MerckMillipore, UK) and primary antibodies used were acquired from Cell Signaling Technology: Akt (#9272), Phospho-Akt (Ser473) (#9271), Phospho-Akt (Thr308) (#2965) and ERK1/2 (#9102), Phospho-ERK1/2 (Thr202/Tyr204).

5.3.2 Sample size calculation

Based on our previous publication characterizing the model (280), the sample size for this experiment was estimated for performing a two-sided test for k-independents samples (ANOVA) following 2 pairwise comparisons (25% minimum expected effect size, common SD of 13%, $\alpha=0.05$ and $\beta=0.20$, ~15% expected losses). Therefore, 21 animals were allocated according to a pre-specified randomization sequence (seed 554, STATA software version 13.1). Three animals were excluded as they failed to meet inclusion criteria, hence each group encompassed 6 animals.

Sample size was not estimated beforehand for Western blot analyses, in which five animals per group were used in line with convention (257). However, hearts were randomly assigned to each rig using the same pre-specified randomization sequence.
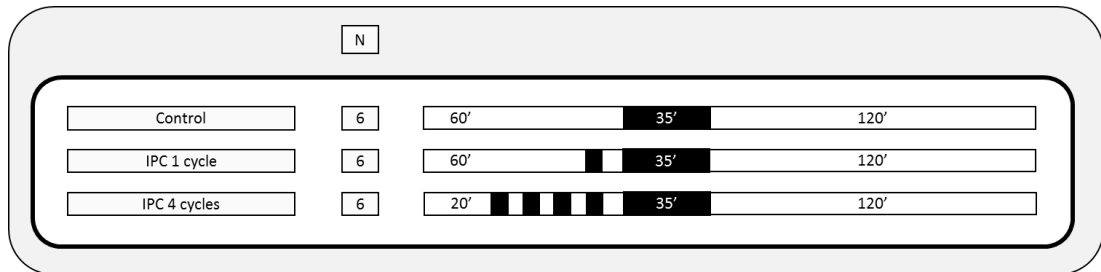
Figure 5-1: Study design for perfusion protocols to determine the effect of two different ischaemic preconditioning (IPC) protocols on myocardial infarct size.

Three different experimental protocol were tested: 1) control; 2) IPC 1 cycle of 5 min ischaemia and 5 min reperfusion; and 3) IPC 4 cycles of 5 min 5 min ischaemia and 5 min reperfusion. Black boxes represent periods of ischaemia and white boxes represent periods of perfusion with Krebs-Henseleit buffer at 80 mm Hg.
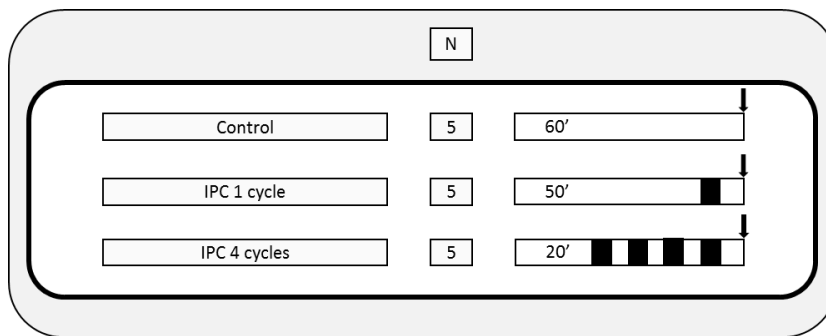
Figure 5-2: Study design for Akt and ERK phosphorylation analysis using Western blot

Three different experimental protocol were tested: 1) control; 2) IPC 1 cycle of 5 min ischaemia and 5 min reperfusion; and 3) IPC 4 cycles of 5 min 5 min ischaemia and 5 min reperfusion. Black boxes represent periods of ischaemia and white boxes represent periods of perfusion with Krebs-Henseleit buffer at 80 mm Hg. Black arrows depict the time-point of sample collection.

5.3.3   Data analysis

Infarct size data is expressed as mean ± SEM, whilst Western blot data is described as increase fold. Normal distribution of the data was determined using the Kolmogorov-Smirnov test for both data sets. One-way ANOVA parametric analyses were performed for the overall comparisons between groups, whilst a Dunnet's multiple comparison post-test was used to compare the groups of interest to the control group. IBM SPSS Statistics software, v.20.0 (IBM Corp. Armonk, NY) was used for the statistical analysis. STATA software, version 13.1 (Stata Corp, College Station, TX, USA) was used to generate the randomization sequence. GraphPad Prism version 6.00 (GraphPad Software, La Jolla California, USA) was used to perform the graphics.

5.4   Results

IPC was examined using 2 different patterns to determine the optimal strategy (1 vs 4 cycles of 5 min ischaemia and 5 min reperfusion). As described in Figure 5-3, IPC 4 cycles resulted in significant reduction of IS when compared to control group (21.1 ± 3.23 % vs 38.0 ± 1.72 %, P=0.006), whilst IPC 1 cycle did not demonstrate to be effective (34.3 ± 4.60, P=0.665 compared to control group).

Phospho-serine 473 and –threonine 308 Akt levels were increased in IPC 4 cycles compared to control group (P=0.019 and 0.041 respectively), whilst IPC 1 cycle Akt phosphorylation did not demonstrate significant phosphorylation in any of these two substrates (P=0.562 and P=0.325 respectively). Figure 5-4 and Table 5-1 present this Western blot data.

Phospho-Thr202/Tyr204 ERK 1/2 was increased in IPC 4 cycles compared to control group (P=0.045), but no significant effect was detected when a protocol of 1 cycle was used (P=0.780).
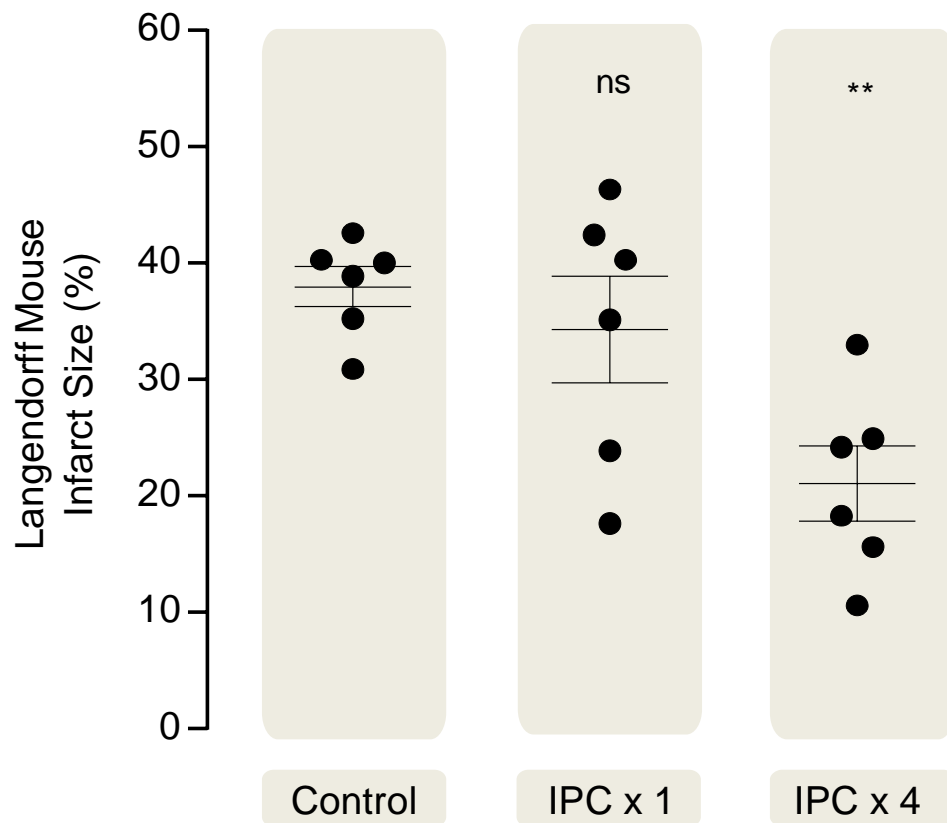
Figure 5-3: Effect of two ischaemic preconditioning protocols on myocardial infarct size (%)

Scatter dot blots: black lines represent mean± SEM and circles represent individual animal data. Myofarcial infarct size was significantly smaller with IPCx4 (mean ± SEM): control 38.0 ± 1.72%, IPC 1 cycle 34.3 ± 4.60 %, IPC 4 cycles 21.1 ± 3.23%. The overall P-value for the ANOVA was 0.008, whilst the Dunnett's multiple comparison test demonstrated a significant difference for IPC 4 cycles (P=0.006) and a non-significant result for IPC 1 cycle (P=0.665) compared to control group.
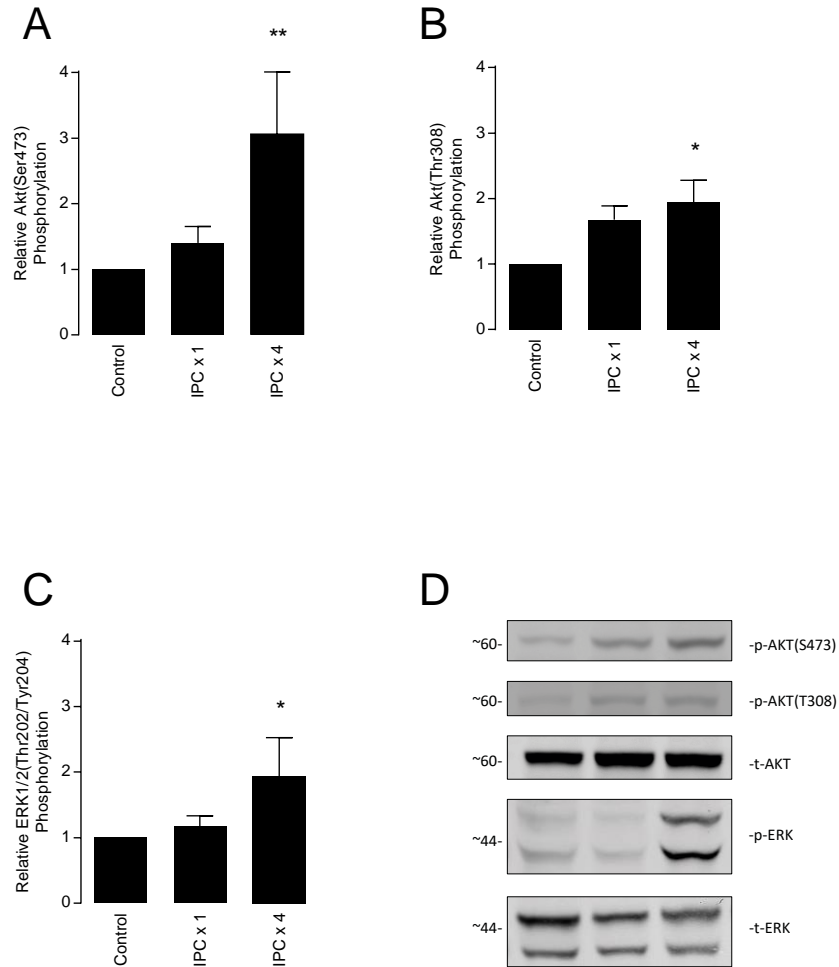
Figure 5-4: Effect of IPC protocols on Akt and ERK 1/2 phosphorylation

Bar graph shows the percentage of phosphorylation in all groups compared to the control group, expressed as mean ± SEM (% of relative phosphorylation, normalized by its total), n=5 per group.

	Control	**IPC 1 cycle**	**IPC 4 cycles**
Akt (Ser473) phosphorylation	1	1.4 ± 0.2	3.1 ± 0.9*
Akt (Thr308) phosphorylation	1	1.7 ± 0.2	1.9 ± 0.3*
ERK (T202/Y204) phosphorylation	1	1.2 ± 0.2	1.9 ± 0.6*

Table 5-1: Raw data for IPC protocol selection

Raw data related to Figure 5-4. Asterisks refers to comparison with control group: * P<0.05, ** P<0.01, *** P<0.001.

5.5 Discussion

The aim of this short study was to examine whether the number of cycles affected the efficacy of IPC, and whether this effect was also mirrored by the activation of pro-survival kinases at the "trigger" phase. The protocol of IPC 4 cycles resulted in a robust means of preconditioning the mouse isolated perfused heart, whilst IPC 1 cycle failed to consistently reduce myocardial IS. Accordingly, the protocol with higher number of cycles was associated with a significant increased phosphorylation of Akt and ERK, whilst IPC did not show pro-survival kinases activation. As a result, the IPC 4 cycle algorithm was selected to be subsequently used in the remaining experiments of this thesis.

5.5.1 The larger the number of IPC cycles, the higher the protection rendered

The ischaemic preconditioning regime of 4 cycles of 5 min ischaemia/reperfusion was selected to be tested. This was all based on the original protocol of IPC reported by Murry et al. in their first description of the phenomenon (33), as well as on the remote ischaemic conditioning algorithm reported in the original study by Przyklenk et al. (35). Other subsequent publications have also demonstrated its efficacy (281). In previous thesis undertaken at The Hatter Cardiovascular Institute, Dr Mark Sumeray (282,283) and Dr Robert Bell (266), both found a protocol of 2 cycles of 10 min ischaemia and 10 min reperfusion to be protective. Li *et al.* demonstrated in anaesthetized dogs that preconditioning with one brief ischaemic interval was as effective as preconditioning with multiple ischaemic periods (277). In 1997, Iliodromotitis *et al.* demonstrated in anaesthetized rabbits that there is little difference in the degree of protection obtained with either a protocol of 1, 2 or 4 cycles, whilst the protection was proved to be lost when applying 6 or 8 cycles (279). In the first-in-man experiment of IPC in humans, Yellon et al. reported an effective 2-cycle protocol of 2 min ischaemia and 3 min reperfusion (67), whilst in the first publication using the human atrial trabeculae model, Yellon and colleagues also successfully applied a protocol of 3 minutes simulated ischaemia and 7 minutes reoxygenation (284). Therefore, both protocols being tested in this chapter, 1-cycle vs 4-cycle, and many others, have shown to be protective in several experimental settings.

Further to the discussion above it is also important to appreciate that there is a general consensus demonstrating that the number of cycles follow a steep dose-response relationship (71,285,286) until reaching a turning point where the protection can be lost (78,276,287). Furthermore, a study published in 2014 demonstrated further increase in IS-sparing effect of IPC with a novel stepwise ischaemic preconditioning protocol applied in rabbits (278). In accordance with the steep dose-response relationship, the results being presented in this chapter shows a small but non-significant reduction of myocardial IS in IPC 1 cycle, compared to a solid significant reduction in IPC 4 cycles. Within the IPC 1 cycle group, two hearts seem to be protected, whilst four show a similar IS in comparison to the control heart. One might speculate that this "biological variability" might be explained by these hearts to be on the threshold of reaching the preconditioning status (see next section for this hypothesis). Also, based on the experience accumulated in The Hatter Cardiovascular Institute, it seems that the *ex vivo* heart model is hardest to precondition when compared to the *in vivo* model (unpublished data), probably due to the nature of their experimental conditions (denervation, lack of immune response…).

5.5.2 Triggering the preconditioning response: the IPC threshold hypothesis

To confer protection, IPC is associated with the release of "triggers", including adenosine, bradykinin, opioids and angiotensin that act upon cell sarcolemmal receptors to activate the preconditioning response. These triggers have been administered exogenously to pharmacologically mimic the IPC response (129,275). In characterizing these "preconditioning mimetics", a non-linear dose-response relationship has been observed between the exogenous trigger (or receptor agonist) and myocardial IS reduction. As a matter of fact, triggering of preconditioning seems to have dose-response threshold: if the duration or numbers of transient ischaemia/reperfusion cycles, or the dose of the preconditioning mimetic is insufficient, no resistance to lethal IRI is observed. By progressive increment of the stimulus, a threshold is crossed and the IPC response involving protein kinase cascades consequently activated. This hypothesis, known as "preconditioning threshold", is supported by observations in both rabbit (71,288) and human myocardium (289).

A "classic" example of the "preconditioning threshold" theory is based on the role of bradykinin in IPC. It is well established that bradykinin is released endogenously by the endothelium under ischaemic conditions (290). It is also known that angiotensin converting enzyme (ACE) inhibitors attenuate bradykinin breakdown, therefore increasing their levels in the extra cellular milieu. In the human atrial trabeculae model, Yellon's group demonstrated that either a subthreshold preconditioning stimulus or the administration of ACE inhibitors (captopril and lisinopril) were not able to confer protection in terms of contractile function recovery when administered separately, whilst the combination of both interventions (ACE inhibitors captopril + subthreshold preconditioning stimulus) showed a significant protective effect (289). This protection was abolished with the use of Hoe 140, a specific bradykinin B2 receptor antagonist, hence suggesting an increase in bradykinin levels as responsible to breach the preconditioning threshold (289). Miki *et al.*, presented similar results using captopril and a sub-threshold protocol of 2 min ischaemia/5 min reperfusion in an in vivo rabbit model of IRI, backing the idea that the sub-threshold level achieved by a short IPC protocol can be augmented by the increase in bradykinin levels.

5.5.3 Similar findings in remote ischaemic preconditioning

Our results are in agreement with previous publications regarding remote ischaemic preconditioning (RIPC). In an *in vivo* mouse model testing for RIPC, Botker's group observed no protection with two cycles, but similar robust protection either with four, six or eight cycles of 5 min. In the same vein, Lu *et al.* did not find cardioprotection by one, but by three cycles when applied remotely in the hind-limb in an in vivo rat model (291). Interestingly, they were able to demonstrate that morphine reduces the threshold of cardioprotection produced by RIPC, as the addition of morphine 0.1 mg/Kg to RIPC 1 cycles significantly reduced myocardial IS, whilst any of these interventions did not when applied separately. These results seem to prove the threshold hypothesis also in remote preconditioning.

5.5.4   Limitations

The influence of other protocols with either different number of cycles, or distinct ischaemic duration within each IPC cycle, was not investigated. Therefore, a steep dose-response relationship cannot be drawn from these results. Mouse hearts obtained for Western blot analysis were not collected at reperfusion ("mediator" phase), but immediately after finishing the IPC protocol. Although it is known that both Akt and ERK are activated at both phases (66), we cannot strictly speak of activating the RISK pathway as this cascade was described to be recruited at reperfusion (36,90).

5.5.5   Conclusions

The protocol of IPC 4 cycles demonstrated to reduce myocardial infarct size and activate both Akt and ERK pro-survival kinases, whilst IPC 1 cycle showed neutral results in both endpoints. Therefore, an algorithm of IPC 4 cycles was selected to be subsequently used in this thesis.

Chapter 6   ROLE OF PI3K IN MYOCARDIAL ISCHAEMIC PRECONDITIONING

Most of the content of this chapter has been already published as a single piece of research:

Rossello X, Riquelme J, Davidson S, Yellon DM. Role of PI3K in myocardial ischemic preconditioning: mapping prosurvival cascades at the trigger phase and at reperfusion. Journal of Cellular and Molecular Medicine (2017, in press).

6.1   Background

Ischaemic preconditioning, consisting of transient cycles of coronary occlusion and reperfusion, is considered the most powerful intervention available to protect the heart against myocardial ischaemia/reperfusion injury, other than reperfusion itself, and has become the paradigm for cardioprotection (66).

IPC results in the recruitment of signalling pathways comprising protein kinases and phosphatases that converge on the mitochondria (41). The Reperfusion Injury Salvage Kinase (RISK) pathway is considered a central prosurvival kinase cascade mediating the IPC-induced protective effect. This pathway actually encompasses two parallel signalling cascades: PI3K-Akt and MEK1-ERK1/2 (66). RISK recruitment not only mediates the protection induced by IPC, but also by other forms of conditioning (pre-, post-, remote and pharmacological conditioning), and therefore appears to be a universal signalling paradigm for cardioprotection. Other alternative pathways, such as the Survivor Activator Factor Enhancement (SAFE) pathway comprising TNFα/STAT3, have also been proposed as IPC-induced protection mediators (92).

The RISK pathway has demonstrated a biphasic activation pattern, occurring first during the preconditioning cycles (or "trigger" phase) and then again during the first few minutes of reperfusion ("mediator" phase) (10,66,292). Although the relevance of the RISK pathway is a well-established concept in cardioprotection, the relative importance of the activation of these kinases and phosphatases, either at the trigger phase or exclusively at the mediator phase, remains to be fully elucidated. In IPC studies, the activation of both RISK and SAFE pathways has been demonstrated to occur at these two time-points (88),

but there is a lack of comprehensive studies assessing the integrative role of these signalling cascades in this setting. Cardioprotective signalling cascades have mostly been simplified in the literature, and crosstalk between the two components of the RISK pathway (101,102) and between the RISK and the SAFE pathways (95,103) have been described in only a few studies.

Once having selected the IRI protocol as per Chapter 4 and the IPC protocol as per Chapter 5, the focus was next turned to the description of the PI3K-Akt cascade following an IPC stimulus. The central scope of this thesis is to elucidate the role of PI3Kα to confer protection against myocardial IRI, therefore a thorough description of PI3K downstream and parallel kinases and phosphatase activation appears to be a necessary and inevitable point to be addressed before further studying specific PI3K isoforms.

6.2   Research objectives and aims

What role plays PI3K in the protective effect elicited by IPC?

<u>**Hypothesis**</u>

PI3K activity is required during both the trigger and mediator phases for IPC to limit the infarct size**.**

<u>**Experimental aims**</u>

The aim of this chapter is to systematically characterize the role of the PI3K-Akt component of the RISK pathway, as well as its counter-regulatory protein, PTEN in mediating the IPC cardioprotective effect during both the trigger phase and the reperfusion stage. In principle, both the activation (phosphorylation) of PI3K and the inactivation (also through phosphorylation) of PTEN should be expected to activate Akt and thus induce cardioprotection. Besides, the aim is to further investigate the interplay between the PI3K-Akt pathway and their parallel ERK and STAT3 cascades at both time-points. Below is an overview of the main aims, including the proposed models related to the research question of this chapter:

Aim 1: Investigate whether the pan-specific PI3K inhibitor wortmannin abrogate the cardioprotective effect of IPC when applied either during the trigger phase or the mediator phase.

- Subject mouse Langendorff to ischaemia-reperfusion and subsequently quantify myocardial infarct size.

Aim 2: mapping prosurvival cascades at the trigger phase and at reperfusion following PI3K inhibition in preconditioned hearts

- Collect protein from mouse hearts subjected to IPC protocols and use Western blot analysis to measure levels of Akt, PTEN, GSK3β, ERK and STAT3 phosphorylation.

6.3   Methods

6.3.1   Experimental design and study protocols

A total of 58 animals were used, although 3 hearts were excluded before randomization as they failed the predefined exclusion criteria (see below). Therefore, 55 animals were randomly allocated to treatment groups in two separate experiments:

1) **Study of infarct size.** The effect on myocardial IS following IPC 4 cycles was studied using wortmannin 100 nM administered during the IPC protocol, or at reperfusion. The IPC protocol consisted of 4 cycles of 5 min ischaemia and 5 min reperfusion and was chosen based on previous publications demonstrating a reduction of myocardial IS (252,280) (Figure 6-1).

2) **Study of phosphorylated protein levels.** Activated levels of kinases and phosphatases involved in IPC-induced cardioprotection were systematically studied in two blocks, as summarized in Figure 6-2. In the first, phosphorylated proteins were measured at the trigger phase in three treatment groups: a) control; b) IPC; and c) wortmannin plus IPC. In the second block, phosphorylated kinases were measured after 5 min reperfusion in four treatment groups: a) control; b) IPC; c)

IPC plus wortmannin during trigger phase; and d) IPC plus wortmannin during mediator phase.

In comparison to the protocols aimed at assessing myocardial IS, for those experiments aimed to evaluate protein phosphorylation levels the period of ischaemia was reduced to 15 minutes and reperfusion shortened to 5 min. Shorter protocols are well accepted in the literature (293) on the basis of (1) obtaining enough non-necrotic tissue to evaluate kinase activation, and (2) the protection elicited by kinase activation and mPTP opening delay occurs at the onset of reperfusion (86,112).

Animals used were male C57BL/6 mice (9-12 weeks, 24-28 g weight), all of them obtained pathogen free from one supplier and housed under identical conditions. Wortmannin, CAS 19545-26-7, the PI3 kinase inhibitor, was purchased from Merck Millipore (Nottingham, UK) and its concentration dose was chosen based on previous publications (95,155). Dimethyl sulfoxide from BDH (Poole, UK) was used as the solvent for Wortmannin at a final concentration in the perfusion buffer of not more than 0.01%, as well as a vehicle control for the rest of the groups.

Details on the protocol of both the ex vivo Langendorff-perfused mouse model and the Western blot technique being developed can be found in section 0 and section 3.9, respectively. Regarding the latter, proteins were transferred onto nitrocellulose blotting membrane (GE Healthcare Life Sciences, UK) using wet transfer and primary antibodies used were acquired either from Abcam, in the case of the loading control anti-GAPDH (mAbcam, #9484), or from Cell Signaling Technology: Akt (#9272), Phospho-Akt (Ser473) (#9271), Phospho-Akt (Thr308) (#2965), ERK1/2 (#9102), Phospho-ERK1/2 (Thr202/Tyr204) (#9101), GSK-3β (#9315), Phospho-GSK-3β (Ser9) (#5558), Stat3 (#9139), Phospho-Stat3 (Tyr705) (#9145) and Phospho-PTEN (Ser380) (#9551).
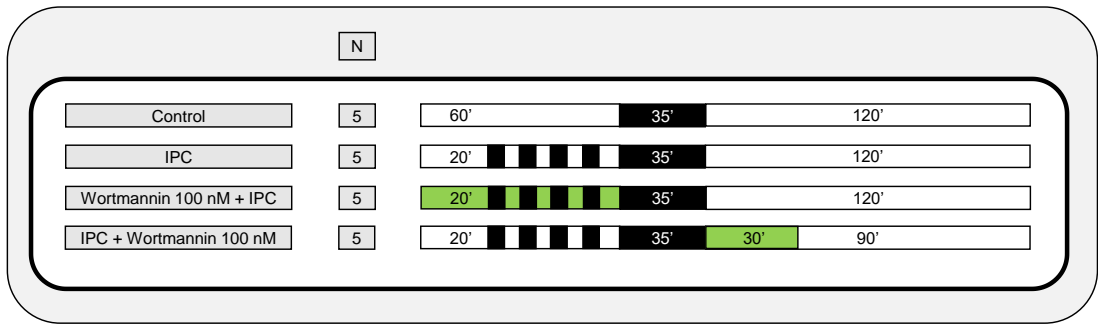
Figure 6-1: Study design for IS determination protocols in the context of IPC and wortmannin before and after index ischaemia.

Four different experimental protocols were tested: 1) control; 2) IPC 4 cycles of 5 min ischaemia and 5 min reperfusion per cycle; 3) IPC 4 cycles in the context of Wortmannin administered during the stabilization period and IPC protocol; and 4) IPC 4 cycles plus the administration of Wortmannin upon reperfusion. Black boxes represent periods of ischaemia, white boxes represent periods of perfusion with Krebs-Henseleit buffer at 80 mm Hg and green boxes represent the perfusion of Wortmannin 100 nM.
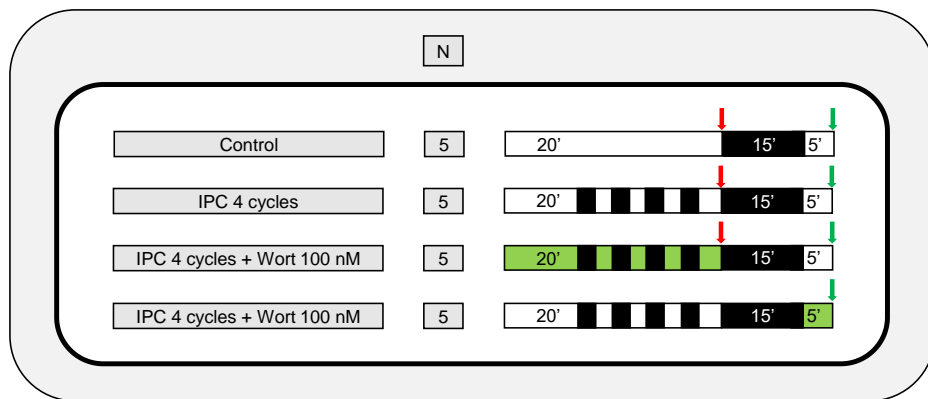
Figure 6-2: Study design to compare Akt and ERK phosphorylation analysis using Western blot

Four different experimental protocols were tested: 1) control; 2) IPC 4 cycles of 5 min ischaemia and 5 min reperfusion per cycle; 3) IPC 4 cycles in the context of Wortmannin administered during the stabilization period and IPC protocol; and 4) IPC 4 cycles plus the administration of Wortmannin upon reperfusion. Black boxes represent periods of ischaemia, white boxes represent periods of perfusion with Krebs-Henseleit buffer at 80 mm Hg and green boxes represent the perfusion of Wortmannin 100 nM. Arrows represent the moment where samples were collected (in red all samples collected after IPC protocol, in green all samples collected at reperfusion).

6.3.2 Sample size

The necessary sample size for the evaluation of the potential abolition of cardioprotective effect using wortmannin either before ischaemia or upon reperfusion was calculated using a two-sided test for k-independents samples (ANOVA) following 3 pairwise comparisons.

Based on the results of the comparison between IPC protocols and considering the literature regarding the effect of wortmannin, a 18% minimum expected effect size and a common SD of 8% were applied, in addition to a significance level of 5% (α=0.05) and 80% power (β=0.2). Hence, the estimated sample size required was 20 animals, five per group. Mice were subsequently allocated by blocks according to a pre-specified randomization sequence (seed 4857, STATA software version 13.1) which assigned each animal into its correspondent experimental group and rig.

Sample size was not calculated beforehand for Western blot experiments, in which five animals per group were used in line with convention (257). However, hearts were randomized by blocks for each rig using a reproducible randomization sequence (seed 45733, STATA software version 13.1).

6.3.3 Data analysis

Normal distribution of each data subset was tested using graphical methods and the Kolmogorov –Smirnov method. All values are presented as mean ± standard error of the mean. If normally distributed, continuous data were compared using one-way analysis of variance followed by post hoc pairwise comparisons to the control group using the Dunnett's test. If highly skewed distributed, the non-parametric Kruskal–Wallis test was used with subsequent post hoc pairwise comparisons to the control group adjusted by the Dunn's test. A P value of less than 0.05 was considered statistically significant. STATA software, version 13.1 (Stata Corp, College Station, TX, USA) and GraphPad Prism version 6.00 (GraphPad Software, La Jolla California, USA) were used to perform the analysis and the graphics.

6.4 Results

6.4.1 PI3K mediates the IPC protective effect against myocardial IRI

To investigate the role of the PI3K-AKT cascade in IPC-induced cardioprotection, isolated perfused mouse hearts were subjected to 35 min global ischaemia, followed by 2 h reperfusion. IPC resulted in reduction in IS compared with the control group (21 ± 4 *vs* 59 ± 5 %, P < 0.001). Administration of wortmannin either during the trigger phase of the IPC protocol or during the mediator phase abrogated its IS-limiting effect when compared to control group (46 ± 5 %, P = 0.158; and 46 ± 4, P = 0.154, respectively) (Figure 6-3).
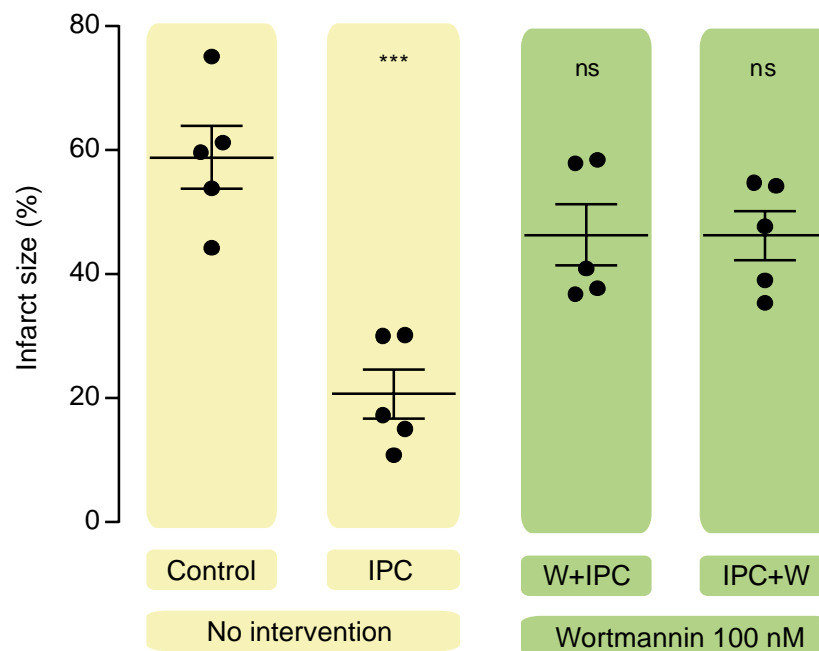
Figure 6-3: Role of PI3K in the protective effect of IPC at the trigger phase and at reperfusion.

Scatter dot blots: black lines represent mean ± SEM and circles represent individual animal data. Myofarcial infarct size was significantly smaller with IPC compared to control group and either the administration of Wortmannin before or after ischaemia index abolished the cardioprotective effect of IPC.

6.4.2 PI3K mediates IPC-induced activation of Akt

To confirm the activation of Akt during the IPC trigger phase and the early phase of reperfusion both of the main Akt phosphorylation sites (Ser-473 and Thr-308) were studied. As expected, IPC increased Akt phosphorylation in the trigger phase at both sites (Ser-473: 2.6 ± 0.5 –fold, P=0.010 *vs* control; and Thr-308: 2.6 ± 0.5 –fold *vs* control, P=0.034), and phosphorylation was prevented when wortmannin was applied during the IPC trigger phase (Figure 6-4A and Figure 6-4C). Likewise, IPC increased the phosphorylation of Akt during the mediator phase of reperfusion (Ser-473: 2.4 ± 0.6 –fold *vs* control, P<0.001; and Thr-308: 3.1 ± 0.7 -fold *vs* control, P=0.001). However, this phosphorylation was consistently abrogated when wortmannin was administered either during the trigger or mediator phase (Figure 6-4B and Figure 6-4D).

6.4.3 IPC phosphorylates GSK3β at the trigger phase, but not the mediator phase

To explore the downstream targets activated by PI3K-Akt both at the trigger phase and upon reperfusion, we investigated the role of the serine/threonine kinase GSK3β. In contrast to many protein kinases, GSK3β is active in resting cells and inactivated by phosphorylation(164). On stimulation, GSK3β is phophorylated at serine 9, resulting in inhibition of its kinase activity. As illustrated in Figure 6-4E, IPC caused an increase in GSK3β phosphorylation during the trigger phase (3.2 ± 0.9 -fold *vs* control, P = 0.007), which was blocked by wortmannin. On the contrary, GSK3β was not significantly phosphorylated at reperfusion in preconditioned hearts compared to control group (1.4 ± 0.2 -fold *vs* control, P = 0.742). Moreover, the administration of wortmannin either during the trigger phase or mediator phase did not affect GSK3β phosphorylation levels when measured at reperfusion (Figure 6-4F).

6.4.4 IPC phosphorylates PTEN through a PI3K-independent mechanism

PTEN is a phosphatase that counter-regulates the PI3K/AKT signalling pathway(294). While phosphorylation of PIP2 by PI3K stimulates Akt activity, PTEN dephosphorylates PIP3 and downregulates Akt activity. The phosphorylated form of PTEN is

considered inactivated, and is therefore a major negative regulator of the RISK pathway(186). PTEN phosphorylation was significantly increased immediately following an IPC stimulus (1.5 ± 0.2 -fold *vs* control, P=0.009). Interestingly, this was not affected by the administration of wortmannin (Figure 6-4F). IPC was also associated with an increase in phosphorylated PTEN during the mediator phase (1.6 ± 0.1 -fold *vs* control, P = 0.047), and again, this was not affected by the presence of wortmannin during either the trigger or mediator phases (Figure 6-4H).

6.4.5 IPC-induced ERK activation involves PI3K during the trigger phase, but not at reperfusion

During the IPC trigger phase, phosphorylated levels of ERK were significantly increased by IPC (3.0 ± 0.6 -fold times compared to control, P = 0.016). This was partially abrogated by wortmannin, such that there was no longer a significant increase in ERK phosphorylation during the trigger phase after IPC (Figure 6-5A). IPC also caused a significant increase in ERK phosphorylation during the mediator phase (2.6 ± 0.6-fold increase for IPC, P=0.016), but in contrast to the response observed in the trigger phase, this was unaffected by the administration of wortmannin (IPC + wortmannin during trigger phase: 2.7 ± 0.2 -fold, P=0.009; IPC + wortmannin during the mediator phase: 2.3 ± 0.3 -fold, P = 0.031) (Figure 6-5B).

6.4.6 IPC activates STAT3 through a PI3K-independent mechanism

The SAFE pathway, which involves TNFα, JAK and STAT3, has been described as an alternative RISK-independent cascade that may be important for mediating the cardioprotective effect elicited by IPC in some circumstances(95,99). We found that IPC significantly increased levels of phosphorylated STAT-3 (Tyr705) during the trigger phase (3.6 ± 1.6 -fold *vs* control, P = 0.009) (Figure 6-5C). The administration of wortmannin during the trigger phase did not alter levels of phosphorylated STAT3. Similarly, IPC increased levels of phosphorylated STAT3 in the mediator phase (2.6 ± 0.5 -fold *vs* control, P = 0.049), and this was unchanged by the administration of wortmannin during either the trigger or mediator phase (Figure 6-5D).
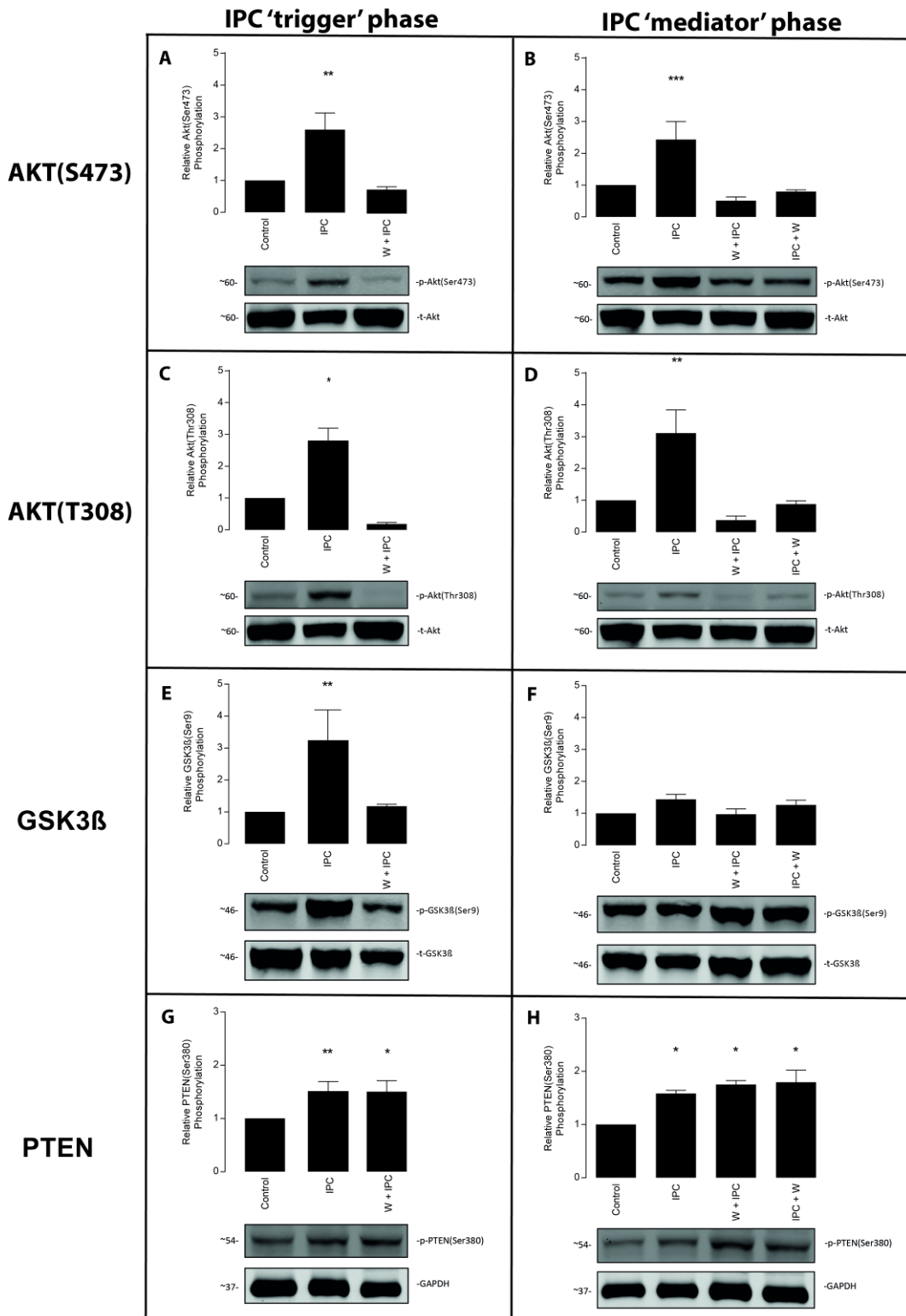
Figure 6-4: Impact of PI3K inhibition in IPC activated signalling cascades

Bar graph shows the percentage of phosphorylation in all groups compared to the control group, expressed as mean ± SEM (percentage of relative phosphorylation), n=5 per group.
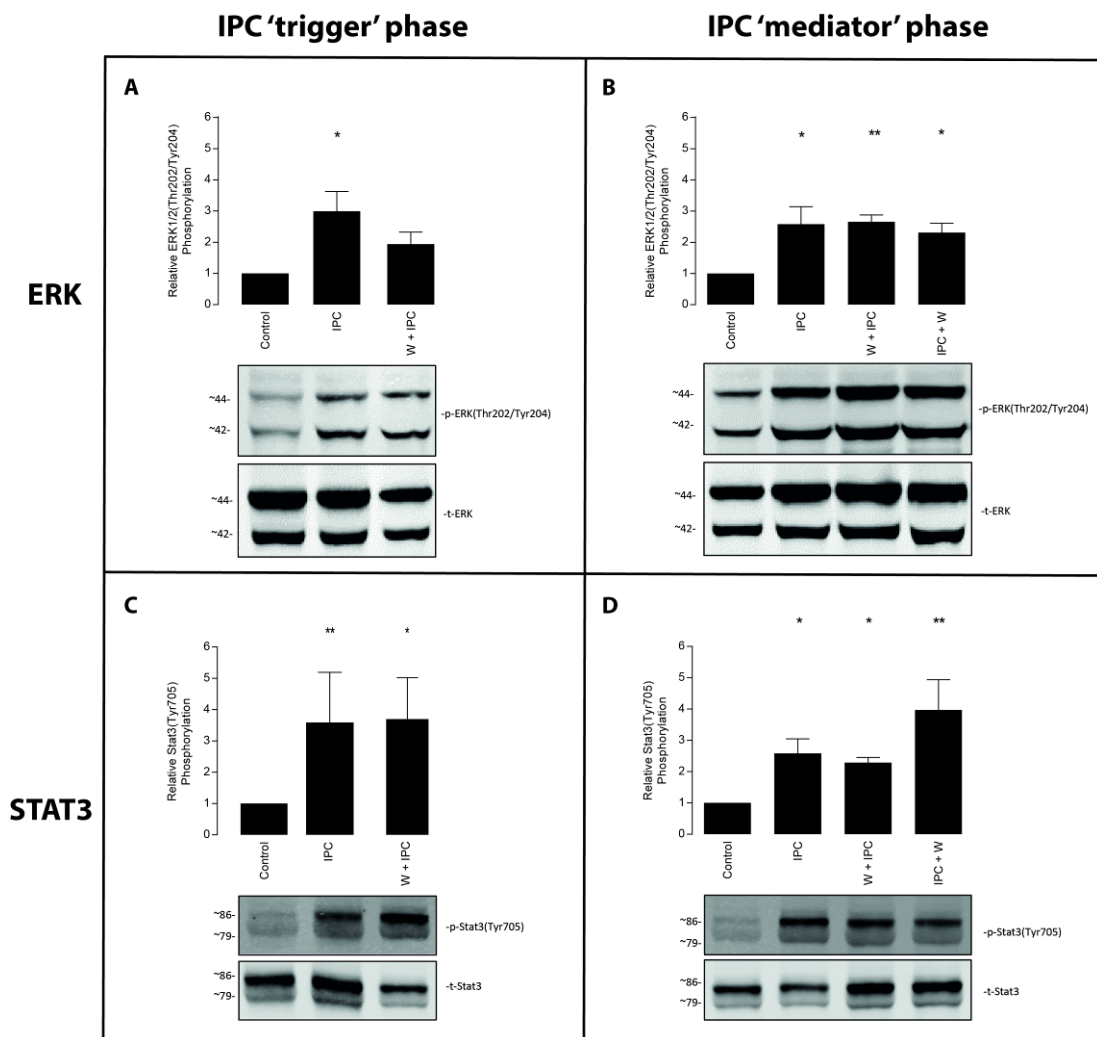
Figure 6-5: Impact of PI3K inhibition in IPC activated ERK and STAT3

Bar graph shows the percentage of phosphorylation in all groups compared to the control group, expressed as mean ± SEM (percentage of relative phosphorylation, normalized by its total), n=5 per group.

6.5 Discussion

In the first block of experiments, PI3K activity was confirmed to be required during both the trigger phase and mediator phase in order for IPC to reduce the infarct size. As expected, IPC increased the levels of Akt phosphorylation during both the trigger and mediator phases. Interestingly, Akt phosphorylation during both phases was completely abrogated by PI3K inhibition during just the trigger phase or the mediator phase, suggesting the existence of a memory effect. In contrast, one of the kinases downstream of the PI3K/Akt pathway, GSK3β, was phosphorylated only during the trigger phase after IPC. PTEN was phosphorylated during both the trigger and mediator phases after IPC, but this was independent of PI3K. IPC increase ERK phosphorylation during both phases, but was only PI3K-dependent during the trigger phase. Finally, STAT3, the kinase mediator of the SAFE pathway, was activated by IPC in both the trigger phase and mediator phase, and this phosphorylation was independent of PI3K activity. All these observations are as summarized in Figure 6-6.
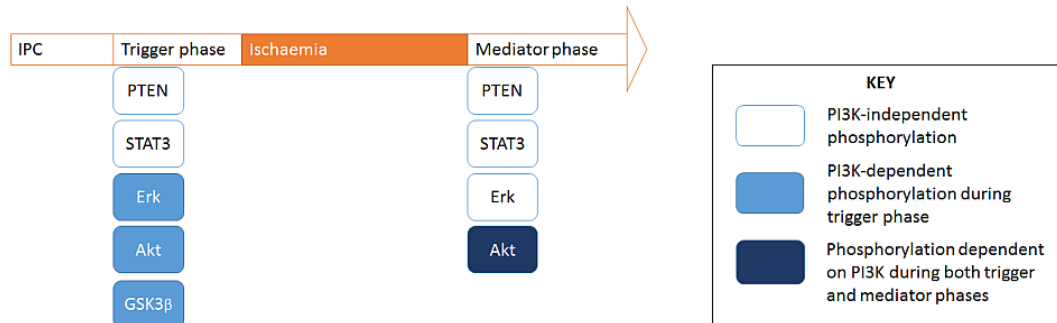
Figure 6-6: Summary of findings

IPC increased the levels of Akt phosphorylation during both the trigger and mediator phases. On the contrary, GSK3β was phosphorylated only during the trigger phase. The phosphatase PTEN was phosphorylated during both the trigger and mediator phases after IPC and this was not affected by PI3K inhibition. ERK was phosphorylated by IPC during both phases, but was only PI3K-dependent during the trigger phase. STAT3 was activated in both the trigger and mediator phases, and this phosphorylation was independent of PI3K.

6.5.1 Role of PI3K in the protective effect of IPC at the trigger phase and at reperfusion

IPC protects against myocardial IRI by activation of the RISK pathway (10,155). The data being presented in this chapter confirm the pivotal role of PI3K as mediator of IPC, as its pharmacological inhibition either during the trigger phase or at reperfusion abolished the IS-limiting effect provided by IPC. It has to be taken into account that wortmannin is a cell-permeable fungal metabolite that acts as a selective and irreversible inhibitor of the PI3K catalytic activity that has been widely used in pharmacological cardioprotection studies, although similar results have also been demonstrated using the reversible inhibitor LY294002 (155).

The fundamental concept of the RISK pathway has been described previously in section 1.6, but may be briefly summarized in two ways: 1) short-term activation of these kinases is protective triggering prosurvival pathways, whilst long-term activation may have detrimental effects such as cell growth and hypertrophic tissue (101,181); and 2) their activation occurs both during the preconditioning phase (275) and at reperfusion (89), also known as the trigger and the mediator phase, respectively. These two phases are crucial to mediate protection, as both of them can be pharmacologically intervened (159). Dissecting the key signalling events occurring at both phases when abolishing PI3K phosphorylation is of the utmost importance, as the IPC signalling architecture can be extrapolated to most cardioprotective therapies (78).

PI3K-Akt, one of the parallel cascades involving the RISK pathway, has been described to activate a kinases cascade. The activation of PI3K by IPC promotes the phosphorylation of PDK1, which in turn activates Akt in order to subsequently recruit a wide range of pro-survival downstream targets such a GSK3β, p70s6k and eNOS(186). On the contrary, PTEN counter-regulates the action of PI3K by dephosphorylating its product phosphatidylinositol(3,4,5)phosphate (abbreviated as PIP3). The activation of the PI3K/Akt pathway inhibits mPTP opening, which is considered the major downstream end-effector of the RISK pathway (10,254).

6.5.2 Impact of PI3K inhibition in IPC activated signalling cascades

Akt is a serine/threonine protein kinase. Its activity is primarily controlled by PI3K and PTEN through the modulation of PIP3 levels (295). Full activation of Akt happens through the phosphorylation of a Thr-308 residue in the catalytic domain by PDK1 and a Ser-473 residue by mTOR2, whilst its inactivation is mediated through dephosphorylation of the two regulatory sites by the serine-threonine phosphatase PP2A. Akt is widely used as surrogate marker for PI3K activation (296). Akt phosphorylation at both residues was abrogated in all preconditioned hearts treated with wortmannin. Therefore, in preconditioned hearts treated with the PI3K inhibitor, the abolishment of IS-sparing effect was mirrored by lack of Akt phosphorylation.

GSK3β is a serine/threonine kinase that has been proposed to be the downstream point of convergence of the RISK pathway, which when phosphorylated (and thus inhibited) at serine-9 by Akt in reponse to PIP3 increase (293), inhibits the mPTP opening (297), enhancing cell survival (164). Our data suggest that GSK3β is phosphorylated at the trigger phase as a consequence of PI3K activation, but this status is lost at the early reperfusion stage. In agreement with our results, *Tong et al.* (293) have reported increased phosphorylated levels of GSK3β following an IPC protocol, which were blocked by wortmannin. Moreover, pretreatment with GSK3β inhibitors also mimicked the protective effect of IPC through a reduction on myocardial IS(293). In another study, pharmacologic and genetic ablation of GSK3β failed to abrogate the protective effect conferred by IPC (297). Similarly, mice with a knockin of GSK3β mutated at ser9 and ser21 remained amenable to protection by IPC (164). Overall, it seems that inhibition of GSK3β may play an important role during the IPC protocol, but not afterwards. However, future studies need to clarify the contentius role of GSK3β in cardioprotection.

The activation of prosurvival protein kinases has been studied in great detail in the context of preconditioning. However, little is known about the role of their counter-regulator phosphatases in this setting. Inhibition of phosphatases (PP1 and PP2A) during the preconditioning phase have been shown to abolish the protective effect of preconditioning, whilst its activation during reperfusion improved protection in preconditioned hearts (298). The ability to up-regulate the RISK pathway by the use of

phosphatase inhibitors during the early reperfusion phase remains largely unexplored despite being an attractive approach to limiting IRI.

PTEN is a dual lipid and protein phosphatase that antagonizes PI3K/AKT signalling pathway. Whereas PI3K activity results in an increased production of the second messenger PIP3 to activate the prosurvival downstream cascade, PTEN dephosphorylates PIP3 to PIP2 to downregulate Akt activation. The phosphorylated form of PTEN is considered inactive. After dephosphorylation, PTEN is activated but is also degraded rapidly as its half-life is substantially reduced (299). Therefore, the phosphorylation (inactivation) of PTEN should be expected to induce cardioprotection following an IRI insult, whilst its dephosphorylated (activated) status should be expected to be detrimental (300,301). Interestingly, our results suggest that IPC induces inhibition of negative regulator PTEN. This observation is in line with the study of Cai and Semenza who identified a reduction in the activity of PTEN following IRI in an isolated perfused rat heart model (299). In the same vein, PTEN haploinsufficiency in mice has been shown to reduce the threshold of protection in IPC (281). This result suggest that IPC not only activates prosurvival kinases, but also inhibits their major counter-regulators (i.e. PTEN, PP1 and PP2A). Additionally, IPC-mediated PTEN phosphorylation appears to be independent of PI3K, suggesting that both activation of PI3K and inactivation of PTEN can work in unison, which may suggest that pharmacological intervention of both proteins at the same time may produce synergistic effect in the context of cardioprotection. Further studies need to be undertaken to explore this hypothesis.

6.5.3   Impact of PI3K inhibition in IPC activated ERK and STAT3

The molecular pathways involved in cardioprotection have been reported as highly interactive (78). Within the RISK pathway, some studies have suggested crosstalk between the PI3K-Akt and the ERK1/2 cascades (102). Our study shows that ERK is phosphorylated following an IPC stimulus, but partially inhibited after the administration of the PI3K inhibitor wortmannin. These results may suggest a PI3K predominance during the IPC early trigger phase, although they contrast with the lack of effect of the PI3K inhibitor on ERK phosphorylated levels at reperfusion. Using a pharmacological approach with specific PI3K and ERK inhibitors in the isolated perfused rat heart model, a previous study from The

Hatter Cardiovascular Institute suggested that the crosstalk between PI3K/Akt and ERK is not balanced, with the PI3K cascade playing a more determinant role in mediating cellular survival (102).

The SAFE pathway, which involves TNFα, JAK and STAT3, has been described as an alternative RISK-independent cascade mediating the cardioprotective effect elicited by IPC. The cardioprotective effect of STAT3 is believed to be partly related to its translocation to the mitochondria by modulating respiration and inhibiting the mPTP opening (302). Like the RISK pathway, the SAFE pathway is also activated during both the trigger and the mediator phase of reperfusion to protect the heart against IRI (92,95). In this study, STAT3 have demonstrated to be activated following an IPC protocol, irrespective of the inhibition of PI3K. Interestingly, upon administration of wortmannin, the protective effect elicited by IPC was lost despite the STAT3 pathway being activated. Our results differ from those described by Lecour *et al.* (95) in adult mouse cardiomyocytes, in which wortmannin administered during the IPC trigger phase decreased STAT-3 phosphorylation, abolishing the protection afforded by IPC. However, in our study the mean infarct size for hearts treated with PI3K inhibitor are approximately in a half-way point between the preconditioned and the non-preconditioned hearts. This difference has been enough to lose statistical significance, but one might speculate on the involvement of more than one signalling cascade to promote maximal protection, as it has been demonstrated that using a STAT-3 inhibitor also abolishes the protective effect of IPC (95). Rather than contradict, our results seems to complement the concept of having multiple protective pathways, namely both RISK and RISK-independent pathways. This may be important in establishing a multi-pathway stratagem when testing for further cardioprotective therapies (174).

In the light of these data, PI3K can be hypothesized to have a dominant role to mediate the IPC protective effect. On close examination, the activation of the p85 regulatory subunit of PI3K has been demonstrated to control the serine phosphorylation of STAT3, a critical step for the formation of stable STAT3 homodimers (303). STAT3 activation has also been shown to be dependent on PI3K recruitment in endothelial cells subjected to hypoxia/reoxygenation injury (304), thus suggesting that PI3K can regulate STAT3 phosphorylation. Despite this evidence supporting a predominant role for PI3K in IPC, other alternative pathways have been demonstrated to be involved in a RISK-independent

manner. Hence, cardiac-specific STAT3 deficient mice have been unable to show Akt phosphorylation following an IPC stimulus, and the pharmacologic preconditioning induced by TNFα have demonstrated protection through STAT3 phosphorylation without involving PI3K activation (92). It is understood that the activation of both the RISK and the SAFE pathway can occur concomitantly either in IPC or in pharmacologic conditioning (103), as they are not mutually exclusive., although it is unknown whether activation of the two pathways provides an additive effect to maximize the protection.

6.5.4 Limitations

Only one PI3K inhibitor was used, and its effect when administered alone was not tested, although many previous studies have shown that wortmannin does not have major effects on myocardial IS and protein phosphorylation by itself (86,155). Wortmannin may inhibit other kinases such as myosin light chain kinase or PI 4-kinase at concentrations higher than that required for inhibition of PI3K. With regard to protein analyses, not all phosphorylated residues were studied here - ie PTEN possesses three phosphorylation sites (Ser380, Thr382, and Thr383) and STAT3 can be phosphorylated at both its serine 727 and tyrosine 705 residues. Of note, many other kinases and proteases remain to be explored in this setting.

6.5.5 Summary and conclusions

In summary, PI3K activity is required during both the trigger and mediator phases for IPC to limit the infarct size. IPC increased the levels of Akt phosphorylation during both the trigger and mediator phases and this effect was fully abrogated by PI3K inhibition in both phases, whilst its downstream GSK3β was phosphorylated only during the trigger phase after IPC. Both PTEN and STAT3 were phosphorylated during both the trigger and mediator phases after IPC, but this was independent of PI3K. IPC increase ERK phosphorylation during both phases, being only PI3K-dependent during the trigger phase.

In conclusion, the PI3K cascade has a central role within the RISK pathway. This pro-survival kinase cascade is considered a unifying signalling pathway, as it is recruited not only by IPC, but also by pharmacological agents (66). Further elucidation of the

mechanisms underlying the IPC-induced protective effect is expected to reveal novel targets to promote myocardial salvage. Particularly, those mediators activated at early reperfusion have the potential to be modulated by pharmacological agents to benefit patients undergoing acute myocardial infarction (174). In this respect, the study of PI3K isoforms appears of utmost importance to identify specific molecular targets in cardioprotection. As it happens with PKC isoforms, where the ε isoform has demonstrated to be protective in the mediator phase (hence targetable using a ε-PKC activator) and the δ isoform has shown to be deleterious in the trigger phase (then targetable using δ-PKC inhibitors) (165,166,305), it may well be that PI3K isoforms have also an antagonistic role in this setting. The next chapters focus on the role of a specific isoform of PI3 kinase in cardioprotection, namely the PI3Kα

Chapter 7   DIFFERENTIAL PI3Kα AND PI3Kβ EXPRESSION IN HEART TISSUE AND CELLS

7.1   Background

Class I PI3K is a family of isoenzymes which produce the lipid second messenger PIP3 and transduce signals that control a variety of cellular events, such as cell survival. In the heart, multiple PI3K isoforms are expressed, each playing potentially distinct roles (207). Isoform selective inhibition is likely to show future important outcomes in the field of cardioprotection. Once the central role of PI3K in the cardioprotective effect conferred by IPC is elucidated (see Chapter 6), the question arises as to whether there is any isoform-specificity for the protective effect mediated by the RISK pathway.

As discussed in section 1.5.4, cardiomyocytes have largely been assumed to have a central role in cardioprotection. However, the premise of other cardiac cells either providing protection or being protected through cardioprotective interventions (i.e. conditioning phenomenon) seems equally plausible (121). Although the protective effect of the RISK pathway activation have been mostly attributed to occur in cardiomyocytes, little is known about its impact on other relevant cell types.

PI3K isoform expression in the heart has received little attention so far. The most highly expressed PI3K isoforms in the heart seems to be the α and β isoforms (206). Both α and β variants are expressed in both mouse cardiomyocytes (306) and endothelial cells (217), but no protein quantification have been reported to adequately compare their expression levels between cell types. Further, specific information is needed with regard to whether PI3K isoform expression pattern is comparable between species. In order to extrapolate future findings, differences in the expression of PI3K isoforms in mouse and human heart tissue should be brought into comparison; this information being largely lacking in the literature.

This chapter focuses upon the characterization of mouse PI3K α and β isoforms expression under basal conditions in both cardiomyocytes and mouse cardiac endothelial cells (MCECs), as well as in both mouse and human heart tissue.

7.2   Research objectives and experimental aims

Are PI3Kα and PI3Kβ expressed in mouse and human tissue? In which cell types?

<u>Hypothesis</u>

To support the hypothesis that a given PI3K isoform protects the myocardium from IRI, it is necessary to first demonstrate its expression in the mouse cardiac tissue. To determine whether these results can potentially be extrapolated to the clinical arena, it is important to ascertain whether its expression occurs in heart human tissue. Furthermore to evaluate whether this effect is cardiomyocyte or endothelial cell-dependent, it is also required to study PI3K isoforms expression in both cell types.

<u>Experimental aims</u>

The aim of this section is to characterize the protein levels of both PI3Kα and β in both mouse and human heart tissue as well as in both primary isolated cardiomyocytes and mouse cardiac endothelial cells. Below is an overview of the main aims, including the proposed models related to the research question of this chapter:

<u>Aim 1</u>: Investigate the percentage of PI3Kα protein in mouse and human heart tissue as well as in primary isolated cardiomyocytes and endothelial mouse cardiac cells

- Collect protein from tissue/cell types not being subjected to IRI protocols and use Western blot analysis to measure protein basal levels of PI3Kα, running the samples alongside p110α purified protein to extrapolate and quantify the amount of protein in each sample.

<u>Aim 2</u>: Investigate the percentage of PI3Kβ protein in mouse and human heart tissue as well as in primary isolated cardiomyocytes and endothelial mouse cardiac cells

- Collect protein from tissue/cell types not being subjected to IRI protocols and use Western blot analysis to measure protein basal levels of PI3Kβ, running the samples alongside p110β purified protein to extrapolate and quantify the amount of protein for each sample.

7.3 Methods

7.3.1 Experimental design and study protocols

A total of 20 samples were used for this experiment:

1) **Mouse heart tissue.** Five animals were perfused for 30 min in the Langendorff apparatus with modified Krebs-Henseleit buffer, without applying any intervention.

2) **Human heart tissue.** Five right atrial appendage samples were collected from patients undergoing cannulation for cardiopulmonary bypass either for CABG or valve replacement at submerged in Tyrode's buffer for 30 min (see section 3.8 for further details).

3) **Adult mouse ventricular cardiomyocytes.** Five samples of primary cardiomyocytes were isolated using liberase according to the protocol described in section 3.6. Once stabilized for 30 min, cells were collected for protein analysis.

4) **Mouse cardiac endothelial cells (MCEC, immortalized line).** Five samples of MCEC were passaged and subsequently cultured for 30 min according to the protocol described in section 3.7.

Once collected, samples were added to protein lysis buffer and the tissues were homogenized before proceeding to BCA protein quantification. Details on the Western blot protocol be found in section 3.9. A nitrocellulose blotting membrane (GE Healthcare Life Sciences, UK) was used for protein transfer. Primary antibodies used were: PI3 Kinase p110α (Cell Signaling, #4249), PI 3-Kinase p110β (Santa Cruz Biotechnology, sc-602) and anti-GAPDH (mAbcam 9484). The dilutions being used for each antibody were 1:1000, 1:500 and 1:20000, respectively.

7.3.2 Quantification of basal PI3Kα and PI3Kβ protein expression

In order to quantify protein expression, three different amounts of purified protein (1, 3 and 10 ng for PI3Kα and 5, 10, 20 ng PI3Kβ, both obtained from Merck Millipore) were loaded in the gels alongside the samples of interest. After being transferred, membranes were probed with specific primary antibodies for PI3Kα and PI3Kβ. For each membrane, PI3Kα and PI3Kβ values were extrapolated using a linear regression. Results were expressed as a percentage of the amount of protein studied out of total protein loaded.

7.3.3 Data analysis

Western blot data is described as the percentage of the PI3K isoform ± SEM. Normal distribution of the data was determined using the Shapiro-Wilk test for both data sets. Highly skewed distributed data was compared using the non-parametric Mann-Whitney test, whilst normal distributed data were compared using unpaired t test. STATA software, version 13.1 (Stata Corp, College Station, TX, USA) was used for the statistical analysis. GraphPad Prism version 6.00 (GraphPad Software, La Jolla California, USA) was used to perform the graphics.

7.4 Results

The basal expression of α and β isoforms of PI3K were studied in several tissues and cells. In order to assess the potential translation ability of subsequent results, we first examined the myocardial content of PI3Kα and PI3Kβ in both mouse and human heart tissue. PI3Kα is expressed in a similar proportion in both tissues (0.019 ± 0.003 % of total protein in the mouse sample was PI3Kα, whilst 0.021 ± 0.004 % of total protein in the human tissue was PI3Kα, P=0.767). In contrast, the myocardial content of PI3Kβ differed between groups, this isoform being having a ~3-fold increase in the mouse tissue (0.019 ± 0.005% of total protein was PI3Kβ for mouse tissue *vs.* 0.006 ± 0.003% for human tissue, P=0.039). Therefore, the α isoform of PI3K seem approximately equally abundant in both

tissues, unlike the β isoform which is further expressed in the mouse tissue. Figure 7-1 graphically illustrates these data, whilst Table 7-1 shows the overall raw data.

In order to study the type of cells potentially involved in PI3Kα- and/or PI3Kβ- mediated protective effect, the levels of these isoforms were then compared between adult ventricular mouse cardiomyocytes and mouse cardiac endothelial cells. Using this quantitative approach, mouse cardiac endothelial cells demonstrated higher PI3Kα levels in comparison to adult ventricular mouse cardiomyocytes (0.012 ± 0.001% of total protein was PI3Kα for cardiomyocytes vs. 0.029 ± 0.009 % for endothelial cells, P=0.047). Interestingly, PI3Kβ levels in mouse cardiac endothelial cells were also higher when compared to adult ventricular mouse cardiomyocytes, following a similar pattern (0.010 ± 0.004 % of total protein was PI3Kβ for cardiomyocytes vs. 0.036 ± 0.004 % for endothelial cells, P=0.001).
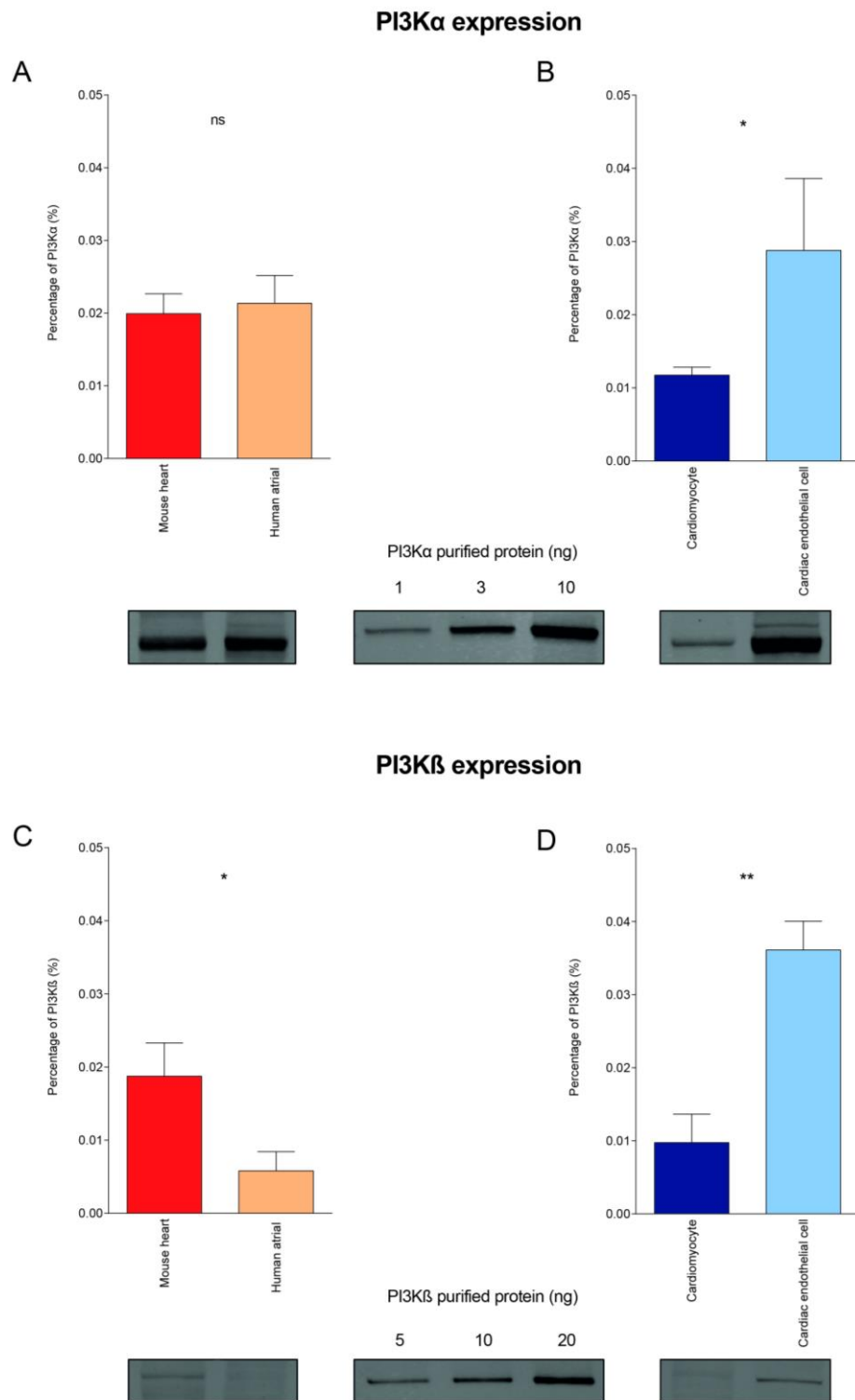
Figure 7-1: PI3Kα and PI3Kβ expression

PI3Kα expression in mouse and human tissues is depicted in panel A, whilst illustrated in panel B for cells. Panel C and D refer for PI3Kβ expression in tissues and cells respectively. Each bar represents an n=5. *P<0.05, **P<0.01, ***P<0.001, and ns, non-significant.

	Mouse heart tissue	Human atrial tissue	P-value	Adult mouse ventricular cardiomyocite	Mouse cardiac endothelial cell line	P-value
PI3Kα (%)	0.019 ± 0.003	0.021 ± 0.004	0.767	0.012 ± 0.001	0.029 ± 0.009	0.047
PI3Kβ (%)	0.019 ± 0.005	0.006 ± 0.003	0.039	0.010 ± 0.004	0.036 ± 0.004	0.001

Table 7-1: Raw data for protein expression of PI3Kα and PI3Kβ

Raw data related to Figure 1A to D.

Results are presented as mean ± SEM and expressed as percentage of protein (PI3Kα or β) relative to total protein in the sample.

7.5   Discussion

The main findings of this study can be summarized as follows: (1) PI3Kα is expressed in the same proportion in both mouse heart and human atrial tissue; (2) PI3Kα levels are higher in mouse cardiac endothelial cells than in adult ventricular mouse cardiomyocytes; (3) PI3Kβ protein content is higher in mouse heart tissue than in human atrial tissue; and (4) PI3Kβ is expressed in higher amount in mouse cardiac endothelial cells than in adult ventricular mouse cardiomyocytes.

As this PhD does not involve transgenic mice models (such as caPI3Kα or dnPI3Kα), but a more physiological approach using pharmacological inhibitors, it is important to actually quantify the expression of PI3Kα and PI3Kβ isoforms in the heart. Future results on the α isoform can be reasonably extrapolated from mouse to human based on the presence of similar amounts of the isoform, whilst caution should be taken when extrapolating the results obtained exploring the role of PI3Kβ. In subsequent experiments, Akt instead of PI3K will be assessed to evaluate PI3K activation. This is because it would be needed a convenient read-out of PI3K activation, and expression levels do not necessarily reflect the activity. Moreover, the catalytic subunit (p110) of PI3K, which determines the

isoform of the protein, is actually regulated by the regulatory subunit p85, which is phosphorylated when activated. Akt is a well-characterized shared target of PI3K, which is not isoform-specific. Therefore, Akt can be used routinely as a surrogate for PI3K activation and inhibition in line with most publications (194,212,225,307), and the PI3K isoform activity will be determined either using specific activators or inhibitors.

Beyond the importance of the comparison in the expression of PI3Kα and PI3Kβ between mouse and human tissue, other relevant subjects needs to be discussed, such as the differential expression of PI3K isoforms in non-cardiac and cardiac cells and tissues, as well as the novel findings on PI3K isoforms specific sub-cellular location.

7.5.1 Differential PI3K isoform expression in non-cardiac cells and tissues

Vanhaesebroeck and colleagues have tried to gain insight into the apparent non-redundant biological roles of class IA PI3K isoforms assessing their absolute protein amount in several mouse tissues (206) including muscle, liver, fat brain and spleen. They determined that the most abundant catalytic subunits were p110β (in liver, brain and fat) or p110δ (in spleen), with a lower abundance of 110α in most tissues tested (206). Unfortunately, they did not evaluate heart tissue. However, they observed two interesting findings in muscle tissue: (1) the absolute protein amount of PI3K in the muscle was clearly lower when compared with the rest of the tissues; and (2) the muscle was the only tissue showing PI3Kα as the predominant isoform, followed at short distance by PI3Kβ. Taking this observation with caution due to the gap between skeletal and cardiac muscle tissue, our results can be considered in the same line. In a separate study examining the specificity of insulin for the PI3Kα-Insulin receptor substrate activation, Foukas *et al.* performed quantitative immunoblot analysis of p110α and p110β expression in mouse liver, muscle and fat (202). They demonstrated that p110α levels were twofold higher than p110β in liver and muscle, but similar in adipose tissue.

7.5.2 Differential PI3K isoform expression in cardiac cells

Few publications have specifically determined the expression of PI3K isoforms in cardiac cell types. Two main studies have assessed differential PI3K isoform expression, one

in primary cardiomyocytes and the other in endothelial cells. Wenzel *et al.* demonstrated that all four class I PI3K isoforms are expressed in cardiomyocytes on the mRNA and protein level, although neither quantifications nor comparisons between levels of each isoform were performed (306). Vanhaesebroeck's group studied both the kinase activity and expression for each class IA isoform in mouse endothelial cells. They reported that p110α kinase activity is particularly high when compared to other isoforms, and preferentially induced by tyrosine kinase ligands (insulin), whilst p110β signals downstream of GPCR ligands, such as SDF1α (217). Using a qualitative assessment, p110α, p110β and p110δ protein expression was studied in both MCECs and HUVECs, demonstrating a similar qualitative expression of p110α and p110β, and a very low expression of p110δ, particularly when these cells were compared with leukocytes (217).

In comparison to the aforementioned studies, our results offer a quantitative assessment of the PI3Kα and PI3Kβ isoform and demonstrate that both isoforms are expressed in higher amount in mouse cardiac endothelial cells that in primary isolated cardiomyocytes.

7.5.3 Differential PI3K isoform sub-cellular location

Further layers of complexity can be added to PI3K isoform expression in cells by the existence of differences in their subcellular location and its degradation along an IRI insult.

Some publications suggest a differential subcellular location of PI3K isoforms. In mouse sympathetic and sensory neurons, p110α have been found predominantly localized at the plasma membrane, whilst p110β have been localized in the perinuclear region (308), therefore suggesting that the PI3K isoforms may be targeted to different subcellular organelles and may then mediate different physiological functions. In an *in vitro* cell model, Kumar et al. have suggested that nuclear, but not cytosolic PI3Kβ has an essential role in cell survival, through the association of the p85β regulatory subunit with the p110β catalytic subunit (220). Unfortunately, we did not test for the sub-cellular location of the PI3Kα and PI3Kβ isoforms in our study.

The protein levels of the catalytic subunit p110γ have been studied in a Langendorff mouse perfusion model at two time-points: following an

ischaemia/reperfusion insult and following an IPC stimulus. Interestingly, there was a drastic reduction in p110γ following IRI. In contrast, there was less protein levels reduction with IPC, suggesting that a potential mechanism of myocardial IRI is the selective degradation of p110γ, given that no changes in protein levels of other catalytic subunits p110α and p110β, or the class IA regulatory subunit p85 were observed following IRI or IPC (309).

7.5.4   Potential implications of the findings on cardioprotection

Temporally controlled overexpression of cardiac-specific PI3Kα have demonstrated to enhance contractility in the Langendorff-perfused mouse model (228). Moreover, the PI3Kα-Akt pathway can promote physiological exercise-induced growth (225). In a myocardial infarct-induced heart failure model, constitutively active PI3Kα has demonstrated to improve left ventricular function when compared to controls (227). All these observations, on top of the results being exposed in this chapter, suggest that PI3Kα may have a relevant role in cardioprotection. PI3Kβ is less expressed in human tissue, and therefore its future related results should be taken more cautiously if the aim is to translate the findings to the clinical setting.

7.5.5   Limitations

Western blot analyses were performed separately by two blocks according to isoform groups: samples for PI3Kα were run in the same gels, whilst samples for PI3Kβ were run in separate gels. This means that, despite being a quantitative assessment, only samples which were run in the same gels should be strictly compared – hence the amount of protein can be compared within cells and tissues for each isoform, but not between groups (i.e. the amount of PI3Kα should not be compared with PI3Kβ levels in a given cell or tissue). Moreover, it is needed to take into account that the PI3K antibodies used might have had different affinities for mouse and human PI3K isoforms, which would impact on the accuracy of the comparisons of isoform expression between human and mouse tissues presented above.

The method of protein quantification is based on a linear regression relationship between the amount of purified protein being loaded and their corresponding band intensity in the membrane. The values are extrapolated from this linear correlation and potentially subjected to some kind of systematic error. Therefore, differences between groups (i.e. mouse vs human heart tissue) should be more accurate than the absolute quantification of protein levels (i.e. $0.019 \pm 0.003\%$ for PI3Kα levels in mouse heart tissue).

Human heart tissue was obtained from the right atrium. Patients with arrhythmias were excluded based on a previous publication demonstrating that PI3K activation in atrial appendages from patients with atrial fibrillation was lower compared with tissue from patients in sinus rhythm (310). Despite carefully selecting patients without arrhythmias, some might argue that left ventricular tissue would better represent the human model.

Caution should be taken when extrapolating the results on an immortalized cell line (mouse cardiac endothelial cells) to a more physiological setting.

In this chapter, the focus was on the comparison of the class IA PI3K isoforms p110α and p110β. There is nonetheless an additional class IA isoform, p110δ, and the closely related class IB p110γ isoform that should be also taken into account. Although interesting, this is beyond the scope of this thesis.

7.5.6   Summary and conclusions

To our knowledge, this is the first study reporting comparisons in protein levels of PI3Kα and PI3Kβ between mouse and human heart tissue. PI3Kα is expressed in a similar proportion in both mouse heart and human atrial tissue, whilst PI3Kβ protein content is higher in mouse heart tissue than in human atrial tissue. These observations set PI3Kα as a potential target with translational value in cardioprotection.

Differences in protein levels between cardiomyocytes and mouse cardiac endothelial cells have also been reported. In general, cardiac endothelial cells present a higher content of either PI3Kα or PI3Kβ when compared with cardiomyocytes.

Chapter 8   ROLE OF PI3Kα IN ISCHAEMIC PRECONDITIONING

8.1   Background

Ischaemic preconditioning is a potent cellular endogenous protective mechanism consisting of transient cycles of sub-lethal coronary occlusion (ischaemia) and reperfusion (33). Its signalling architecture is considered the paradigm for cardioprotection (66) and has been extrapolated to most cardioprotective interventions.

The cellular mechanisms underlying the protection elicited by IPC against myocardial IRI have been the subject of intense research. Work has focused on identifying novel targets at different levels e.g triggers, mediators and end-effectors of IPC has been studied in the hope of producing agents mimicking their action. The mediators affording IPC have received special attention. Although several signal transduction pathways have been described (78,92), the RISK pathway is considered the central prosurvival kinase cascade mediating the IPC-induced protective effect. This pathway actually encompasses two parallel signalling cascades: PI3K-Akt and MEK1-ERK1/2 (66).

Within the RISK pathway, PI3K plays a dominant role in mediating the IPC protective effect, as illustrated in Chapter 6. Inhibitors of PI3K such as wortmannin (89) and LY294002 (155) have been very helpful for probing the function of PI3K in IPC, but as with all pharmacological inhibitor studies questions of specificity as to which specific isoform are being inhibited at the dosages being used are a concern. In recent years, isoform-specific PI3K inhibitors have been developed in the field of oncology (190) but these yet to been explored in the context of cardioprotection, which could aid in the development of PI3K isoform-specific agents enhancing such protection.

Class I PI3K is a family of lipid and protein kinases that phosphorylate PIP2 to form PIP3 to mediate cellular actions. Within this class, PI3Kα and PI3Kβ are well expressed in the heart (see Chapter 7), the former binding RTKs and the latter both RTK and GCPRs. PI3K isoforms convey distinct roles in cardiac physiology. PI3Kα has emerged as a key player in cardiac physiology, enhancing contractility (228,230), and promoting physiological exercise-

induced growth, but not pathological hypertrophy (226), whilst PI3Kβ has been mainly related to platelet biology (311).

In IPC, there is biphasic activation of PI3K signalling, first during the preconditioning cycles (or "trigger" phase) and then again during the first few minutes of reperfusion ("mediator" phase) (10,66,292). Insights into the role of PI3K isoforms in each phase can be gained by the use of isoform-specific pharmacological inhibitors (235–237). As it happens with PKC isoforms, where the ε isoform has demonstrated to be protective in the mediator phase (hence targetable using a ε-PKC activator) and the δ isoform has shown to be deleterious in the trigger phase (then targetable using δ-PKC inhibitors) (165,166,305), PI3K isoforms could potentially have either synergistic or antagonistic roles in IPC phases of cardioprotection.

Once having elucidated the role of PI3K in IPC as per Chapter 6 and the PI3K isoforms protein content in the heart as per Chapter 7, this chapter focuses on the identification of the specific role of PI3Kα in the protective effect elicited by IPC.

8.2   Research objectives and experimental aims

Is PI3Kα involved in the cardioprotective effect elicited by IPC either in the "trigger" or the "mediator" phase?

<u>Hypothesis</u>

As demonstrated in Chapter 6, PI3K is involved in the IS-sparing effect of IPC in both the "trigger" and the "mediator" phase. The hypothesis is that PI3Kα may have a different role (even antagonistic) in each crucial phase where the PI3K-Akt is activated following an IPC stimulus.

<u>Experimental aims</u>

The aim of this section is to systematically evaluate the role of PI3Kα in IPC at both time-points in both the *ex vivo* and *in vivo* models. Below is an overview of the main aims, including the proposed models related to the research question of this chapter:

Aim 1: Select PI3Kα inhibitors dose

- Collect protein from mouse hearts exposed to the PI3Kα canonical activator (insulin) and to several doses of two inhibitors to measure levels of Akt activation.

Aim 2: Investigate the role of PI3Kα in the IPC "trigger" phase in the ex vivo model

- Subject Langendorff-perfused mouse heart to ischaemia-reperfusion and subsequently quantify myocardial infarct size.

Aim 3: Investigate the role of PI3Kα in the IPC "mediator" phase in the ex vivo model

- Subject mouse Langendorff-perfused heart to ischaemia-reperfusion and subsequently quantify myocardial infarct size.

Aim 4: Investigate the role of PI3Kα in the IPC "mediator" phase in the in the in vivo model

- Subject mouse Langendorff-perfused heart to a non-recovery in vivo ischaemia-reperfusion and subsequently quantify myocardial infarct size.

Aim 5: Evaluate Akt activation following PI3Kα inhibition in both the IPC "trigger" phase the IPC "mediator" phase

- Collect protein from Langendorff-perfused mouse hearts subjected to IPC protocols and use Western blot analysis to measure levels of Akt activation.

8.3 Methods

8.3.1 Experimental design and study protocols

Below is an outline of the experimental designs being presented in this chapter. Further details and sample size estimations can be found subsequently in their corresponding figures and footnotes.

1) **Selection of PI3Kα inhibitors dose.** In order to study the role of PI3Kα in IPC using a pharmacologic approach, it is first necessary to determine the appropriate concentration of inhibitors that is effective in the Langendorff-perfused mouse heart model. The effect of PI3Kα inhibition was evaluated through its impact on Akt phosphorylation in response to the PI3Kα canonical activator, insulin. Langendorf-isolated mouse hearts were perfused during 15 min with modified Krebs and during 15 min more with one the following protocols using PI3Kα activator and inhibitors (3 animals per group): (1) Control (only modified Krebs); (2) Insulin 100 nM; (3) Insulin 100 nM with the co-administration of wortmannin 100 nM; (4) Insulin 100 nM with the co-administration of G326 1 µM; (5) Insulin 100 nM with the co-administration of G326 3 µM; (6) G326 3 µM; (7) Insulin 100 nM with the co-administration of BYL719 1 µM; (8) Insulin 100 nM with the co-administration of BYL719 3 µM; and (9) BYL719 3 µM. Samples were collected after finishing the protocol and processed to study Akt phosphorylation by Western blot analysis. Figure 8-1 illustrates the related study protocols.

2) **PI3Kα inhibition to abolish the IPC-induced cardioprotective effect on myocardial infarct size (*ex vivo*).** In the Langendorff-perfused mouse model, isolated hearts subjected to 35 min ischaemia and 2 h reperfusion received either G326 or BYL719 during the IPC protocol (which consisted of 4-cycles of 5 min ischaemia and 5 min reperfusion), or at reperfusion. G326 and BYL719 concentrations (3 µM) were chosen based on both previous publications (312–314) and in our dose-response characterization. Figure 8-2 and Figure 8-3, and their relevant footnotes summarize the study design for each set of experiments.

3) **PI3Kα inhibition to block the IPC-induced cardioprotective effect (*in vivo*).** We used an *in vivo* mouse model of myocardial infarction (40 min ischaemia and 120 min reperfusion) subjected to a 3-cycle IPC protocol, in the presence or absence of the PI3Kα inhibitor G326, administered through external jugular vein at reperfusion. Figure 8-4 outlines all the relevant information.

4) **Impact of PI3Kα inhibition in IPC-induced Akt phosphorylation (*ex vivo*).** In the Langendorff-perfused mouse model, hearts subjected to 15 min ischaemia and 5 min reperfusion, receiving either G326 or BYL719 during the IPC protocol or at

reperfusion. Akt phosphorylation were assessed separately for each set of experiments. Figure 8-5 and its corresponding footnote outlines the study design.
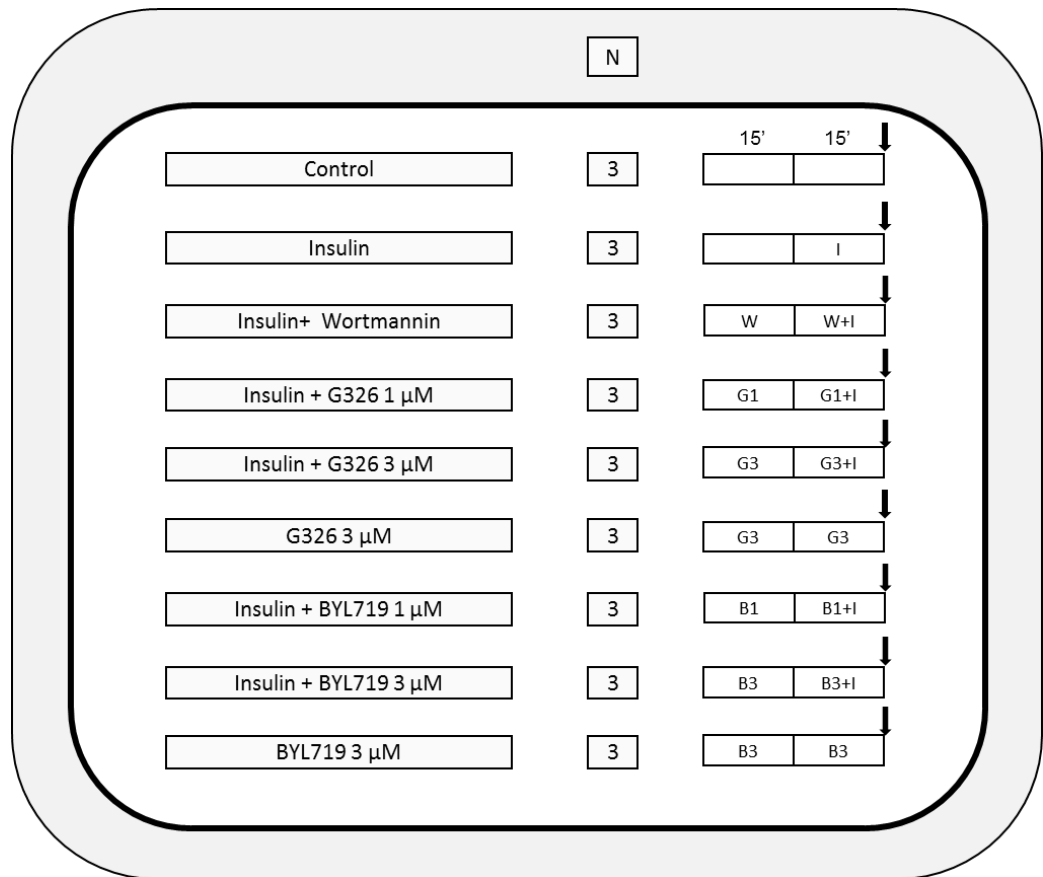
Figure 8-1: Overview of protocols aimed to select PI3Kα inhibitors dose

Overview of protocols performed to assess Akt phosphorylation using Western blot analysis. White empty boxes represent periods of perfusion with modified Krebs-Henseleit buffer at 80 mm Hg, whilst white written boxes represent periods of perfusion with the same buffer and the corresponding drug. No protocol of ischaemia/reperfusion injury was applied. Arrows represent the moment where samples were collected.

Akt and ERK phosphorylation levels were systematically studied in nine separate groups: (1) vehicle control; (2) insulin 100 nM; (3) insulin 100 nM in hearts pre-treated with wortmannin 100 nM; (4) insulin 100 nM in in hearts pre-treated with G-326 1 μM; (5) insulin 100 nM in in hearts pre-treated with G-326 3 μM; (6) BYL7193μM without insulin; (7) insulin 100 nM in in hearts pre-treated with BYL719 1 μM; (8) insulin 100 nM in in hearts pre-treated with G-326 3 μM; and (9) BYL719 3μM without insulin.

A sample size of 3 animals/group was pre-defined considering both the exploratory purpose of the experiment and the principles of the 3Rs (Replacement, Reduction and Refinement) for humane animal research.
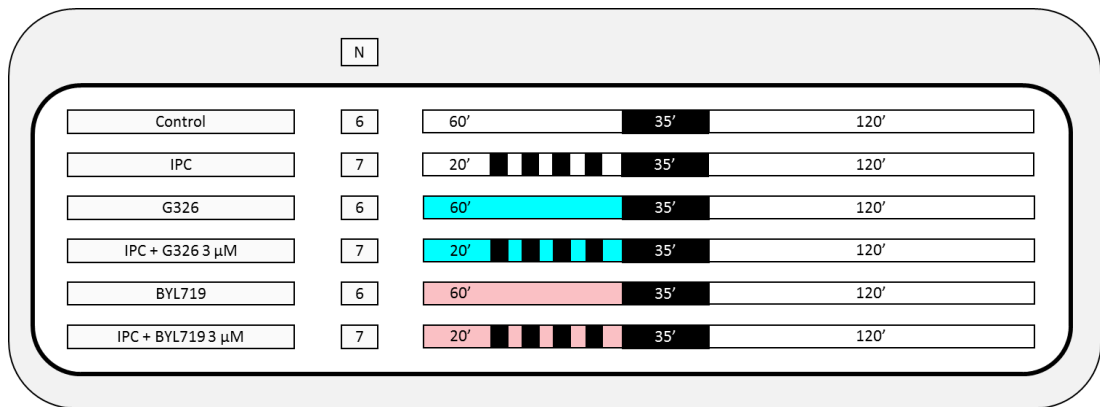
Figure 8-2: Study design for the assessment of PI3Kα inhibition during the IPC protocol

Overview of the Langendorff-perfused mouse protocols aimed to determine the effect on infarct size of PI3Kα inhibition at the "trigger phase" in preconditioned hearts. A black box represents a period of ischaemia and a white box represents a period of perfusion with modified Krebs-Henseleit buffer at 80 mm Hg.

Following 20 min stabilization, six different experimental protocols were tested: (1) vehicle control; (2) IPC 4 cycles of 5 min ischaemia and 5 min reperfusion per cycle; (3) G326 3 μM administered during 60 min; (4) IPC 4 cycles in the context of G326 3 μM administered during the stabilization period and IPC protocol; (5) BYL719 3 μM administered during 60 min; and (6) IPC 4 cycles in the context of BYL719 3 μM administered during the stabilization period and IPC protocol.

Based on previous results from our laboratory, the sample size for this experiment was estimated for performing a two-sided test for k-independents samples (ANOVA) following 5 pairwise comparisons (18% minimum expected effect size, common SD of 8%, α=0.05 and β=0.15, ~15% expected losses). Therefore, 42 animals were allocated according to a pre-specified randomization sequence (seed 588130, STATA software version 13.1). Three animals were excluded as they failed to meet inclusion criteria, hence each group included 6-7 animals.
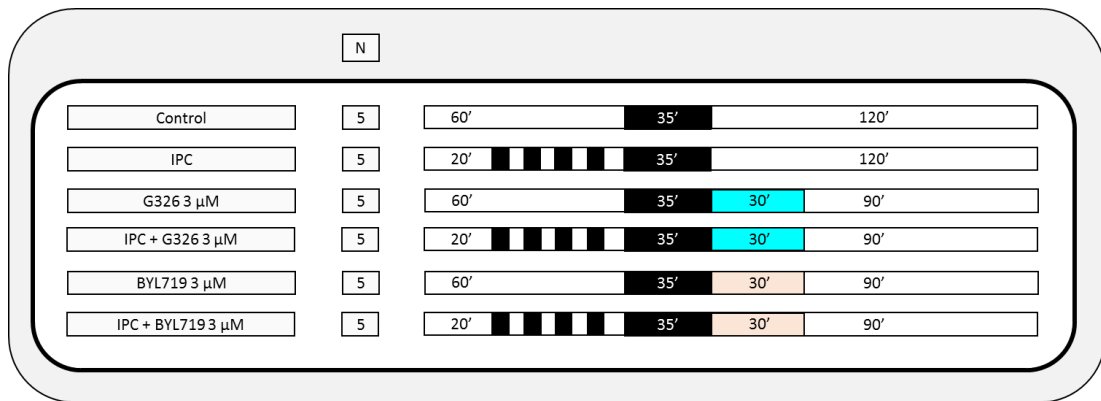
Figure 8-3: Study design for the assessment of preconditioned hearts with PI3Kα inhibition at reperfusion

Overview of the Langendorff-perfused mouse protocols aimed to determine the effect on infarct size of PI3Kα inhibition at the "mediator" phase in preconditioned hearts. A black box represents a period of ischaemia and a white box represents a period of perfusion with modified Krebs-Henseleit buffer at 80 mm Hg.

Following 20 min stabilization, six different experimental protocols were tested: (1) vehicle control; (2) IPC 4 cycles of 5 min ischaemia and 5 min reperfusion per cycle; (3) G326 3 µM administered upon reperfusion for 30 min; (4) IPC 4 cycles plus G326 3 µM administered at reperfusion for 30 min; (5) BYL719 3 µM administered upon reperfusion for 30 min; and (6) IPC 4 cycles in the context of BYL719 3 µM administered at reperfusion for 30 min.

Based on previous results from our laboratory, the sample size for this experiment was estimated for performing a two-sided test for k-independents samples (ANOVA) following 5 pairwise comparisons (18% minimum expected effect size, common SD of 8%, α=0.05 and β=0.20). In order to produce groups with equal numbers, animals were randomized after checking for the exclusion criteria. Therefore, 30 animals (n=5/group) were allocated according to a pre-specified randomization sequence (seed 93055, STATA software version 13.1). Two animals were excluded as they failed to meet inclusion criteria.
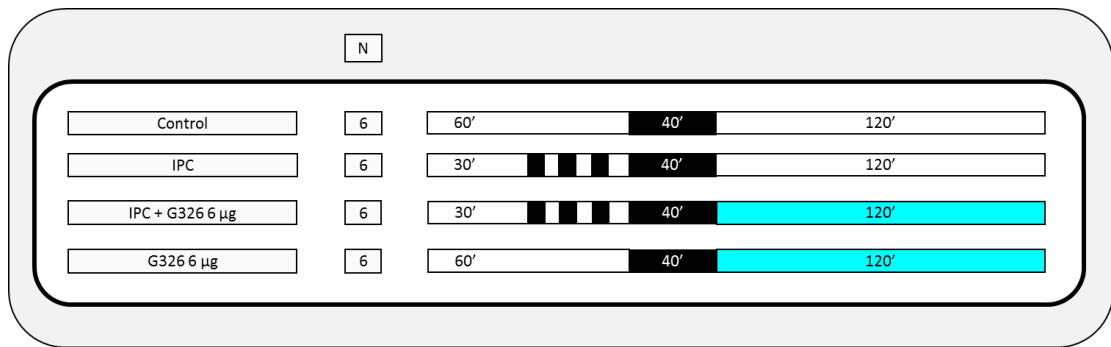
Figure 8-4: Study design for infarct size assessment protocols in preconditioned hearts in the context of PI3Kα inhibitors at the "mediator" phase (in vivo model)

Overview of the protocols related to the in vivo mouse model of ischaemia/reperfusion injury aimed to determine the effect on infarct size of PI3Kα inhibition at the "mediator" phase in preconditioned hearts. A black box represents a period of LAD occlusion (ischaemia) and a white box represents a period of LAD-non-occlusion (perfusion/reperfusion).

Following 20 min stabilization, four different experimental protocols were tested: (1) vehicle control; (2) IPC 3 cycles of 5 min ischaemia and 5 min reperfusion per cycle in the LAD; (3) IPC 3 cycles plus G326 3 µM administered at reperfusion; and (4) G326 3 µM administered at reperfusion.

IPC was induced by applying 3 cycles of 5 minutes ischaemia and 5 minutes reperfusion in the LAD. Vehicle control and IPC mice received 50 µl of 6% DMSO in saline (vehicle). G326 was dissolved in DMSO and injected via external jugular vein (6 µg per ~25g mouse) at reperfusion in groups 3 and 4.

Based on our previous experience and the results in the *ex vivo* model, the sample size for this experiment was pre-defined in six animals per group. Animals were randomized accordingly until reaching the planned sample size. Seven animals were excluded as they failed to meet inclusion criteria. A total of 33 mice were used for infarct experiments and were randomly assigned to treatment group. Seven mice died during the procedure and were therefore excluded from analyses (3 in control group, 3 in IPC and 1 in IPC+G326), hence each group included six animals.
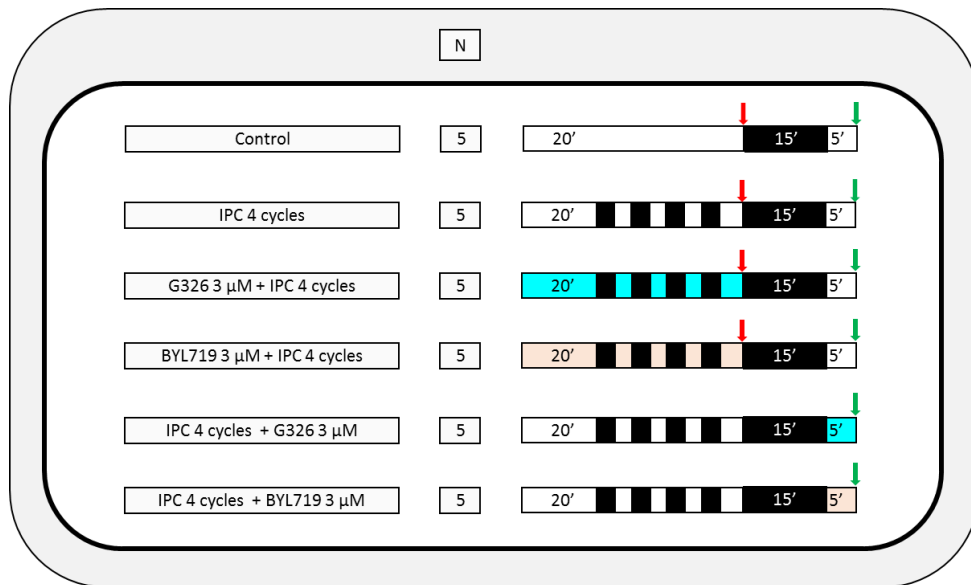
Figure 8-5: Study design and protocols to collect tissue for Western blot analyses to evaluate the role of PI3Kα in ischaemic preconditioning

Overview of protocols performed to assess Akt and ERK phosphorylation using Western blot analysis. Black boxes represent periods of ischaemia, white boxes represent periods of perfusion with Krebs-Henseleit buffer at 80 mm Hg and coloured boxes represent the perfusion of a given drug (turquoise for G326 and salmon for BYL719). Arrows represent the moment where samples were collected (in red all samples collected after IPC protocol, known as "trigger" phase; in green all samples collected at reperfusion, known as "mediator" phase).

Akt and ERK phosphorylation levels were systematically studied in two sections. In the first, samples were collected after finishing the IPC protocol (see red arrows in figure) and were classified into four groups: (1) vehicle control; (2) IPC 4 cycles; (3) IPC 4 cycles in the context of G326 3 µM; and (4) IPC 4 cycles in the context of BYL719 3 µM. In the second section, samples were collected after finishing a short protocol of ischaemia-reperfusion injury (see green arrows in figure) and were classified into six groups: (1) control; (2) IPC 4 cycles; (3) IPC 4 cycles with the administration of G326 3 µM during the "trigger" phase; (4) IPC 4 cycles with the administration of BYL719 3 µM during the "trigger" phase; (5) IPC 4 cycles with the administration of G326 3 µM during the "mediator" phase; and (6) IPC 4 cycles with the administration of BYL719 3 µM during the "mediator" phase.

A sample size of 5 animals/group was pre-defined in line with convention (257). Hearts were randomly assigned to each rig using a reproducible randomization sequence (seed 224466 for STATA version 13.1).

8.3.2 Data analysis

Infarct size data is expressed as mean±SEM, whilst protein phosphorylation data is reported in increase fold. Normal distribution of the data was determined using the Shapiro-Wilk test. Continuous data were compared either using one-way analysis of variance if normally distributed, or using the non-parametric Kruskal–Wallis test if highly skewed distributed. P-values for post hoc pairwise comparisons to the control group were adjusted using the Dunnett's test if normally distributed, or the Dunn's test if non-normally distributed. A P value of less than 0.05 was considered statistically significant. STATA software version 13.1 (Stata Corp, College Station, TX, USA), SPSS Statistics version 21 (IBM, Armonk, NY, USA) and GraphPad Prism version 6.00 (GraphPad Software, La Jolla California, USA) were used to perform the analyses and the graphics.

8.4 Results

8.4.1 Selection of PI3Kα inhibitors dose

To select adequate doses of PI3Kα inhibitors to be used, we applied a dose-response curve using G326 and BYL719, two distinct PI3Kα-selective inhibitors in the context of stimulation with insulin, a known canonical activator of PI3Kα. The results of these experiments indicate that the higher dose (3µM) of each drug is enough to inhibit Akt(Ser473) and Akt(Thr308) phosphorylation, when co-administered with its activator insulin. Hence, a similar phosphorylation to that observed without insulin or in presence of a pan-PI3K inhibitor, is observed when applying 3µM of the specific PI3Kα inhibitors (Figure 8-6). We therefore used these concentrations in the next experiment, either before or after global ischaemia/reperfusion to determine the relevance of the PI3Kα isoform phosphorylation in preconditioning.
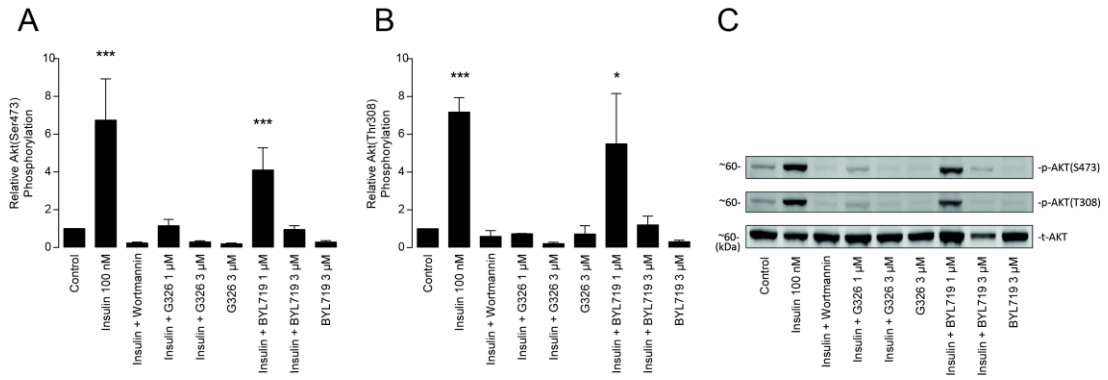
Figure 8-6: Effect of PI3Kα inhibitors on Akt phosphorylation

Panel A and B displays the effect of PI3Kα inhibitors on Akt phosphorylation(Ser473) and Akt phosphorylation(Thr308), respectively. Panel C displays a representative example of Western blot

Bar graph showing the percentage of phosphorylation increased in all groups compared to the control group, expressed as mean ± SEM (% of relative protein phosphorylation, normalized by its total), n=3 mice per group. Non-statistical differences were detected after using the Dunnett's post-hoc test. **P<0.01; ***P<0.001

Abbreviations: B, BYL719; C, control; G, G326; I, insulin, W, wortmannin.

8.4.2   PI3Kα inhibition in IPC

In the isolated Langendorff-perfused *ex vivo* mouse model, IPC reduced myocardial IS compared to control (49±4% vs 23±2%, P<0.001). When PI3Kα inhibition was undertaken <u>during the IPC protocol</u>, protection was not abolished neither with G326 (26±3%, P<0.001) nor with BYL719 (25±3%, P<0.001) (Figure 8-7). Neither drug affected IS on its own (G326: 52±4%, P<0.959; BYL719: 48±5%, P<0.997). In contrast, these same isoform inhibitors did block protection when given <u>at reperfusion</u> (G326: 50±3% and BYL719 47±4%, both non-significant when compared to their control group) (Figure 8-8).

This latter observation was confirmed in the *in vivo* setting when using the PI3Kα isoform inhibitor at reperfusion to block IPC protection (control 56±5% vs IPC+G326 60±5%, P=0.931, whilst IPC 29±6%, P=0.003) (Figure 8-9).

Table 8-1 summarizes the results of this section.

	Control	IPC	G326 3µm	IPC + G326	BYL719 3µm	IPC + BYL719
PI3Kα inhibition during IPC *ex vivo* ()	49.4±3.5	23.0± 1.8***	52.3±3.5	25.5±3.2***	47.9±4.5	25.0±3.0***
PI3Kα inhibition at reperfusion ex *vivo* ()	47.4±4.8	19.2±2.7	46.5±4.7	50.2±3.2	49.9±3.7	46.9±3.9
PI3Kα inhibition at reperfusion *in vivo* ()	56.3±4.6	29.4±5.8**	59.7±4.9	56.7±4.9		

Table 8-1: Raw data on myocardial infarct size following IPC and PI3Kα inhibition

Results are presented as mean ± SEM and comparisons were made against the corresponding control group (*P<0.05, **P<0.01, ***P<0.001).
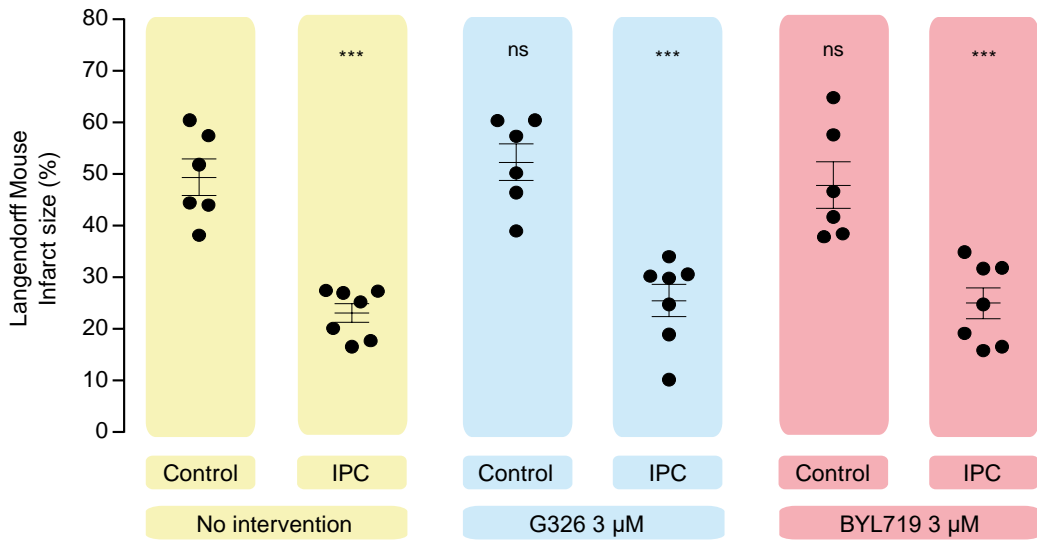
Figure 8-7: Impact on myocardial infarct size of PI3Kα inhibition during the IPC protocol *(ex vivo)*

Scatter dot blots: black lines represent mean ± SEM and circles represent individual animal data.
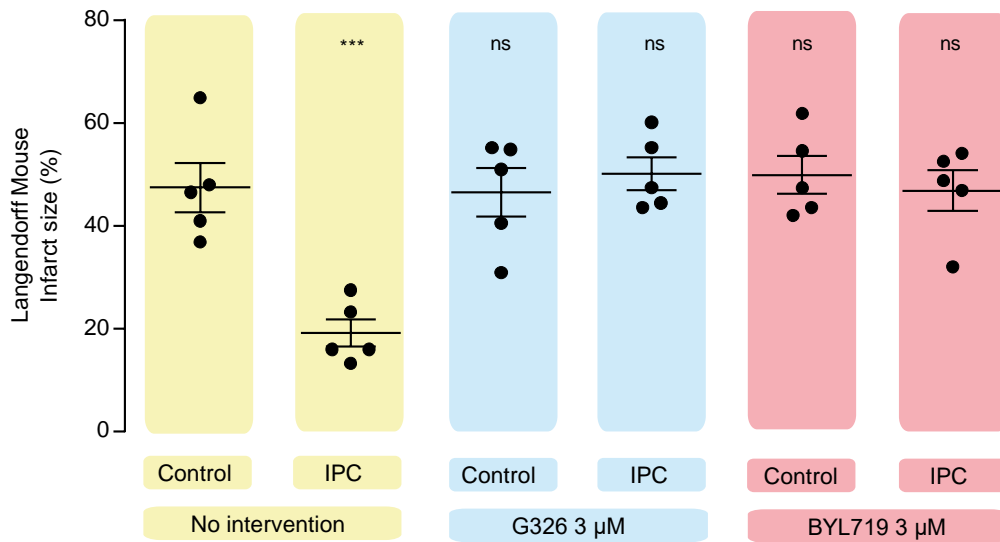
Figure 8-8: Impact on myocardial infarct size of PI3Kα inhibition at reperfusion *(ex vivo)*

Scatter dot blots: black lines represent mean ± SEM and circles represent individual animal data.
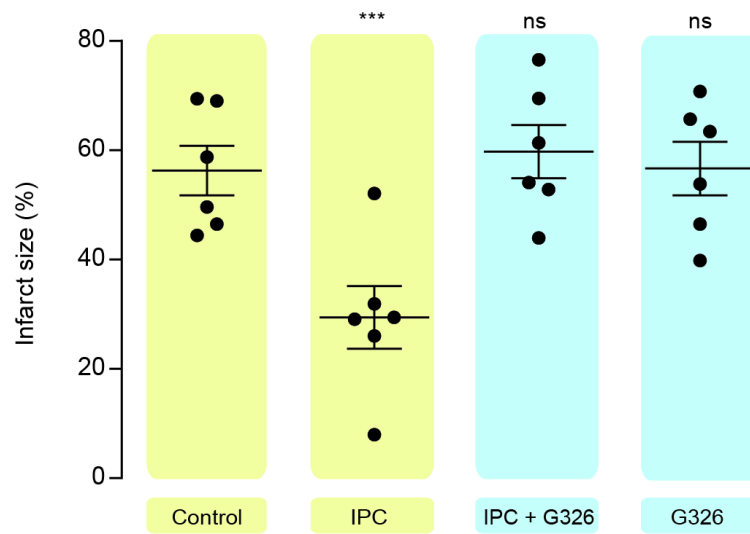
Figure 8-9: Impact on myocardial infarct size of PI3Kα inhibition at reperfusion *(in vivo)*

Scatter dot blots: black lines represent mean ± SEM and circles represent individual animal data.

8.4.3 Akt phosphorylation following PI3Kα inhibition in preconditioned hearts

In isolated Langendorff-perfused hearts, IPC increased Akt phosphorylation both after finishing the IPC protocol and at reperfusion (Figure 8-10). Unlike the results observed with regard to myocardial infarct size, PI3Kα inhibition blocked Akt phosphorylation also at both phases of PI3K activation.
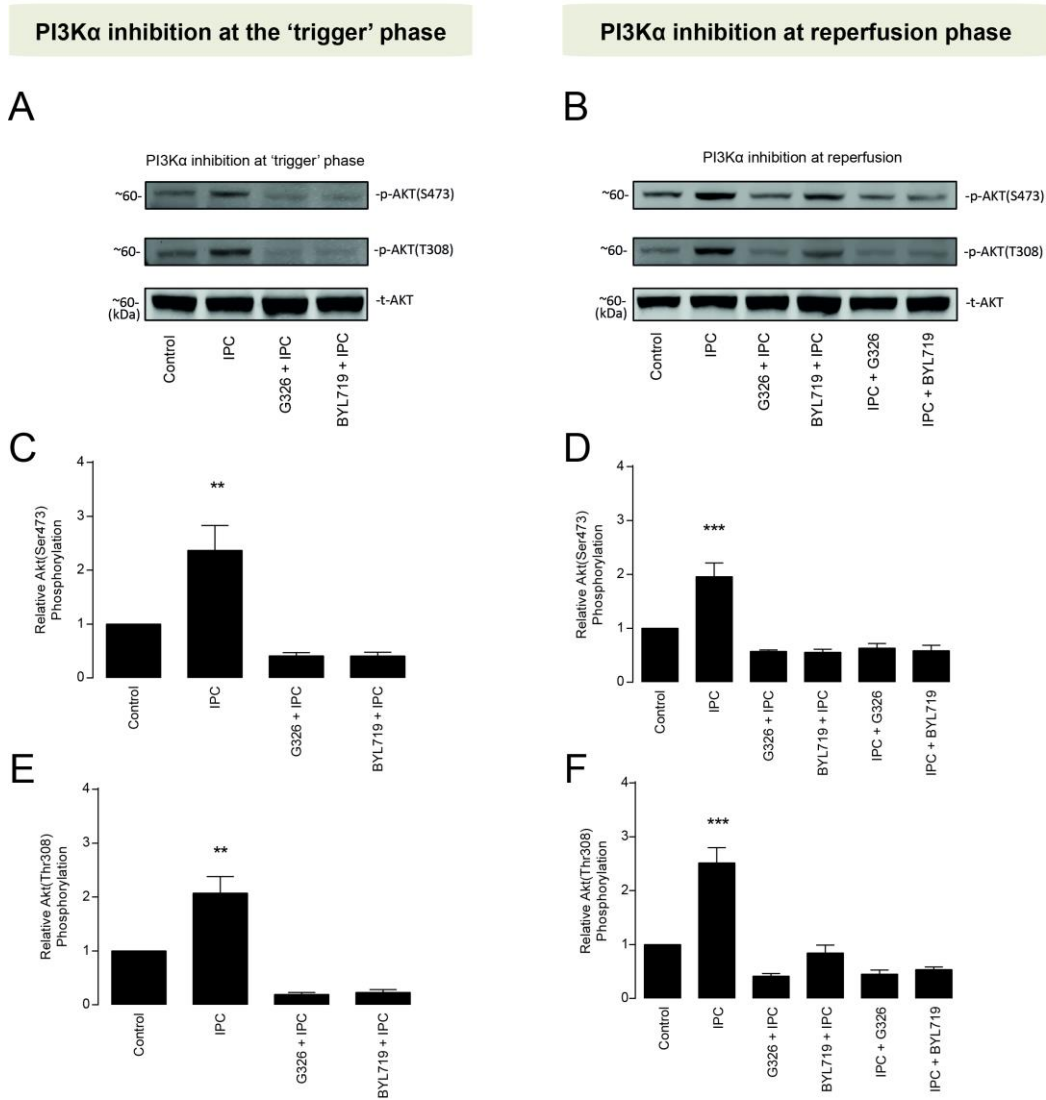
Figure 8-10: Akt phosphorylation following PI3Kα inhibition in preconditioned hearts

Panels A, C and E illustrate Akt activity measured *after IPC* in the presence or absence of PI3Kα inhibitors (ie: during the "trigger" phase), whilst Panels B, D and F illustrate Akt activity measured *at reperfusion* following PI3Kα inhibition during either the trigger phase only ("drug + IPC"), or at reperfusion ("IPC + drug").

8.5   Discussion

The main objective of this chapter was to assess whether PI3Kα inhibitors BYL719 and G326 abrogate the cardioprotective effect of IPC either in the trigger or the mediator phase of its biphasic response. Using a targeted pharmacological approach, we have shown that the PI3Kα isoform is required during the reperfusion phase to reduce myocardial infarct size, as it has been demonstrated with IPC in both the *ex vivo* and the *in vivo* model of myocardial infarction. Interestingly, IPC-induced protection was not abolished when PI3Kα inhibitors were administered during the trigger phase despite the phosphorylation of AKT being inhibited.

8.5.1   PI3K isoforms in IPC

PI3K activation is crucial in IPC-induced cardioprotection (Chapter 6). Despite the great body of evidence demonstrating the importance of PI3K as mediator of IPC, very little information is available on the particular roles played by its different isoforms in ischaemic conditioning. In the past, the signalling pathways recruited by IPC have been dissected using pharmacological approaches with broad-spectrum PI3K inhibitors wortmannin and LY294002, which when applied abrogated the IPC-induced protective effect (74,155). Both agents are highly selective for PI3K (315), although neither drug is isoform-specific. The specific role of the PI3K isoforms initiating receptor-mediating signalling in IPC have received little attention to date, these studies being based on genetically modified animal models. The number of pharmacological PI3Kα inhibitors that have been developed to treat cancer (231) have provided a unique tool to explore the role of this isoform in myocardial IRI and IPC.

In the field of cardiovascular physiology and disease, PI3Kα has been the focus of research in the subject of cardiac growth. McMullen's group have actively investigated this topic, concluding that PI3Kα-Akt promotes physiological exercise-induced growth, but antagonize pathological growth in a model of pressure overload or a transgenic mouse model of dilated cardiomyopathy (223–226). Interestingly, both the cardiac growth and pro-survival actions have been intimately related in the kinases being recruited in cardioprotection. In fact, Akt and ERK awoke the interest by Yellon's group about 15 years

ago due to their dual activity (see the origins of the RISK pathway in section 1.6.1). Following the same reasoning, PI3Kα isoform, which is known to have an important role in cardiac growth, becomes an attractive target to be used acutely in the setting of cardioprotection.

To our knowledge, there is only one study evaluating the importance of PI3Kα in the context of myocardial infarction. In a heart failure model induced by myocardial infarction, Lin *et al.* have demonstrated that increased PI3Kα activity can protect the heart against left ventricular dysfunction following a chronic myocardial infarction (227). Of note, this study was not evaluating PI3Kα after an IRI insult, but its role in improving the subsequent pathological remodelling and heart failure that results from the chronic coronary occlusion without reperfusion (227). In this study, caPI3Kα mice demonstrated less function impairment when compared to controls (227). Our observations showing that PI3Kα can reduce myocardial infarction, and their observation demonstrating that the activation PI3Kα can improve ventricular remodelling in the infarct tissue appears to be additive. In a sense, PI3Kα has been demonstrated to be protective acutely (against IRI) and chronically (against negative myocardial remodelling).

It is important to take into account that other PI3K isoforms have been postulated to play a relevant role in IPC. PI3Kγ has been shown to mediate the IPC-protective effect elicited by IPC. First, Tong *et al.* observed that transgenic mice with cardiac-specific overexpression of a catalytically inactive mutant PI3Kγ lose the protective effect conferred by IPC (232). Later, Ban *et al.* confirmed the importance of the PI3Kγ signalling cascade in IPC using PI3Kγ knockout mice (PI3Kγ$^{-/-}$) in a Langendorff-based model. They observed that mice lacking the γ isoform which underwent IPC resulted in a poorer functional recovery and greater tissue injury (measured by LDH release) compared to their wild-type and PI3Kγ$^{-/+}$ counterparts. Based on the importance of GPCR in IPC and the well-known protective effect of adenosine through GPCRs, they also studied the adenosine-mediated pharmacological preconditioning, suggesting PI3Kγ as its main mediator (309). To put the counterpoint, they also demonstrated in the same article that mice expressing a cardiac-specific kinase-deleted PI3Kα (PI3KαDN) were resistant to IRI, showing an already "preconditioned" status. This paper, which contradicts most of the results being exposed in these chapter, has several limitations: (1) the endpoint demonstrating myocardial injury

was not infarct size, but a less robust endpoint of functional recovery and LDH release, which cannot be fully correlated with the gold-standard infarct size as reported in Chapter 4; (2) In PI3Kγ$^{-/-}$ mice, IPC resulted in a statistically significant increase in LDH release compared to their PI3Kγ$^{-/-}$ littermates not subjected to IPC, therefore suggesting that IPC is deleterious in the absence of the γ isoform; and (3) the compensatory role of other isoforms cannot be ruled out in such a transgenic model, as the targeted isoform is chronically removed from their natural environment. Unlike genetic approaches, pharmacological strategies are able to provide novel information by permitting temporally restricted inhibition during either the trigger phase or at reperfusion. As IPC is mostly mediated by GPCR ligands at the trigger phase and the genetically modified animals have "chronically" deleted PI3Kγ, we might speculate that PI3Kγ, which involves GPCR-ligand activation, may mediate IPC in the "trigger phase", whilst PI3Kα, which involves tyrosine kinase activation, mediates IPC at reperfusion. Further studies are needed to confirm this hypothesis.

8.5.2 PI3Kα activity is abrogated at both IPC phases

Due to the technical challenges of directly measuring the PI3K product, PIP3, the phosphorylation of the downstream effector Akt has historically been used to indirectly assess the activity of PI3K, based on the assumption that this readout is proportional to PIP3 levels (312). In this study, Akt is used as a surrogate for PI3K activation and inhibition in line with published previous publications (194,212,225,307).

In this study, PI3Kα inhibitors did not abrogate the myocardial IS-limiting effect of IPC when used before index ischaemia, although they abolished its effect when used at reperfusion. In contrast, Akt phosphorylation was abrogated when PI3Kα was inhibited either before or after index ischaemia. This apparent conflicting data deserves further discussion. Assuming that the specific-PI3Kα inhibitors do not block other isoforms, it might happen that PI3Kα-induced Akt phosphorylation is not relevant to promote myocardial salvage during the trigger phase.

According to the results reported in Chapter 6, PI3K mediates both the trigger and the mediator phase of IPC. The primary action of PI3K is to rise PIP3 levels, which in turn

activates PDK1 and MTORC2 to actually phosphorylate Akt. It may well happen that the increase of PIP3 following PI3K activation by IPC do not only translate in Akt phosphorylation (which might be irrelevant in this phase), but in other unrevealed cellular mechanisms.

8.5.3   Limitations

These data predominantly rely on pharmacological manipulation, with all the inherent problems of target selectivity (199). Although it could be argued that genetically-modified animal models could also have been used to further examine the role of PI3Kα this would not have been possible due to the chronic deletion of the protein which would not allow us to focus on specific phases of the conditioning process - i.e. before ischemia and at reperfusion. To overcome the risk of off-target effects with inhibitors, we used two structurally unrelated inhibitors and saw the same results.

8.5.4   Conclusions

Our present results on the isoform specificity of PI3K in IPC strongly suggest that PI3Kα is critical for IPC-induced heart protection against IRI. In the *ex vivo* model of myocardial infarction, it has been demonstrated that PI3Kα is required during the IPC reperfusion phase to reduce myocardial infarct size, whilst this same isoform is not mediating the effect during the trigger phase. The importance of PI3Kα activation at reperfusion in IPC was confirmed in the *in vivo* setting.

Chapter 9   PI3Kα PHARMACOLOGICAL ACTIVATION AT REPERFUSION

9.1   Background

The ability to manipulate and up-regulate the RISK pathway during the early reperfusion phase may provide a potential approach to limiting reperfusion-induced cell death. From early studies, insulin has been shown to to mimic the IPC protective effect, hence becoming a promising pharmacological agent in the field of cardioprotection (316,317).

Insulin has been demonstrated to promote myocardial salvage when administered at reperfusion through the activation of the pro-survival kinase PI3K (86). The broad-spectrum PI3K inhibitor wortmannin has been useful to implicate PI3K as the main mechanism of the insulin-induced cardioprotective effect, either in *in vitro* model using rat neonatal cardiomyocytes subjected to simulated hypoxia/reoxygenation (318), the *ex vivo* model of Langendorff-perfused rat heart (86) and the *in vivo* rat model of acute myocardial infarction (319).

Importantly PI3Kα (and not PI3Kβ) has been identified as primarily responsible for the effects of insulin signalling (311). Despite PI3Kβ often being expressed at higher levels in insulin-responsive tissues (202), Foukas *et al.* reported an isoform-selective role of PI3Kα in insulin-signalling using mice expressing a kinase-dead version of the endogenous PI3Kα. The underlying mechanism proposed was a selective recruitment and activation of the catalytic subunit p110α over p110β to insulin receptor complexes (202). Knight *et al.* (203) used pharmacological tools to reach similar conclusions regarding the selectivity of insulin for PI3Kα signalling   - when adipocyte cells were stimulated with insulin, Akt phosphorylation still occurred despite PI3Kβ pharmacological inhibition (203). Further, Jia *et al.* observed little impact on Akt phosphorylation in response to insulin stimulation in mouse embryonic fibroblasts lacking PI3Kβ (212). Taken together, the findings of these studies support the concept of insulin activating Akt through PI3Kα, with no involvement of PI3Kβ.

After having elucidated the central role of PI3Kα in IPC in Chapter 8, this chapter focuses on the ability to protect the heart against IRI with PI3Kα activation at reperfusion.

9.2   Research aims and objectives

Can myocardial infarct size be reduced by targeting PI3Kα through its canonical activator at reperfusion?

Hypothesis

Activating PI3Kα activity upon reperfusion will phosphorylate Akt and therefore reduce myocardial infarct size.

Experimental aims

The overall objective of this chapter was to evaluate whether the pharmacological activation of PI3Kα through the exogenous administration of its canonical activator (insulin) confers a protective effect. Below is an outline of the main aims, including the proposed models related to the research question of this chapter:

Aim 1: *Assess the role of PI3Kα activation to protect the heart against IRI using both its canonical activator and its specific inhibitor*

- Subject mouse Langendorff-perfused hearts to ischaemia-reperfusion and subsequently quantify myocardial infarct size.

Aim 2: *Demonstrate Akt phosphorylation in response to PI3Kα pharmacological activation and inhibition*

- Collect protein from mouse Langendorff-perfused hearts subjected to ischaemia followed by reperfusion, and use Western blot analyses to measure phosphorylation levels of Akt and ERK.

9.3 Methods

9.3.1 Experimental design and study protocols

A total of 43 animals were used, although 3 hearts were excluded before randomization as they failed the predefined exclusion criteria (see below). Therefore, 41 animals were randomly allocated to treatment groups in two separate experiments:

1) **Study of infarct size.** Twenty-six animals were used to study the effect on myocardial IS of PI3Kα activation through insulin and PI3Kα inhibition through G326, at the dose determined in Chapter 8. Figure 9-1 presents an overview of the study protocols.

2) **Study of phosphorylated protein levels.** Fifteen animals were used to study the phosphorylated levels of Akt and ERK following PI3Kα pharmacological activation and inhibition, as summarized in Figure 9-2.

Animals used were male C57BL/6 mice (9-12 weeks, 24-28 g weight), all of them obtained pathogen free from one supplier and housed under identical conditions. G326 was obtained from Genentech through the Cancer Institute at UCL. Insulin solution human was purchased from Sigma-Aldrich and its concentration dose was chosen based on previous publications (74,86). Dimethyl sulfoxide from BDH (Poole, UK) was used as the solvent for G326, as well as a vehicle control for the rest of the groups.

Details on the protocol of both the ex vivo Langendorff-perfused mouse heart model and the Western blot technique being developed can be found in section 0 and section 3.9, respectively. Regarding the latter, proteins were transferred onto nitrocellulose blotting membrane (GE Healthcare Life Sciences, UK) using wet transfer and primary antibodies used were acquired either from Abcam, in the case of the loading control anti-GAPDH (mAbcam, #9484), or from Cell Signaling Technology: Akt (#9272), Phospho-Akt (Ser473) (#9271) and Phospho-Akt (Thr308) (#2965).
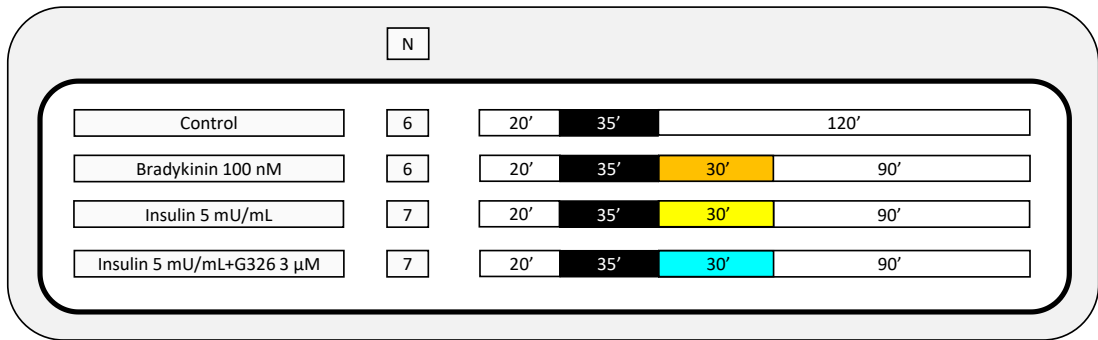
Figure 9-1: Study design and protocols aimed to evaluate the effect on myocardial infarct size of PI3Kα activation and inhibition in an *ex vivo* model of IRI

Overview of the Langendorff-perfused mouse heart protocols aimed to determine the effect on infarct size of PI3Kα activation at reperfusion. A black box represents a period of ischaemia and a white box represents a period of perfusion with modified Krebs-Henseleit buffer at 80 mm Hg. Following 20 min stabilization, four different experimental protocols were tested: 1) control; 2) bradykinin (well accepted pharmacological positive control)(252) for 30 min; 3) insulin (PI3Kα canonical activator) at a 5 mU/mL concentration for 30 min; and 4) insulin 5 mU/mL and G326 3 µM co-administered upon reperfusion for 30 min.

Colour code: coloured boxes represent the perfusion of a given drug (orange for bradykinin, yellow for insulin and turquoise for the co-administration of insulin and G326).
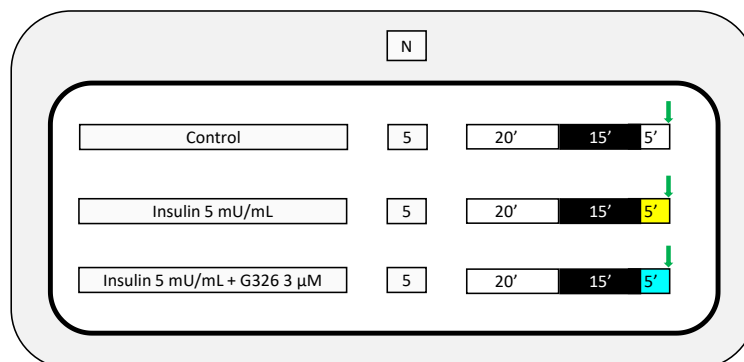
Figure 9-2: Study design for Akt and ERK phosphorylation analysis using Western blot

Three different experimental protocol were tested: 1) control; 2) Insulin 5 mU/mL at reperfusion for 5 min reperfusion; and 3) Insulin 5 mU/mL with the co-administration of the PI3Kα inhibitor G326 3 µM at reperfusion for 5 min reperfusion. Black boxes represent periods of ischaemia and white boxes represent periods of perfusion with Krebs-Henseleit buffer at 80 mm Hg. Green arrows depict the time-point of sample collection.

9.3.2 Sample size

The sample size for the experiment aimed to evaluate myocardial infarct size was estimated for performing a two-sided test for k-independents samples (ANOVA) following 3 pairwise comparisons (15% minimum expected effect size, common SD of 8%, $\alpha=0.05$ and $\beta=0.20$, ~15% expected losses). Therefore, 28 animals were allocated according to a pre-specified randomization sequence (seed 9147, STATA software version 13.1). Two animals were excluded as they failed to meet inclusion criteria, hence each group included 6-7 animals.

For Western blot experiments, a sample size of 5 animals/group was pre-defined in line with convention (257). Hearts were randomly assigned to each rig using a reproducible randomization sequence (seed 333 for STATA version 13.1).

9.3.3 Data analysis

Normal distribution of each data subset was confirmed using the Shapiro-Wilk method and continuous data were therefore compared using one-way analysis of variance followed by post hoc pairwise comparisons to the control group using the Dunnett's test. All values are presented as mean ± standard error of the mean. A P-value of less than 0.05 was considered statistically significant. GraphPad Prism version 6.00 (GraphPad Software, La Jolla California, USA) was used to perform both the analysis and the graphics.

9.4 Results

Once having established that PI3Kα is necessary to mediate the protection provided by preconditioning in Chapter 8, we evaluated whether activation of PI3Kα at reperfusion was sufficient to elicit a protective effect and examined whether insulin would mimic the cardioprotective effect afforded by IPC. When given at reperfusion, the canonical PI3Kα activator (insulin) reduced myocardial IS compared to control (25 ± 2 vs $55\pm4\%$, $P<0.001$) and this protection was abolished by G326 ($48\pm3\%$, $P=0.687$ compared to

control), as depicted in Figure 9-3. Accordingly, Akt was activated by insulin and blocked when G326 was co-administered at reperfusion following a protocol of IRI (Figure 9-4).
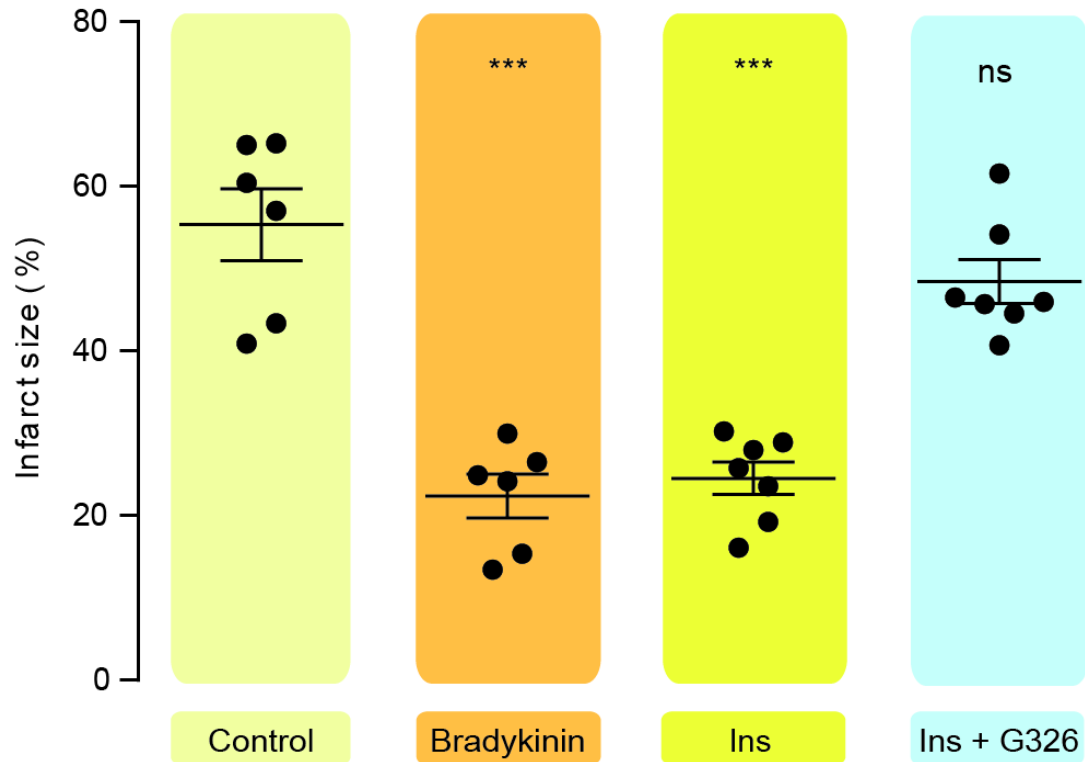
Figure 9-3: Impact of pharmacological PI3Kα activation at reperfusion on myocardial infarct size

Scatter dot blots: black lines represent mean ± SEM and circles represent individual animal data. When compared to control group (55.3±4.37), myocardial infarct size was significantly smaller with the administration of insulin (24.5 ±1.98, P<0.001) or the positive control bradykinin (22.4±2.67, P<0.001). The co-dministration of G326 and insulin abrogated the cardioprotective effect of the latter (48.4±2.66, P=0.687).
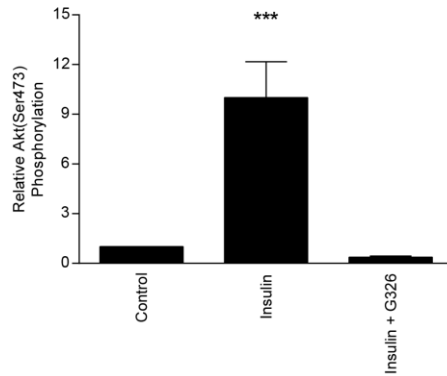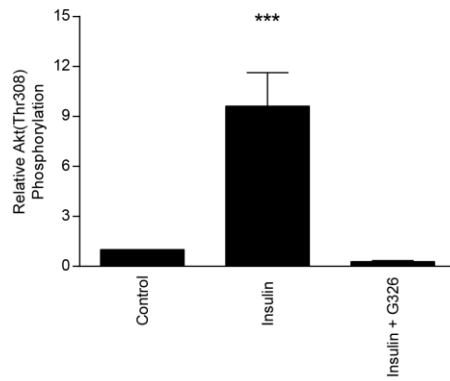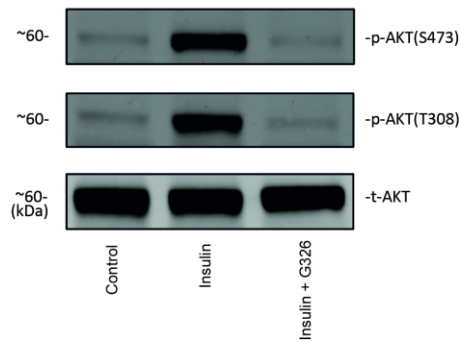
Figure 9-4: Impact of pharmacological PI3Kα activation at reperfusion on Akt phosphorylation

Panels A and B illustrate Western blot analyses for Akt(S473) and Akt(T308) respectively. Compared to control, insulin increased 10.0±2.2 –fold and 9.6±2.0-fold Akt(S473) and Akt(T308) respectively, although this effect was abolished with the co-administration of G326 (0.4±0.1 –fold and 0.3±0.6-fold, respectively) *P<0.05, **P<0.01, ***P<0.001, and ns, non-significant.

9.5 Discussion

The main findings of this study can be summarized as follows: (1) the exogenous administration of the PI3Kα canonical activator (insulin) at reperfusion reduced myocardial infarct size; (2) this cardioprotective effect was abolished with the co-administration of a PI3Kα specific inhibitor; and (3) the PI3Kα-induced protective effect is associated with Akt phosphorylation.

9.5.1 Insulin in previous cardioprotective studies: an historical perspective

The potential therapeutic use of insulin to protect ischaemic cardiomyocytes was proposed several decades ago by Sodi Pallares (320). This protective effect was first attributed to its ability to modulate glucose metabolism. The infusion of glucose-insulin-potassium (GIK), known as metabolic cocktail, was evaluated in acute myocardial infarction experimental models under the hypothesis that GIK reduces free fatty acids metabolism, therefore providing an optimal metabolic milieu to resist both ischaemic and reperfusion injury (321). However, research from Yellon, Jonassen and colleagues demonstrated later a "metabolically-independent" mechanism of GIK in protecting the heart. They hypothesized that the mitogen insulin, itself, promotes tolerance against IRI through the activation of innate cell-survival pathways in the heart, such as PI3K-Akt (316).

The case for insulin in cardioprotection greatly evolved at the turn of the century. In 1999, Baines *et al.* demonstrated that the activation of the insulin receptor reduced myocardial infarct size in the isolated rabbit heart when initiated at reperfusion and suggested the involvement of PI3K (74). In 2000, Jonassen and co-workers compared the temporal effects of administering GIK in an *in vivo* rat model of myocardial infarction and found that GIK therapy given at the onset of reperfusion reduced infarct size to the same extent as when GIK was administered throughout the entire ischaemia/reperfusion period (322). The same year (2000), Jonassen & Yellon demonstrated in simulated ischaemia/reoxygenation experiments in rat neonatal cardiomyocytes that the administration of insulin at the onset of reoxygenation enhances myocardial cell viability through anti-apoptotic effects mediated via tyrosine kinase and PI3K signalling pathways (318). In 2001, the same group demonstrated that insulin (and not glucose) was the major

component of the metabolic cocktail conferring cardioprotection, as they replaced glucose by pyruvate in the buffer of their isolated rat heart model of IRI (86). They also demonstrated that early administration of insulin during reperfusion (first 15 min) is enough to elicit protection against IRI (86). Taken together, these studies brought into question the exclusivity of the metabolic hypothesis concerning GIK combination's cardioprotective effects and placed insulin as an independent cardioprotective therapy.

The contribution of the data being presented in this chapter to this story is modest. In our study, PI3Kα has been revealed to be the specific PI3K isoform mediating the cardioprotective effect of insulin. On one hand, it was already known that insulin phosphorylated Akt through PI3Kα (202,203). On the other hand, it was also known that insulin was a cardioprotective agent (319,323–325). Using broad-spectrum PI3K inhibitors, it was demonstrated that this protective effect was mediated through PI3K in both *in vitro* and *in vivo* models (319,322). To our knowledge, this is the first study using a specific PI3Kα inhibitor to block the protective effect elicited by insulin against IRI.

9.5.2   Insulin: lost in translation?

A number of early clinical studies evaluating the effect of the metabolic cocktail yielded promising results in the "pre-reperfusion era", and a subsequent meta-analysis suggested that GIK therapy may reduce in-hospital mortality after acute myocardial infarction (326). In fact, this meta-analysis suggested that GIK could save 49 lives per 1000 patients treated for myocardial infarction (316,326). However, subsequent clinical trials failed to demonstrate the effect of insulin in the setting of acute myocardial infarction (327).

The cardioprotective potential of the GIK cocktail was examined in the large multi-centred randomized clinical trial comprising 20,201 patients undergoing PCI or thrombolysis for an STEMI, and was found to confer no beneficial effect in terms of mortality, cardiac arrest, cardiogenic shock and re-infarction at 30 days (328). This ambitious attempt to test the efficacy of GIK in acute myocardial infarction patients has been criticised for many reasons (reviewed elsewhere by Apstein & Opie) (329), the main being: (1) 17% of patients did not receive reperfusion therapy, (2) GIK was started late and

often post-reperfusion; and (3) the overall mortality was abnormally high (Killip class I reperfused patients presented with a mortality rate of 7.1%), six-fold higher than in contemporary studies (329).

The Immediate Myocardial Metabolic Enhancement During Initial Assessment and Treatment in Emergency Care (IMMEDIATE) trial recruited patients with suspected acute coronary syndrome and randomized them to GIK or placebo during transfer to the hospital. This trial also failed to demonstrate efficacy in its primary endpoint, although in the subgroup of patients presenting with STEMI, GIK significantly reduced cardiac magnetic resonance-evaluated infarct size (330).

In parallel to these clinical studies, further experimental research has been carried to assess the cardioprotective effect of insulin. In studies published in 2015 and 2017, insulin has been demonstrated to be effective in the swine model of IRI (331,332). Interestingly, one of these studies have shown insulin to have additive effects with remote ischaemic conditioning on infarct size reduction (332). The fact that we have identified PI3Kα to mediate the insulin-induced protective effect can move the focus from the use of insulin (a "dirty" drug with many side-effects, such as hypoglycaemia, hypokalemia, and catecholamine elevation) to the development of pharmacological agents specifically targeting PI3Kα. We are not proposing insulin to come to the fore again, but to take advantage of the the overwhelming evidence demonstrating its protective effect to further improve how to target its downstream signalling.

9.5.3   Limitations

These data entirely rely on a pharmacological approach, with all the inherent problems of target selectivity (199). Glucose could be replaced by pyruvate in the Krebs buffer to ensure insulin was the only protective agent, as Jonassen *et al.* did before (86). However, we preferred to be consistent with our isolated mouse heart model and infuse the same buffer that was administered in other experiments.

9.5.4 Conclusions

Taken together the conclusions drawn from Chapter 8 and Chapter 9, this thesis is the first example to show that PI3Kα is necessary and sufficient to confer cardioprotection: it is necessary for IPC to mediate cardioprotection, and it is sufficient for its activator insulin to promote myocardial salvage against IRI. The development of therapeutic agents that target downstream insulin-activated signalling molecules should be expected to promote myocardial salvage in patients with acute myocardial infarction.

Chapter 10 MECHANISM AND CLINICAL POTENTIAL UNDERLYING THE PROTECTIVE EFFECT OF PI3Kα

10.1 Background

Insulin has cardioprotective effects against myocardial IRI, as demonstrated in Chapter 9. Although the impact of insulin on cell viability has been studied in rat neonatal cardiomyocytes (318) and primary isolated cardiomyocytes (324) subjected to simulated hypoxia/reoxygenation, it has been always assumed that cardioprotective therapies, such as insulin, target mostly cardiomyocytes, although other cardiac cells may play an important role. In the same way, it has been always assumed that the signalling cascade orchestrating the cardioprotective effect of insulin in mouse models can be extrapolated to human models, although little is known in this regard.

Understanding the intracellular signalling pathways that control cardiomyocyte survival has been the focus of research in cardioprotection for many years. The end-effector of cardioprotection, namely the mitochondrial permeability transition pore (mPTP) has been identified as the molecule where several pathways eventually converge to protect against myocardial IRI (83,333). The mPTP is a non-specific pore of the inner mitochondrial membrane, which on opening causes cell death. Opening the mPTP permits water and solutes to enter the mitochondria, increasing matrix volume, and rupturing the outer mitochondrial membrane, leading to release of intermembrane cytochrome C release, which in turn initiates the necrosis and apoptosis. Further, its opening uncouples mitochondria, leading to ATP hydrolysis and collapse of the mitochondrial membrane potential (87). The mPTP is closed during ischaemia and only opens in the first few minutes of reperfusion, when the conditions for its opening are present: high concentration of mitochondrial calcium, ATP depletion, oxidative increase and increased matrix pH (84,109). The mPTP has been considered the central end-effector of the cell death-induced by IRI. It was initially shown that inhibiting mPTP opening at reperfusion with pharmacological agents (cyclosporine A and sanglifehrin-A) was protective (112,113). IPC was then shown to also have a protective effect which was mediated through mPTP inhibition (84,87). Later, it was demonstrated that the activation of PI3K-Akt pro-survival kinase pathway inhibits the

opening of the mPTP (254). Taken together, there is solid evidence demonstrating the link between the activation of these prosurvival kinase pathways (i.e. RISK pathway) and the mPTP to protect from myocardial IRI.

This chapter focuses upon elucidating whether PI3Kα can be manipulated in two cardiac cell types, as well as in human heart tissue. Further, the role of the end-effector mPTP following PI3Kα activation was also evaluated.

10.2 Research objectives and experimental aims

Is PI3Kα activated in both cardiomyocytes and endothelial cells?

Is the mPTP the end-effector of the cardioprotective effect of PI3K?

Are these findings of potential clinical relevance?

<u>Hypothesis</u>

Activating PI3Kα activity upon reperfusion phosphorylates downstream Akt and therefore reduces myocardial infarct size. The hypotheses we investigated are: (1) PI3Kα activation occurs specifically in either cardiomyocytes or MCECs, or non-specifically in both; (2) mPTP is the end-effector of the PI3Kα-Akt cascade; and (3) PI3Kα can be activated not only in mouse hearts, but also in human heart tissue.

<u>Experimental aims</u>

To investigate the pharmacological activation of PI3Kα in several cell types and tissues, as well as mitochondrial Permeability Transition Pore inhibition being a potential end-effector. Below is an outline of the main aims, including the proposed models related to the research question studied:

Aim 1: *Assess whether PI3Kα can be activated in both primary adult mouse cardiomyocyte and mouse cardiac endothelial cells (immortalized cell line)*

- Apply a pharmacological approach with both PI3Kα activator and inhibitor in both cell types, and use Western blot analyses to measure phosphorylation levels of Akt.

Aim 2: *Determine the effect of PI3Kα activation and inhibition on the susceptibility of cardiomyocytes to form the mPTP.*

- Use the TMRM-based confocal assay to quantify the time taken for the mPTP to open in isolated mouse cardiomyocytes in the context of a pharmacological approach with PI3Kα canonical activator and high-specific inhibitor.

Aim 3: *Assess whether the overall results can potentially be extrapolated to humans, activating and inhibiting PI3Kα in both mouse and human heart tissue.*

- Apply a pharmacologic approach with both PI3Kα activator and inhibitor in both cell types, and use Western blot to measure phosphorylation levels of Akt.

10.3 Methods

10.3.1 Experimental design and study protocols

The experiments in these chapter can be outlined in three blocks:

1) **PI3Kα activation in cardiomyocytes and MCECs.** Akt phosphorylation was evaluated by Western blot analyses after the pharmacological activation and inhibition of PI3Kα in the following cell types:

   o **Adult mouse ventricular cardiomyocytes.** Five samples of primary cardiomyocytes were isolated using liberase according to the protocol described in section 3.6. Once stabilized for 30 min, cells incubated for 15 min according to the following interventions: (1) vehicle control (0.01%

DMSO); (2) Insulin (5 mUl/l); (3) Insulin 5 mUl/l with 30 min pretreatment G326 3 µM; and (4) G326 3 µM.

- o **Mouse cardiac endothelial cells (MCEC, immortalized line).** Five samples of MCEC were passaged and subsequently cultured according to the protocol described in section 3.7. Prior to being subjected to pharmacologic stimulation, MCECs were deprived of serum for 2 h. As with cardiomyocytes, MCECs were incubated for 15 min with: (1) vehicle control (0.01% DMSO); (2) Insulin (5 mUl/l); (3) Insulin 5 mUl/l with 30 min pretreatment G326 3 µM; and (4) G326 3 µM.

2) **PI3Kα translational ability.** Akt phosphorylation was evaluated by Western blot analyses after the pharmacological activation and inhibition of PI3Kα in the following tissues:

- o **Mouse heart tissue.** Five mouse Langendorff-perfused hearts were stabilized for 20 min using the Langendorff apparatus and then perfused for 15 min with: (1) vehicle control (DMSO) perfusion; (2) Insulin (5 mUl/l); (3) Insulin 5 mUl/l and G326 3 µM; and (4) G326 3 µM.

- o **Human heart tissue.** Five right atrial appendage samples were collected from patients undergoing cannulation for cardiopulmonary bypass either for CABG or valve replacement (see section 3.8 for further details). After being collected in the operating theatre, human atrial tissue was dissected into four pieces and swiftly submerged in previously oxygenated Tyroids' modified buffer (118.5 mM NaCl, 24.8 mM NaHCO3, 4.7 mM KCl, 1.44 mM MgSO4.7H20, 1.2 mM K2HPO4, 1.8 mM CaCl2, 10 mM pyruvate, 10 mM D-Glucose) for 30 min in the presence of: (1) vehicle control (DMSO); (2) Insulin (5 mUl/l); (3) Insulin 5 mUl/l and G326 3 µM; and (4) G326 3 µM. Of note, the tissue was not perfused, but superfused and under ongoing temporal hypoxia.

  Once collected, samples from the above blocks were added to protein lysis buffer and the tissues were homogenized before proceeding to BCA protein quantification. Details on the Western blot protocol be found in section 3.9. A

nitrocellulose blotting membrane (GE Healthcare Life Sciences, UK) was used for protein transfer. Primary antibodies used were Akt (#9272, Cell Signaling Technology), Phospho-Akt (Ser473) (#9271, Cell Signaling Technology), and anti-GAPDH (#9484, Abcam).

3) **Impact of PI3Kα activation on the end-effector mPTP.** The sensitivity of the mPTP to opening was assayed using a well-characterised and reproducible cellular model of ROS-mediated mPTP opening (334), described in detail in section 3.10. Adult mouse ventricular cardiomyocytes were isolated as described. Live cardiomyocytes were incubated with the fluorescent dye TMRM at 12 μM for 15 min in Hepes based recording buffer (NaCl 156 mM, KCl 3 mM, MgSO4.7H20 2 mM, K2HPO4 1.25 mM, CaCl2 2 mM, HEPES 10 mM and D-Glucose 10 mM; pH 7.4), then washed and randomly treated for 15 min into the following groups: (1) Vehicle control; (2) insulin 5 mU/mL; (3) insulin 5 mU/mL with G326 3μM; and (4) G326 3μM alone. Once washed for a second time, mouse cardiomyocytes were stimulated with laser illumination and imaged using confocal microscopy. The time to reach half peak signal was recorded in seconds and compared across groups. A total of 19 ± 2 cardiomyocytes were analyzed for each intervention in each experiment (n=8 mice).

10.3.2 Sample size and data analysis

The sample size for the assay evaluating the sensitivity of the mPTP to opening was estimated for performing a two-sided test for k-independents samples (ANOVA) following 3 pairwise comparisons (25% minimum expected effect size, common SD of 15%, α=0.05 and β=0.20). Therefore, 8 successful cardiomyocyte isolations were needed to make solid conclusions. Three isolations were needed to set up the model, and three further isolations were excluded to either poor quality or quantity. For Western blot analyses, a sample size of 5 per group was pre-defined in line with convention (257).

Normal distribution of each data subset was tested using graphical methods and the Kolmogorov –Smirnov method. All values are presented as mean ± standard error of the

mean. If normally distributed, continuous data were compared using one-way analysis of variance followed by post hoc pairwise comparisons to the control group using the Dunnett's test. If highly skewed distributed, the non-parametric Kruskal–Wallis test was used with subsequent post hoc pairwise comparisons to the control group adjusted by the Dunn's test. A P value of less than 0.05 was considered statistically significant. GraphPad Prism version 6.00 (GraphPad Software, La Jolla California, USA) was used to perform both the analyses and the graphics.

10.4 Results

10.4.1 PI3Kα can be activated in mouse cardiomyocytes and cardiac endothelial cells

Once the central role of PI3Kα in cardioprotection had been established, we aimed to study whether the activation of the α isoform of PI3K was specific for cardiomyocytes or whether it could be extended to the mouse cardiac endothelial cells. As illustrated in Figure 10-1A and Figure 10-1B, PI3Kα activation was approximately twofold higher in the immortalized endothelial cells compared to the primary isolated cardiomyocytes. These results are in accordance with the higher protein content demonstrated in MCECs in Chapter 7.

10.4.2 PI3Kα can be activated in mouse and atrial human tissue

In order to assess the potential translation ability of PI3Kα activation, we collected human right atrial appendage samples and tested whether the PI3Kα pathway could be stimulated (patient baseline characteristics are outlined in Table 10-1). Human tissue was proven to respond to PI3Kα activation and inhibition, although to a less extent that the mouse heart tissue (Figure 10-1C and Figure 10-1D). Of note, the human tissue was not perfused, but superfused, and under ongoing temporal hypoxia whilst being transferred to the laboratory (Akt was therefore already activated in controls), whilst mouse heart tissue was perfused with modified Krebs buffer and controlled under non-ischaemic conditions.

10.4.3 PI3Kα activation delays the mPTP opening

The sensitivity of the mPTP to opening was assayed in adult mouse ventricular cardiomyocytes using a well-characterized cellular model of ROS-mediated mPTP opening. In the presence of insulin, the time taken to induce mPTP opening (a surrogate for cell death) was significantly increased compared to the control (571 ± 30 s vs 459 ± 25 s, P< 0.013), whilst the treatment with the PI3Kα inhibitor G326 no longer delayed the time taken to induce mPTP opening (455 ± 24 s, P=0.999) (Figure 10-2). The drug had no effect on its own (467 ± 25 s, P=0.993). These results suggest that PI3Kα kinase cascade promotes cardiomyocyte survival through the inhibition of the end-effector, mPTP.
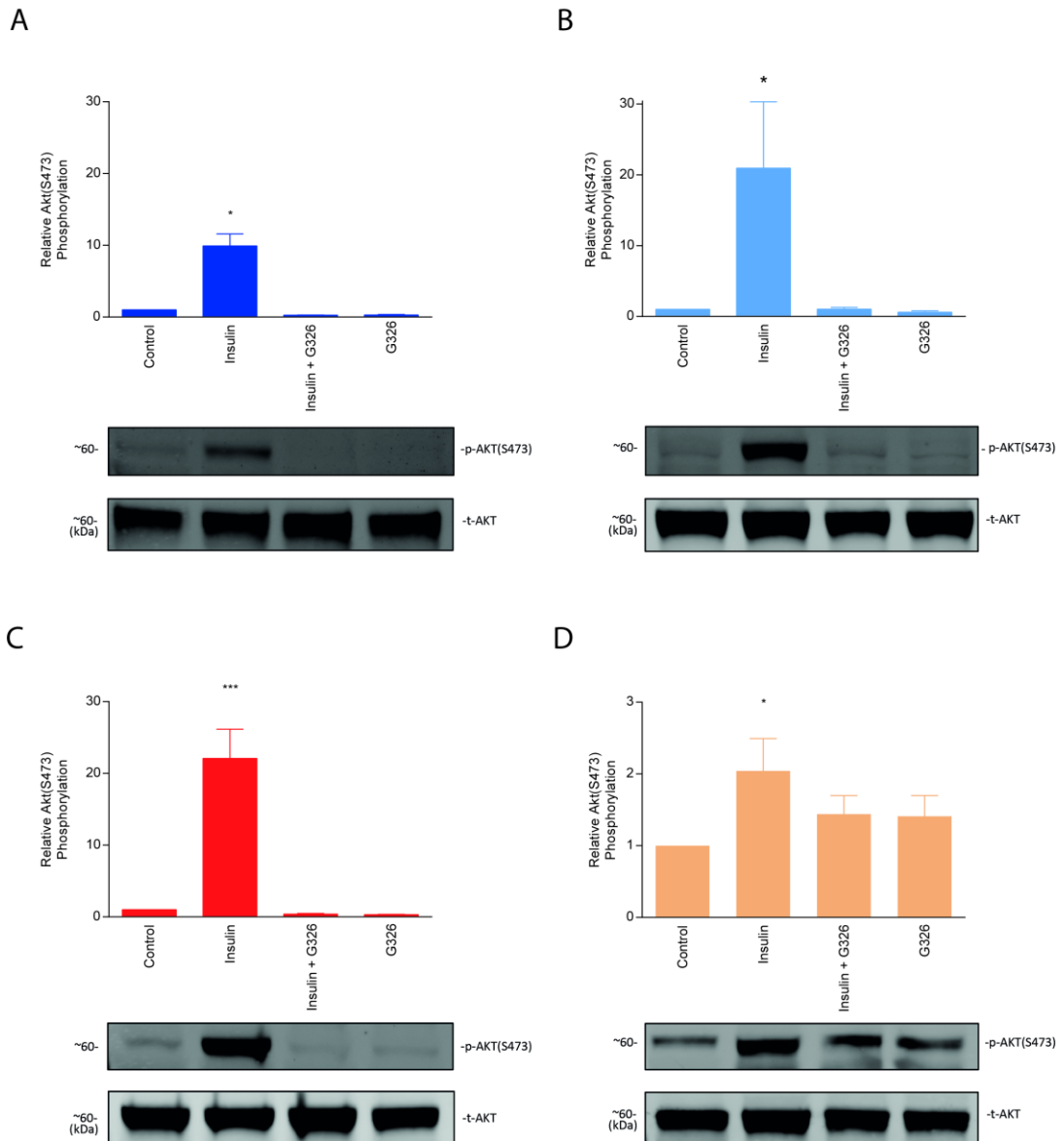
Figure 10-1: Pharmacological PI3Kα activation in cells and tissues

A pharmacological approach with the PI3Kα canonical activator and the G326 α-specific inhibitor was applied in adult mouse ventricular cardiomyocytes (Panel A), mouse cardiac endothelial cells (Panel B), isolated-perfused mouse heart tissue (Panel C) and human atrial tissue (Panel D). *P<0.05, **P<0.01, ***P<0.001, and ns, non-significant.
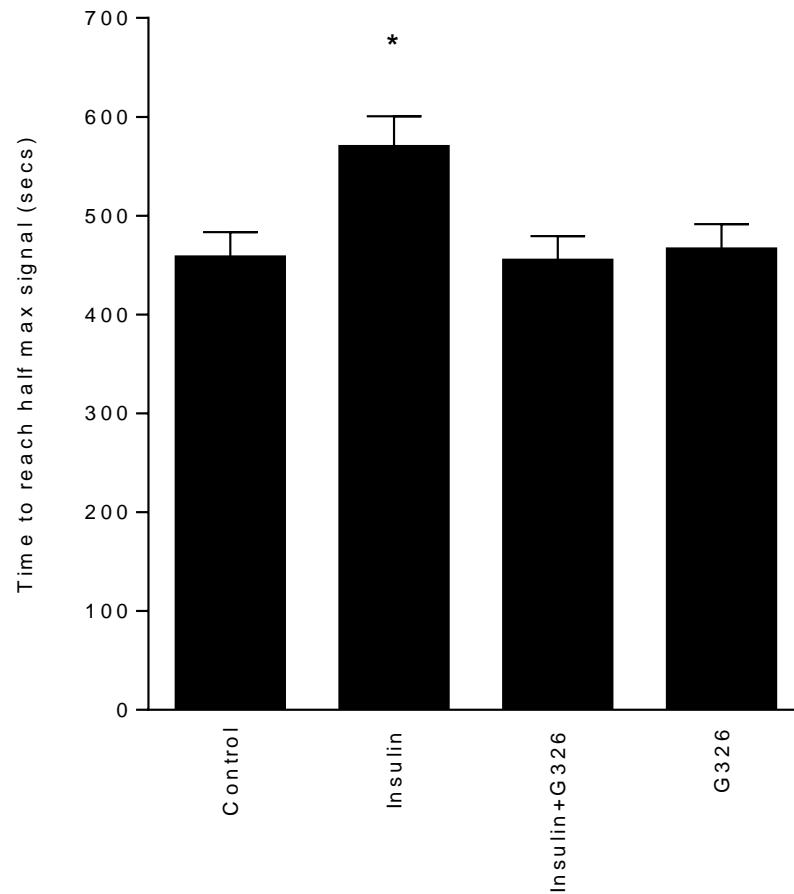
Figure 10-2: Effect of PI3Kα activation and inhibition on mPTP opening in primary isolated mouse cardiomyocytes

This figure depicts the graphical representation of the calculated time for mPTP opening in each group. Isolated mouse cardiomyocytes were loaded with 12 µM TMRM, and randomized into 4 groups (control; insulin 5 mU/mL; insulin 5 mU/mL in G326 3 µM pre-treated cells; and G326 3 µM; n=8/group) and exposed to constant confocal laser illumination. *p<0.05.

	Total patients (n=5)
Age	64.2 ± 18.6
Gender (male)	4 (80%)
Diabetes mellitus	1 (20%)
Dyslipidemia	2 (40%)
Hypertension	5 (100%)
Smoking history	1 (20%)
Prior cardiovascular disease	2 (40%)
Preserved LVEF (>50%)	4 (80%)
Sinus rhythm	5 (100%)
Surgery	
CABG	3 (60%)
AVR	1 (20%)
CABG + AVR	1 (20%)
Medications	
Beta blocker	4 (80%)
ACE inhibitor	2 (40%)
Calcium channel blocker	1 (20%)
Statin	4 (80%)
Antiplatelet therapy	4 (80%)

Table 10-1: Patient baseline characteristics

Data expressed as number (%) or mean ± SD.

AVR, aortic valve replacement; CABG, coronary artery bypass graft; LVEF, left ventricular ejection fraction.

10.5 Discussion

The main findings of this study can be summarized as follows: (1) PI3Kα canonical activator (insulin) increased the phosphorylation of Akt, which was abolished using the specific-PI3Kα inhibitor G326, in both primary cardiomyocytes and mouse cardiac endothelial cells; (2) in adult mouse ventricular cardiomyocytes, PI3Kα activation delays the mPTP opening time, which is abolished using the specific-PI3Kα inhibitor G326; and (3) PI3Kα can potentially be activated in human heart tissue.

10.5.1 PI3Kα can be modulated in both cardiomyocytes and mouse cardiac endothelial cells

As discussed in Chapter 10, insulin has previously demonstrated to mediate its cardioprotective effect through PI3K in *in vitro* (318), *ex vivo* (86) and *in vivo* models (319). Broad-spectrum PI3K inhibitors have been used to evaluate this acute effect. At cellular level, insulin can protect rat neonatal cardiomyocytes (318) and primary isolated cardiomyocytes (324) subjected to simulated hypoxia/reoxygenation. There is little information regarding the specific role of the endothelial cells in the insulin-induced protective effect, although the involvement of the eNOS isoform (highly expressed in these cells) in *in vivo* experiments suggest its potential implication. In this study, we have demonstrated that PI3Kα can be modulated (activated and inhibited) in primary isolated cardiomyocytes and immortalized mouse cardiac endothelial cells, although we lack evidence on to wehter insulin increases cell viability in MCECs.

The next step was to study the impact of PI3Kα activation in cell survival through its end-effector mPTP. In 2006, work carried out in The Hatter Cardiovascular Institute unveiled the relationship between the activation of the pro-survival kinase pathway by insulin and the probability of mPTP opening upon reperfusion. Using TMRM to induce oxidative stress in primary isolated adult rat ventricular cardiomyocyctes, they demonstrated that insulin delays mPTP opening and that this effect is prevented by wortmannin or by LY294002 (broad-spectrum PI3K inhibitors), by SH-6 (Akt inhibitor) and by L-NAME (inhibitor of nitric oxide production) (254). Further, the expression of a dominant negative construct of Akt abolished the effect of insulin in delaying mPTP

opening in a cardiac cell line (HL-1) and the overexpression of constitutively active Akt was sufficient to mimic the insulin-induced effect (254). Taken together, this seminal study linked the pro-survival pathway PI3K-Akt (considered a mediator according to the signal transduction pattern of cardioprotection described in section 1.5.1) and the mPTP (considered an end-effector). Our modest contribution to this story is to have taken advantage of the both the reports linking insulin with PI3Kα (202,203) and the development of PI3Kα specific inhibitors (231) to selectively target this isoform. Our results suggest that PI3Kα signalling promotes cardiomyocyte survival through the inhibition of the end-effector mPTP. In a simulated model of reperfusion injury, PI3Kα activation impacts on mPTP opening, delaying cell death.

10.5.2 PI3Kα fits the translational RISK profile for cardioprotection

The translational perspective of our results are highlighted by the observation that PI3Kα is expressed in human heart tissue and can be stimulated by its canonical activator. Given that this isoform is involved in the cardioprotection rendered by IPC and insulin at reperfusion, future therapeutic strategies could selectively target this α isoform of PI3K to enhance its protective effect against IRI. Thus, further studies with specific PI3Kα activators should be tested in both small- and large-animal models, before being eventually translated in STEMI patients who undergo coronary revascularization.

10.5.3 Limitations

The sensitivity of the TMRM assay simulates the ROS-mediated mPTP opening that occurs at reperfusion. Although this model has been widely used in this context (254,334), it could be argued that does not represent a true physiological scenario. Further, the TMRM model is known to have an usually high biological variation, particularly relying on the quality of the cells being isolated.

Human heart tissue was obtained from the right atrium. Despite carefully selecting patients without arrhythmias, we appreciate that left ventricular tissue would better represent the human model. Caution should be taken when extrapolating the results on an immortalized cell line (mouse cardiac endothelial cells) to a more physiological setting.

10.5.4 Conclusions

PI3Kα activation can be modulated in both primary cardiomyocytes and mouse cardiac endothelial cells, demonstrating its protective effect to be mediated through the delay in mPTP opening. Interestingly, PI3Kα can be activated in human heart tissue.

Chapter 11   IS THERE A ROLE FOR PI3Kβ? A PRELIMINARY INVESTIGATION

11.1 Background

The class IA PI3K family is composed by PI3Kα, PI3Kβ and PI3Kδ. Whilst the two former are ubiquitously expressed, the latter is more abundant in hematopoietic cells (335). Despite the similarity in sequence and regulatory subunits, the catalytic subunit p110α and p110β have distinct functions. Their different role in cell function can be explained by their differential expression between tissues, their subcellular location and their lipid-kinase properties, as explained in section 1.7.4. Importantly, PI3Kβ can be engaged either by RTK and GPCRs, whilst PI3Kα activation is exclusively mediated through RTK receptors. Further, there is growing evidence that PI3Kβ is synergistically activated by both receptors, acting in concert for cooperative signalling of (210).

In previous chapters, insulin has been studied as PI3Kα canonical activator, phosphorylating the p110α catalytic subunit and enhancing Akt activity through RTK signalling. In contrast, in studies in mouse embryonic fibroblasts, PI3Kβ has been shown to be insensitive to activation by growth factors that act through classical RTK signalling, such as insulin, but has been demonstrated to mediate PI3K signalling downstream of certain GCPR ligands, such as Stromal cell-derived factor-1 (SDF1α) (191,208,210,212).

SDF-1α (or CXCL12) is a chemokine of 10 kDa that is induced by hypoxia and recruits stem cells, but also has been demonstrated to be cardioprotective against IRI, particularly through its receptor CXCR4 (336). Using an elegant approach, Yellon and colleagues reported that: (1) remote ischaemic conditioning increased SDF-1α plasma levels; (2) the exogenous administration of SDF1α increases functional recovery in an *ex vivo* rat papillary muscle; and (3) this protective effect was mediated through CXCR4, as it can be blocked using the CXCR4 inhibitor AMD3100 (337). In other studies, Hu *et al.*, not only demonstrated SDF1α release in isolated cardiomyocytes following an hypoxia/reoxygenation insult, but also reported that the exogenous administration of 25 nmol/l of SDF1α for 10 min results in increased phosphorylation of Akt (338). In chronic infarct models (coronary occlusion without reperfusion), the intracardiac injection of SDF1α has been demonstrated to increase angiogenesis through a PI3K-Akt mediated mechanism

(339), whilst the overexpression of SDF1α through adeno-associated virus has been shown to enhance cardiac stem cells migration and engraftment, and reduce infarcted size via CXCR4/PI3K pathway (340). Furthermore, Yellon & colleagues also demonstrated to mimic the protective effect elicited by IPC with the administration of SDF1α in a model of simulated IRI using isolated human atrial trabeculae muscle (341). Altogether, there is a growing body of evidence demonstrating that SDF1α is cardioprotective, and that this protection might be conferred through Akt activation.

As exposed in the previous two paragraphs, SDF-1α has been demonstrated in separate studies to both activate Akt through PI3Kβ and to be cardioprotective, hence suggesting a potential link between PI3Kβ and cardioprotection, SDF1α. This would be reinforced by the fact that IPC is believed to be mostly mediated by GPCRs, rather than RTKs.

The role of PI3Kα in cardioprotection has been described in Chapters 8 to 10. The focus of this chapter is to begin to try and understand whether there is a possible role for PI3Kβ in cardioprotection. We sought to do this by using the potential PI3Kβ agonist, SDF1α, as a means of elucidating a potential role for PI3 kinase β.

11.2 Research objectives and experimental aims

Is SDF1α able to activate AKT through PI3Kβ?

<u>Hypothesis</u>

In order to test the role of PI3Kβ in cardioprotection, it is needed to demonstrate an adequate pharmacological activation and inhibition of this kinase. SDF1α has been described as PI3Kβ activator(191,217), therefore the hypothesis was to demonstrate the activation of Akt (as surrogate for PI3Kβ) following the administration of SDF1α.

Experimental aims

To preliminary investigate PI3Kβ activation with the purpose of subsequently testing PI3Kβ-specific inhibitors when available. Below is an overview of the main aims, including the proposed models related to the research question of this chapter:

Aim 1: *Demonstrate Akt phosphorylation in response to PI3Kβ activation using SDF1α in mouse isolated Langendorff-perfused hearts*

- Apply two separate doses of SDFα in Langendorff-perfused mouse hearts and use Western blot analyses to measure phosphorylation levels of Akt.

Aim 2: *Demonstrate Akt phosphorylation in response to PI3Kβ activation using SDF1α in a mouse cardiac endothelial cell line*

- Apply two separate doses of SDFα in mouse cardiac endothelial cells (immortalized line) and use Western blot analyses to measure phosphorylation levels of Akt.

11.3 Methods

11.3.1 Experimental design and study protocols

Two experimental groups were performed to study Akt phosphorylation:

1) **Mouse heart tissue.** Nine male C57BL/6 mice (9-12 weeks, 24-28 g weight) were perfused for 15 min in the Langendorff apparatus with modified Krebs-Henseleit buffer, and subsequently randomized to 15 min extra perfusion with: (1) vehicle control; (2) SDF 25 ng/mL; and (3) SDF 100 ng/mL.

2) **Mouse cardiac endothelial cells (MCEC, immortalized line).** Three passages of MCEC were cultured and randomized into the following groups (1) vehicle control; (2) SDF 25 ng/mL for 5 min; (3) SDF 100 ng/mL for 5 min; (4) SDF 25 ng/mL for 15 min; and (5) SDF 100 ng/mL for 15 min.

Once collected, the samples were introduced into protein lysis buffer, the tissue was homogenized and the protein extracted from either cells or tissue and analysed via the BCA method. Details on protein quantification and Western blot protocol can be found in section 3.9. Proteins were transferred onto nitrocellulose blotting membrane (GE Healthcare Life Sciences, UK) using wet transfer. The primary antibodies used were acquired either from Abcam, in the case of the loading control anti-GAPDH (mAbcam, #9484), or from Cell Signaling Technology: Akt (#9272), Phospho-Akt (Ser473) (#9271) and Phospho-Akt (Thr308) (#2965). Recombinant human SDF-1α was obtained from Miltenyi Biotec Inc.

The concentration of SDF-1α used in these cardioprotective studies was 25 ng/mL (337,341,342). A higher concentration was also tested based on the assumption that a higher concentration may be required to allow sufficient activation in an *ex vivo* model.

11.3.2 Data analysis

Normal distribution of each data subset was tested using the Shapiro-Wilk method. Data was non-normally distributed, therefore the non-parametric Kruskal–Wallis test was used to compare means. As none of the comparisons resulted in statistical significance, no further post hoc tests were performed. All values are presented as mean ± standard error of the mean. A P-value of less than 0.05 was considered statistically significant. STATA software, version 13.1 (Stata Corp, College Station, TX, USA) and GraphPad Prism version 6.00 (GraphPad Software, La Jolla California, USA) were used to perform the analysis and the graphics.

11.4 Results

The results of the protein analyses are depicted in Figure 11-1 for mouse cardiac tissue and Figure 11-2 for mouse cardiac endothelial cells, and a breakdown of the results can be found in the corresponding footnote. Overall, SDF1α does not increase Akt phosphorylation.

A

B
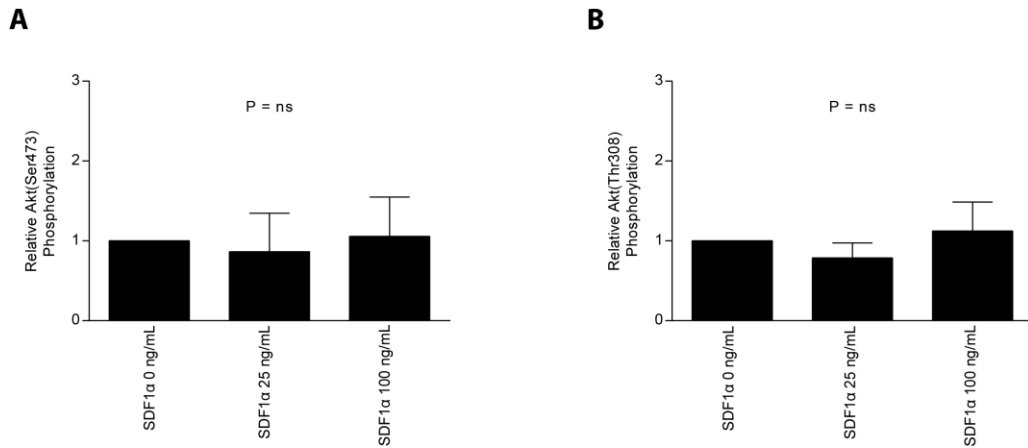
Figure 11-1: Akt response to SDF1α in mouse cardiac tissue

Bar graph shows the percentage of phosphorylation in all groups compared to the control group, expressed as mean ± SEM (percentage of relative phosphorylation), n=3 per group. For Akt(S473) phosphorylation (panel A), the fold-increase of SDF1α groups when compared to vehicle control was: SDF1α 25 ng/mL 0.9 ± 0.5 and SDF1α 100 ng/mL 1.1 ± 0.5 (P=0.301). For Akt(T308) phosphorylation (panel B), the fold-increase of SDF1α groups when compared to vehicle control was: SDF1α 25 ng/mL 0.8 ± 0.2 and SDF1α 100 ng/mL 1.1 ± 0.4 (P=0.252).
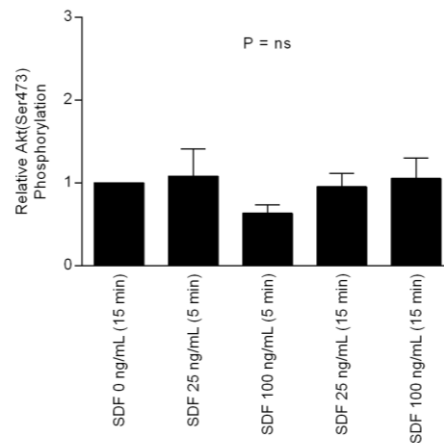
Figure 11-2: Akt response to SDF1α in mouse cardiac endothelial cells

Bar graph shows the percentage of phosphorylation in all groups compared to the control group, expressed as mean ± SEM (percentage of relative phosphorylation), n=3 per group. For Akt(S473) phosphorylation, the fold-increase of SDF1α groups when compared to vehicle control was: SDF1α 25 ng/mL (5 min) 1.1 ± 0.3, SDF1α 100 ng/mL (5 min) 0.6 ± 0.1, SDF1α 25 ng/mL (15 min) 1.0 ± 0.2, and SDF1α 100 ng/mL (15 min) 1.1 ± 0.2 (P=0.429).

11.5 Discussion

This chapter aimed to lay the ground to investigate the role of PI3Kβ in cardioprotection in the future studies. However, these preliminary experiments failed to demonstrate SDF1α as a reliable agonist of PI3Kβ at the doses used in the isolated perfused mouse heart and the mouse endothelial cell line. Further experiments will be needed to determine a reliable PI3Kβ agonist (or an appropriate SDF1α dose), and then to elucidate the appropriate concentration of the PI3Kβ inhibitors (i.e. TGX-155).

Although the sample size for each group is small; these being only preliminary results, there is no hint towards SDF1α being a good PI3Kβ activator. To interpret these results, several considerations need to be taken into account, such as the dose of SDF1α, the tissue and cell type being tested and the features of PI3Kβ activation.

There is solid evidence demonstrating that SDF1α is cardioprotective (336–338,341). Despite the aforementioned study published by Hu *et al.* demonstrating Akt activation following the exogenous administration of SDF1α in primary cardiomyocytes (338), others have involved further mechanisms to justify the protective effect elicited by SDF1α. Huang *et al.* found that SDF1α improves functional recovery in isolated mouse hearts subjected to IRI through the upregulation of STAT3 (342), a central mediator of the SAFE pathway (81). Interestingly, in this study they failed to demonstrate either an increase in Akt phosphorylation or attenuation of the protective effect using the PI3K pan-inhibitor LY294002 (342). In an *ex vivo* Langendorff model of IRI, Jang *et al.* demonstrated both myocardial infarct size reduction and ERK activation at early reperfusion with the administration of SDF1α given 10 min before reperfusion to 30 min after (343). Therefore, these results should not be interpreted as SDF1α not being cardioprotective, but rather not activating Akt at the doses being given.

The doses of SDF1α tested in this study (25 ng/mL and 100 ng/mL) were based on what had previously been reported in the literature. Previous publications can be classified into those proving SDF-1α as a cardioprotective therapy and those demonstrating the interaction SDF-1α-PI3Kβ through a GPCR, but no publications reporting PI3Kβ as mediator for SDF1α-induced protective effect has to date been published. Those studies focused on testing the cardioprotective effect of SDF1α used a dose of 25 ng/mL dissolved in saline

vehicle, although other have used lower amounts of the chemokine when applied to cardiomyocytes (338). Huang et al. (342) in a Langendorff-perfused mouse heart model undertook a dose-response curve using doses of 5, 15 and 25 ng/mL of SDF1α, eventually selecting the latter. In studies undertaken in the Hatter Cardiovascular Institute using a superfused human atrial trabeculae, 25 ng/mL of SDF-1α also proved to be protective (341). On the other hand, those studies using SDF-1α as tool to describe PI3Kβ signalling features have applied a dose of 30 ng/mL in a variety of cells (191,217), which are within the range of the doses being tested here. Interestingly, these studies have shown SDF1α to activate the PI3Kβ pathway in NIH 3T3 mouse embryonic fibroblast cells (191) and in aortic ring tissue (217). In the former study, SDF-1α increased the phosphorylation of Akt, which was abolished by the application of either the pan-specific PI3K inhibitor LY294002 and the PI3Kβ inhibitor TGX-155, therefore suggesting a predominant PI3Kβ effect, whilst in the same setting, insulin also activated Akt, and this effect was abolished by the broad-spectrum PI3K inhibitor, but not by the PI3Kβ inhibitor, therefore suggesting that the β isoform is not involved in the insulin Akt-mediated effect (191). In the latter study , SDF1α was demonstrated to induce microvessel outgrowth of aortic rings through p110β and downstream Akt activation, without involving p110α (217). Although our results do not point towards the same conclusions, it may well be that SDF1α, at these doses, only activates PI3Kβ in certain cell types and tissues (i.e. different expression or receptor sensitivity), whilst different doses are required in the heart.

Since SDF1a is only a GPCR ligand, stimulating this receptor may not be sufficient to activate PI3Kβ. As described in other settings, the synergistic action of both RTK and GPCRs might be required to activate PI3Kβ (209,210,214). Finally, it should not be ruled out that SDF1α does not activate PI3Kβ. Other GPCRs ligands which have demonstrated to mediate the activation of Akt through PI3Kβ could potentially be tested, such as sphingosine-1-phosphate and lysophosphatidic acid (191). If one of them are shown to activate Akt, a set of experiments with pan-specific PI3K inhibitors (i.e. wortmannin) and PI3Kβ-specific inhibitors (i.e. TGX155) should be carried out to determine the specific dose required for PI3Kβ inhibition to be eventually tested, in experiments testing cardioprotective therapies such as ischaemic preconditioning.

If the role of specific PI3K isoforms are determined by their predominant expression in the tissue, rather than by its biochemical differences amongst other isoforms, then PI3Kα should play a central role when compared to PI3Kβ. The relative low levels of the PI3Kβ isoform shown in Chapter 7, and particularly the differences in PI3Kβ expression between mouse and human heart tissues, are not at this stage indicative for PI3Kβ having relevant role of in human tissue.

11.5.1 Limitations

Only two doses were applied in these experiments. It is unknown whether a longer (or shorter) period of SDF1α exposure might change the results. The small sample size should be taken into account when interpreting these results.

11.5.2 Conclusions

SDF-1α does not activate Akt at 25 ng/mL and 100 ng/mL neither in Langendorff-perfused isolated mouse heart nor in mouse cardiac endothelial cells. The potential for the PI3Kβ isoform is still unclear and requires much further studying including a need to identify a reliable PI3Kβ activator in this setting.

Chapter 12   OVERALL CONCLUSIONS

12.1   Summary of findings

Acute myocardial infarction is the leading causes of mortality and morbidity worldwide. To improve clinical outcomes in patients with myocardial infarction, we need to both improve the implementation and timings of reperfusion therapy and to address the paradoxical injury induced by reperfusion. The use of cardioprotective interventions administered as adjunct to reperfusion in this regard are an unmet clinical need.

Ischaemic preconditioning is the most powerful cardioprotective intervention known and its underlying signalling has become the paradigm for cardioprotection. The infarct size-sparing effect elicited by IPC is mediated by endogenous pro-survival signalling pathways, such as the RISK pathway, which encompasses both PI3K and ERK cascades. The aim of this project was to investigate the role of the specific PI3Kα isoform in cardioprotection.

Before studying the role of PI3Kα, we first provide a thorough characterization of the ischaemia/reperfusion model in the *ex vivo* Langendorff-perfused isolated mouse heart model, dissecting the contribution of the length of ischaemia and reperfusion to the final myocardial infarct size. Then, we undertook a systematic analysis of the kinases involved within the PI3K-Akt pathway including an assessment of the phosphatase, PTEN, concluding that PI3K plays a pivotal role in the protective effect elicited by IPC.

Before evaluating the protective effect of PI3Kα against IRI, we quantified the protein levels of the two main cardiac isoforms. To our knowledge, this thesis provides the first quantification of the amount of PI3Kα and PI3Kβ in mouse and human heart tissue, as well as the comparison between endothelial cells and cardiomyocytes. Importantly, the protein levels of PI3Kα are comparable between mouse and human heart tissue, whilst human tissue expresses proportionally less PI3Kβ compared with mouse.

In subsequent experiments, we demonstrated that PI3Kα is critical for IPC-induced heart protection against IRI. In the *ex vivo* model of myocardial infarction, we showed that

PI3Kα is required during the IPC reperfusion phase to reduce myocardial infarct size, whilst this same isoform is not mediating the effect during the trigger phase. The importance of PI3Kα activation at reperfusion in IPC was confirmed in the *in vivo* setting. Using insulin as canonical activator, we also showed that PI3Kα is not only necessary for IPC to confer cardioprotection, but sufficient for its activator to promote myocardial salvage against IRI.

Furthermore, we also confirmed that PI3Kα activation delays mPTP opening in primary isolated cardiomyocytes. Finally, we showed that PI3Kα can be modulated in both cardiomyocytes and immortalized mouse cardiac endothelial cells, as well as in mouse and human heart tissue. This last observation highlights the potential ability of PI3Kα to be translated into the clinical setting.

In regards to SDF1α, further experiments are indeed required to strengthen our preliminary data. Nonetheless, although our results do not challenge the fact that SDF1α has cardioprotective properties, they suggest that this effect is not mediated by Akt, and therefore it seems very unlikely to be mediated by PI3Kβ.

12.2 Clinical implications

The translational outlook of our results is highlighted by the observation that PI3Kα is expressed in human heart tissue and can be modulated by its canonical activator. Taking into account that this isoform is mediating the IPC-induced protective effect against IRI, and its activation through insulin at reperfusion is sufficient to confer cardioprotecton, future therapeutic strategies could selectively target this α isoform of PI3K to enhance its activity promoting myocardial salvage. Further studies with specific PI3Kα activators should be tested in large-animal models, before being eventually translated in STEMI patients who undergo coronary revascularization.

12.3 Further investigations & future directions

Use of genetically modified animal models

This thesis predominantly relies on pharmacological manipulation, with all the inherent problems of target selectivity (199). Confirming these results in genetically-modified animal models would make our conclusions more solid, although this type of approach would not allow us to focus on specific phases of the conditioning process - i.e. before ischemia and at reperfusion.

Linking PI3Kα activation to the protection elicited by remote ischaemic conditioning

We have demonstrated in the current study that, within the RISK pathway, PI3Kα plays a major role in the cardioprotective effect of both ischaemic preconditioning and insulin administration. There is solid evidence demonstrating that remote ischaemic conditioning consistently results in activation of the RISK pathway (344), although the mechanisms underlying the effect afforded by remote conditioning are overall less known. Further research needs to be undertaken to conduct a solid approach demonstrating that this other form of conditioning is also mediated by PI3Kα. This would broaden the clinical interest on this particular isoform, as remote conditioning has a great potential to be translated into the clinical setting.

Development of specific pharmacological agents targeting PI3Kα at reperfusion

In this thesis, insulin has been used as canonical activator for PI3Kα. Taken into account that this metabolic agent has multiple targets and can be considered a "dirty" drug with many side-effects (i.e. hypoglycaemia, hypokalemia, and catecholamine elevation), it would be useful to actually develop a pharmacological agent specifically targeting PI3Kα. The focus of this thesis is not to return life to insulin as a cardioprotective agent, but to take advantage of the overwhelming evidence demonstrating its protective effect to further improve how to target its downstream signalling.

Effects of the upregulation or abolishment of other isoforms on ischaemia/reperfusion experimental models

If an alternative ligand to SDF1α is found to activate Akt through PI3Kβ, it would be interesting to evaluate its potential cardioprotective role, and if proved, whether using a combination of the two isoforms is additive. Considering that mostly GPCRs mediate the protective effect of the autocoids released by IPC, it would be actually very interesting to study whether either PI3Kβ or PI3Kγ (both of them downstream of GCPRs) are also involved in the "trigger" phase of IPC. One might speculate that activating both GPCRs and RTK can act synergistically to promote myocardial salvage against IRI.

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