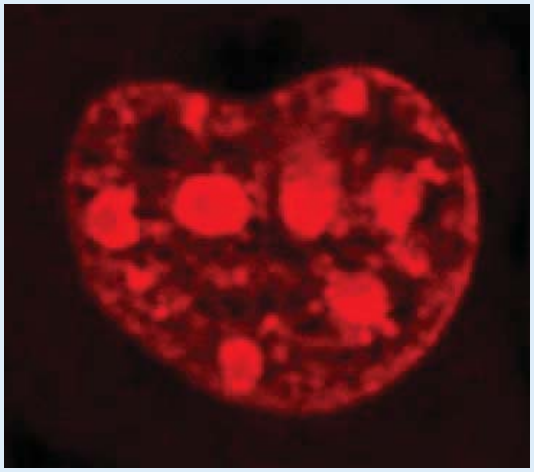
Reducing Infarct Size by Ischemic Preconditioning versus Insulin Treatment in the Heart

Same outcome - similar mechanisms?

Britt Nanny Fuglesteg



A dissertation for the degree of Philosophiae Doctor

UNIVERSITY OF TROMSØ
Faculty of Medicine
Department of Medical Physiology

2008

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You're gone now little dancer

With fur of silver grey

But the memory of you, dancing

Will never fade away

Contents

Acknowledgements

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Tromsø, October 2008 Britt Nanny Fuglesteg

Abbreviations

AG490	tyrphostin AG490
AMI	acute myocardial infarction
AMPK	AMP-activated protein kinase
BAD	bcl2-antagonist of cell death
Bax	bcl-2 associated X-protein
Bcl-2	B-cell lymphoma-2
CREB	cyclic AMP response element binding protein
Csp9	caspase 9
Cyt	cytochrome
DHE	dihydroethidium
DMSO	dimethyl sulfoxide
eNOS	endothelial nitric oxide synthase
Erk	extracellular-signal regulated kinase
4EBP1	4E-binding protein 1
FKBP12	FK506 binding protein 12
GAP	GTPase activating protein
GβL	G-protein β subunit like
GC	guanylyl cyclase
G-CSF	granulocyte colony stimulating factor
GF	growth factor
GIK	glucose-insulin-potassium
GLUT	glucose transporter
GSK-3β	glycogen synthase kinase-3 β
HB-EGF	heparinbinding epidermal growth factor-like growth factor
HIMO	1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2-O-methyl-3-O-octadecylcarbonate]
H_2O_2	hydrogen peroxide
IκBα	inhibitory subunit of NF-κB alpha
IKK	IκB kinase
Ins_R	insulin at reperfusion
IPC	ischemic preconditioning
I/R	ischemia/reperfusion

IRS	insulin receptor substrate
JAK	janus activated kinase
K_{ATP}	ATP-dependent potassium channels
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
mK_{ATP}	mitochondrial ATP-dependent potassium channels
MMP	matrix metalloproteinases
MPG	N-2-mercaptoproprionyl glycine
MPT	mitochondrial permeability transition
mPTP	mitochondrial permeability transition pore
mTOR	mammalian target of rapamycin
mTORC1/2	mammalian target of rapamycin complex 1/2
NADH	nicotinamid adenine dinucleotide
NADPH Ox	nicotinamid adenine dinucleotide phosphate oxidase
NF-$\varkappa$B	nuclear factor $\varkappa$B
NO	nitric oxide
NOS	nitric oxide synthase
O_2^-	superoxide anion
$^{\cdot}OH$	hydroxyl radical
PC	preconditioning
PDK	3-phosphoinositide dependent kinase
PFK2	phosphofructokinase 2
PIKK	phosphatidylinositol kinase-related kinase
PI3-kinase	phosphatidylinositol 3-kinase
PI4,5P2/PIP2	phosphatidylinositol-4,5-bisphosphate
PI3,4,5P3/PIP3	phosphatidylinositol-3,4,5-trisphosphate
PKB/Akt	protein kinase B
PKC	protein kinase C
PKG	protein kinase G
Pro	pro-HB-EGF
p70s6K	p70s6 kinase
PTEN	phosphatase and tensin homolog deleted on chromosome 10
Rapa	rapamycin
Raptor	regulatory associated protein of mTOR

Rheb	ras-homolog enriched in brain
Rictor	rapamycin insensitive companion of mTOR
RISK	reperfusion injury salvation kinase
ROS	reactive oxygen species
SB	SB216763
sK_{ATP}	sarcolemmal ATP-dependent potassium channels
SOD	superoxide dismutase
STAT	signal transducer and activator of transcription
SWOP	second window of protection
TNF-α	tumor necrosis factor-α
TSC1/2	tuberous sclerosis protein complex
TTC	triphenyl-tetrazoliumchloride
TYK	tyrosine kinase
VDAC	voltage dependent anion channel

List of papers

Paper I **Fuglesteg BN, Tiron C, Jonassen AK, Mjøs OD, Ytrehus K.** Pre-treatment with insulin before ischemia reduces infarct size in Langendorff perfused rat hearts. Submitted to *Acta Physiologica* March 2008. Returned for 1. Revision April 2008. Resubmitted June 2008.

Paper II **Fuglesteg BN*, Suleman N*, Tiron C, Kanhema T, Lacerda L, Andreasen TV, Sack MN, Jonassen AK, Mjøs OD, Opie LH, Lecour S.** Signal transducer and activator of transcription 3 is involved in the signalling pathway activated by insulin therapy at reperfusion. *Basic Research in Cardiology* 2008; 103:444-453.

* Both authors contributed equally to this work.

Paper III **Fuglesteg BN, Xi C, Mjøs OD, Ytrehus K.** Cardioprotective pre-treatment with insulin depends on ROS-production and GSK3B blockade via STAT3 signalling. Submitted as rapid communication to *Cardiovascular Research* June 2008.

Introduction

Background

According to the World Health Organization cardiovascular disease is one of the main causes of death in both developed and developing countries. In 2005 the number of people dying from cardiovascular disease was estimated to 17.5 million, accounting for 30% of all global deaths. In Norway the Public Health Institute reported 14537 deaths from cardiovascular disease in 2005, 42% of the deaths were attributed to ischemic heart disease (Statistics Norway).

Acute myocardial infarction (AMI) occurs when the coronary flow is no longer sufficient to meet the oxygen requirements of the heart. Impairment of coronary blood supply to the myocardium can be caused by thrombosis, embolism or other acute alterations of coronary atherosclerotic plaques. The heart can survive a short period of ischemia and exhibit recovery upon reperfusion, but if the ischemic period is too long tissue injury and cell death will occur. Species differences with respect to survival time exist, due to different degree of collateral flow in the different species. Early restoration of blood flow is crucial in order to salvage the ischemic myocardium; however, reperfusion itself may also have deleterious effects and exacerbate the damage occurring during the ischemic period. This is also known as reperfusion injury (Braunwald & Kloner 1985) and clinical manifestations of this injury can be multifactorial, including myocardial apoptosis, arrhythmias, myocardial stunning, microvascular dysfunction, and irreversible cell damage (Zhao et al. 2000; Kloner & Jennings 2001).

There are two main models of cell death; oncosis and apoptosis, which both ultimately lead to necrosis – changes secondary to cell death by any mechanism (Majno & Joris 1995). Oncosis is defined as cell injury with swelling (Majno & Joris 1995), and represents the major damage to an ischemic heart. Apoptosis or programmed cell death describes cell injury with shrinkage (Majno & Joris 1995), and there is emerging evidence that myocytes around the periphery of the infarct die of apoptosis, contributing to lethal reperfusion injury (Gottlieb et al. 1994; Freude et al. 2000; Zhao et al. 2003). Understanding the basic mechanisms of myocardial ischemic injury and finding methods to prevent ischemic and reperfusion injury are of major clinical importance, and this thesis is focused on two protective treatments against cell death in myocardial ischemia; ischemic preconditioning (IPC) and insulin therapy.

Ischemic preconditioning

Exposing the heart to one or several brief episodes of ischemia followed by reperfusion generates increased resistance to the effects of a subsequent prolonged episode of ischemia and reperfusion. This phenomenon was first described by Murry et al. (1986). In their classic study performed in dogs, four cycles of 5 min of ischemia alternating with 5 min of reperfusion prior to a prolonged ischemic insult of 40 min was shown to limit myocardial infarct size by 75%. Since then IPC has been confirmed in pig (Schott et al. 1990; Van Winkle et al. 1994), rabbit (Cohen et al. 1991; Thornton et al. 1993), rat (Lawson et al. 1993), mouse (Sumeray & Yellon 1998), sheep (Bukhari & Levitsky 1995), and even in human isolated myocytes (Ikonomidis et al. 1994), as well as in-vivo human hearts (Deutsch et al. 1990; Yellon et al. 1993; Nakagawa et al. 1995). Other organs like kidney (Bonventre 2002), gut (Ishida et al. 1997), skeletal muscle (Pang et al. 1995; Hopper et al. 2000) and liver (Peralta et al. 2000) can also be preconditioned. Additionally it has been shown that remote organ ischemia can trigger preconditioning of the myocardium (Takaoka et al. 1999; Schoemaker & van Heijningen 2000).

The protection offered by IPC is biphasic; an early phase of preconditioning emerges immediately following the ischemic stress and persists for 2-3 hours, and a late phase occurs 12-24 hours after the IPC stimulus, lasting for up to 3 days (Kuzuya et al. 1993; Marber at al. 1993; Baxter et al. 1997). This delayed phase is also termed second window of protection (SWOP) (Yellon & Baxter 1995). The focus in this thesis is on the "early" or "classic" phase of preconditioning.

Triggers, mediators and end-effectors of IPC

A sequence of events involving triggers activated during the preconditioning (PC) phase prior to the prolonged period of ischemia, mediators exerting their activity after onset of the index ischemia and end-effectors constituting the termination point of the signal transduction pathways for protection were suggested by Yellon & Downey (2002), illustrated in Fig. 1. The PC protocol is thought to create a cardiac memory somewhere between the trigger and end-effector in the signalling transduction pathways, keeping the heart in a preconditioned state (Yellon & Downey 2003) (Fig. 1).

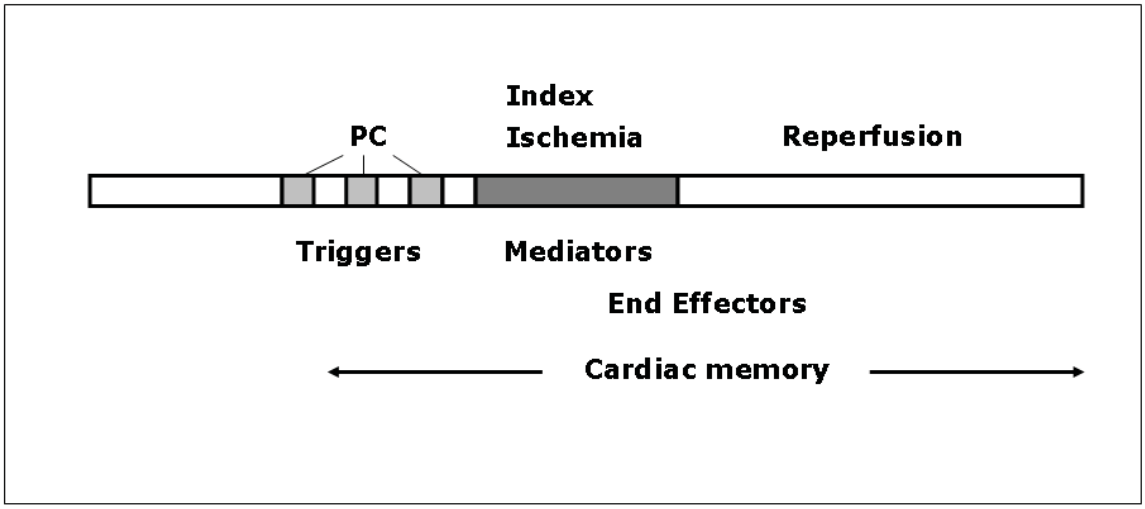

Figure 1 A schematic illustration of a classical preconditioning (PC) protocol and the sequence of events involved in myocardial resistance to infarction by preconditioning.

The major endogenous triggers of IPC are adenosine (Liu et al. 1991), bradykinin (Wall et al. 1994) and opioids (Schultz et al. 1995); all of them classified as G coupled protein receptor dependent triggers. By using antagonists of the different receptors, these investigators were able to show that protection induced by IPC was lost. Also it has been shown that preconditioning can be triggered by pharmacological interventions prior to index ischemia, as adenosine (Liu et al. 1991), bradykinin (Wall et al. 1994; Goto et al. 1995; Bugge & Ytrehus 1996) and opioid agonists (Schultz et al. 1996; Bell et al. 2000) can each precondition the heart when administered exogenously by infusion. However, species differences with respect to agonists that can induce IPC have been observed. In the rat heart for instance, the cardioprotective effect of IPC does not depend on adenosine production (Liu & Downey 1992; Li & Kloner 1993; Bugge & Ytrehus 1995). Free radicals also act as triggers of IPC (Baines et al. 1997; Das et al. 1999) and proposed mechanisms are activation of G-proteins (Nishida et al. 2000), protein kinases (Das et al. 1999) and ATP-dependent potassium channels (K_{ATP}) (Lebuffe et al. 2003).

One of the first potential mediators of IPC-induced protection to be identified was protein kinase C (PKC) (Armstrong et al. 1994; Ytrehus et al. 1994). Ytrehus et al. (1994) showed that pharmacological activation of PKC mimicked protection induced by IPC; however detrimental effects of PKC activation have also been reported (Vogt et al. 1996). Isoform specificity of PKC could explain different results with respect to cardioprotection, as activation of PKC-δ has been shown to be detrimental whereas activation of PKC-ε is protective in mouse and rat hearts (Inagaki et al. 2003). The unravelling of the cardioprotective signalling pathways involved in IPC is still ongoing, and numerous upstream activators of PKC, for instance PI3K-Akt (Tong et al.

2000), nitric oxide (Ping et al. 1999) and the mitochondrial K_{ATP} channel (mK_{ATP}) (Wang & Ashraf 1999) as well as downstream targets of PKC like the sarcolemmal K_{ATP} channel (sK_{ATP} channel) (Hu et al. 1996), the mK_{ATP} channel (Sato et al. 1998), and p38 MAPK (Maulik et al. 1996) have been suggested as mediators in IPC. It is not always easy to distinguish between triggers and mediators however; adenosine production has been shown to be necessary both during PC as well as the prolonged ischemia in order to offer protection (Thornton et al. 1993).

Receptor tyrosine kinases activated by IPC represent a possible parallel signalling pathway to the PKC pathway (Vahlhaus et al. 1998; Fryer et al. 1999). Furthermore, members of the mitogen-activated protein kinase family, the MEK 1/2-Erk 1/2 pathways have been demonstrated to contribute to the protection afforded by IPC at the time of reperfusion (Hausenloy et al. 2005). The PI3K-Akt pathway, the JAK-STAT pathway, GSK-3β and ROS are focused upon in the present work, and will therefore be described in more detail later.

Although extensive research over the past 20 years has been dedicated to elucidate the intracellular signalling cascades involved in mediating the protective effects of IPC, the ultimate end-effector(s) of this cardioprotective phenomenon is still under investigation. A point of convergence for many signalling pathways is the mitochondria, and the mitochondria have therefore been focused on as a target for preconditioning. Numerous studies have suggested a role for the mK_{ATP} channel in IPC. Several theories on how this channel could act as an end-effector exist, one of them suggesting that opening of mK_{ATP} inhibits mitochondrial calcium uptake (Holmuhamedov et al. 1999), another that entry of potassium causes swelling of the matrix and thereby maintains the voltage dependent anion channel (VDAC) in a low permeability state (Dos Santos et al. 2002). However, mK_{ATP} channels have also been described as triggers (Pain et al. 2000; Krenz et al. 2002) and mediators (Gross & Auchampach 1992) of IPC, and some authors have even suggested that mK_{ATP} channels act as both a trigger and an end-effector of IPC (Gross & Peart 2003; Yellon & Downey 2003). Oldenburg et al. (2003) hypothesize that mK_{ATP} participate in a positive feedback pathway in which opening of the channels during IPC may generate ROS and activate PKC, which subsequently phosphorylates the mK_{ATP} channels and keep them in an open state. In a recent review by Hanley & Daut (2005) the role of mK_{ATP} channels in IPC is contested although the authors state that there is no doubt that the sK_{ATP} channels contribute to preconditioning.

Other mitochondrial targets of IPC are anti-apoptotic proteins like B-cell lymphoma-2 (Bcl-2). Cardiac specific overexpression of Bcl-2 has been shown to reduce myocyte death after ischemia and reperfusion (Chen et al. 2001; Chatterjee et al. 2003), and it is suggested that overexpression of Bcl-2 modulates cardioprotection via inhibition of VDAC (Imahashi et al. 2004) and possibly blocking the formation or opening of a cytochrome c release pathway (Shimizu et al. 1999; 2000). Preconditioning has also been reported to reduce the levels of the pro-apoptotic protein Bcl-2-associated X-protein (Bax) in the heart (Nakamura et al. 2000).

Hausenloy et al. (2002; 2003) have suggested that the final step of the cardioprotective signalling pathway in IPC is inhibition of the mitochondrial permeability transition pore (mPTP) upon reperfusion after the index ischemia. The mPTP is a mitochondrial channel mediating cell death at myocardial reperfusion by uncoupling oxidative phosphorylation and inducing mitochondrial swelling (Hausenloy & Yellon 2003). The mPTP has been reported to be inhibited by GSK-3β (Juhaszova et al. 2004), by eNOS (Costa et al. 2005) and other components in the reperfusion injury salvage kinase (RISK) pathway (Bopassa et al. 2006; Davidson et al. 2006). Fig. 2 illustrates a simplified overview of some of the proposed signalling mechanisms involved in myocardial preconditioning.

Other suggested end-effectors of IPC are modifications in the cytoskeleton (Vanderheite & Ganote 1987), gap-junctions (Schwanke et al. 2002), and the sodium/proton exchanger (Xiao & Allen 2000). It is possible that there are several end-effectors of preconditioning, both mitochondrial and non-mitochondrial targets (Murphy 2004).

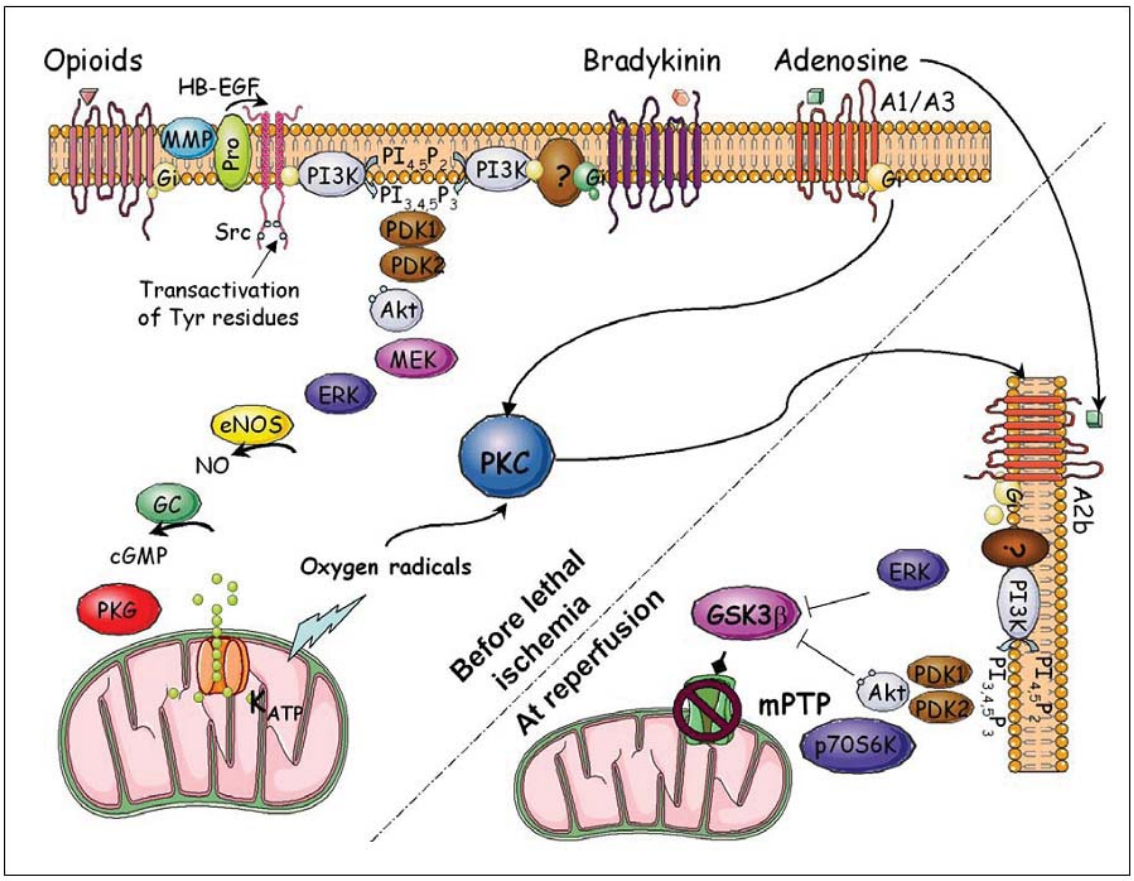

Figure 2 Simplified schematic overview of signalling pathways of myocardial preconditioning activated before the lethal ischemia or upon reperfusion. eNOS= endothelial NOS; ERK= extracellular-signal regulated kinase; GC= guanylyl cyclase; GSK-3β= glycogen synthase kinase-3β; HB-EGF= heparinbinding epidermal growth factor-like growth factor; MEK= mitogen activated protein kinase kinase; MMP= matrix metalloproteinases; mKATP= mitochondrial ATP-dependent potassium channel; mPTP= mitochondrial permeability transition pore; NO= nitric oxide; NOS= NO synthase; PI3K= phosphatidylinositol 3-kinase; PI3,4,5P3= phosphatidylinositol trisphosphate; PI4,5P2= phosphatidylinositol bisphosphate; PKC= protein kinase C; PKG= protein kinase G; Pro= pro-HB-EGF; p70S6K= p70S6 kinase (Copied from Tissier et al. 2008).

Insulin in cardioprotection

In 1962 Sodi-Pallares et al. showed that treatment with glucose-insulin-potassium (GIK) after AMI was beneficial. Almost 30 years later, a meta-analysis of nine trials revealed that in-hospital mortality after AMI was reduced by GIK therapy (Fath-Ordoubadi & Beatt 1997), and one study reported a 66% reduction in the relative in-hospital mortality risk when adding GIK upon reperfusion during AMI (Diaz et al. 1998). GIK therapy has also been found to expedite recovery and prevent myocardial infarction after coronary artery bypass grafting (Lazar et al. 1997), and enhance left ventricular function during AMI (Whitlow et al. 1982). However, negative studies with regards to the beneficial effects of GIK in relation to myocardial ischemia also exist. In a large multi-centered randomized clinical trial by Mehta et al. (2005), GIK was found to offer no beneficial effect with respect to mortality, cardiac arrest, cardiogenic shock and re-infarction at 30 days. However, as Apstein & Opie (2005) pointed out, timing of GIK-administration is of importance as experimental studies have shown that GIK confers protection from ischemia when it is present prior to reperfusion (de Leiris et al. 1975; Opie et al. 1975; Jonassen et al. 2000).

Insulin was later demonstrated to mediate cardioprotection independently of the presence of glucose at ischemic reperfusion (Baines et al. 1999; Jonassen et al. 2001), but it was crucial that insulin was present at onset of reperfusion in order to reduce infarct size (Jonassen et al. 2001). Gao et al. (2002) showed that administration of GIK or insulin alone during the last minutes of ischemia and at reperfusion in an in vivo myocardial ischemia-reperfusion model reduced myocardial apoptotic death in rat hearts, whereas treatment with glucose or potassium alone, or a combination of the two did not protect against ischemia/reperfusion-induced myocardial apoptosis. Moreover, GIK or insulin alone was also able to significantly reduce infarct size (Gao et al. 2002). In vivo studies in rabbits showed that infusion of GIK starting 30 min before ischemia and continuing throughout the reperfusion period exerted cardioprotection against postischemic myocardial injury and improved cardiac functional recovery following myocardial ischemia/reperfusion (Zhang et al. 2004). Furthermore, it was demonstrated that insulin elicited cardioprotection independently of glucose and potassium (Zhang et al. 2004), identifying insulin as the key component in GIK-induced myocardial protection. The same cardioprotective effect of GIK or insulin alone was later shown in dogs (Zhang et al. 2006).

Common signalling proteins for IPC and insulin

Extensive research over the past years has revealed that signal transduction pathways activated by different ligands converge on common targets. Both IPC and insulin seem to activate common proteins like Akt (Tong et al. 2000, Jonassen et al. 2001, Mocanu et al. 2002, Kis et al. 2003), STAT3 (Smith et al. 2004; Zecchin et al. 2005) and GSK-3β (Juhaszova et al. 2004). Also activation of ROS seems to be common for IPC and insulin (Baines et al. 1997; Goldstein et al. 2005).

Akt

In mammals three isoforms of Akt, also called protein kinase B (PKB), sharing a high degree of amino acid identity have been recognized, Akt1, 2 and 3 (PKBα, β and γ). Akt1 is ubiquitously expressed with predominant expression in brain, heart and lung (Coffer & Woodgett 1991). When referring to Akt in the following, it is Akt1 that is described. Activation of Akt by insulin and growth factors involves phosphorylation on the kinase domain (Thr^{308}) and the C-terminal regulatory domain (Ser^{473}), and phosphorylation of both residues is essential for maximal activation of Akt (Alessi et al. 1996). However, whereas phosphorylation of Thr^{308} alone is able to stimulate Akt activity, phosphorylation of Ser^{473} alone does not significantly increase the kinase activity (Alessi et al. 1996; Bellacosa et al. 1998). Phosphorylation of Thr^{308} on Akt by phosphatidylinositol 3-kinase (PI3K) is mediated by a 3-phosphoinositide-dependant kinase (PDK1) (Alessi et al. 1997). Upon activation of PI3K, membrane bound phosphatidylinositol-4,5-bisphosphate (PI4,5P2), abbreviated as PIP2, is converted to phosphatidylinositol-3,4,5-trisphosphate (PI3,4,5P3), abbreviated as PIP3, illustrated in Fig. 3. PIP3 has a high binding affinity for Akt and PDK1. Activation of PI3K induces translocation of Akt from the cytosol to the membrane, where it is anchored to PIP3 and exposed to phosphorylation and activation by PDK1 at Thr^{308} (Alessi et al. 1997). The mechanism of phosphorylation of Ser^{473} was unresolved for a long time, but recently mammalian target of rapamycin complex 2 (mTORC2) was reported as an activator of Akt at Ser^{473} (Sarbassov et al. 2005) (Fig. 3).

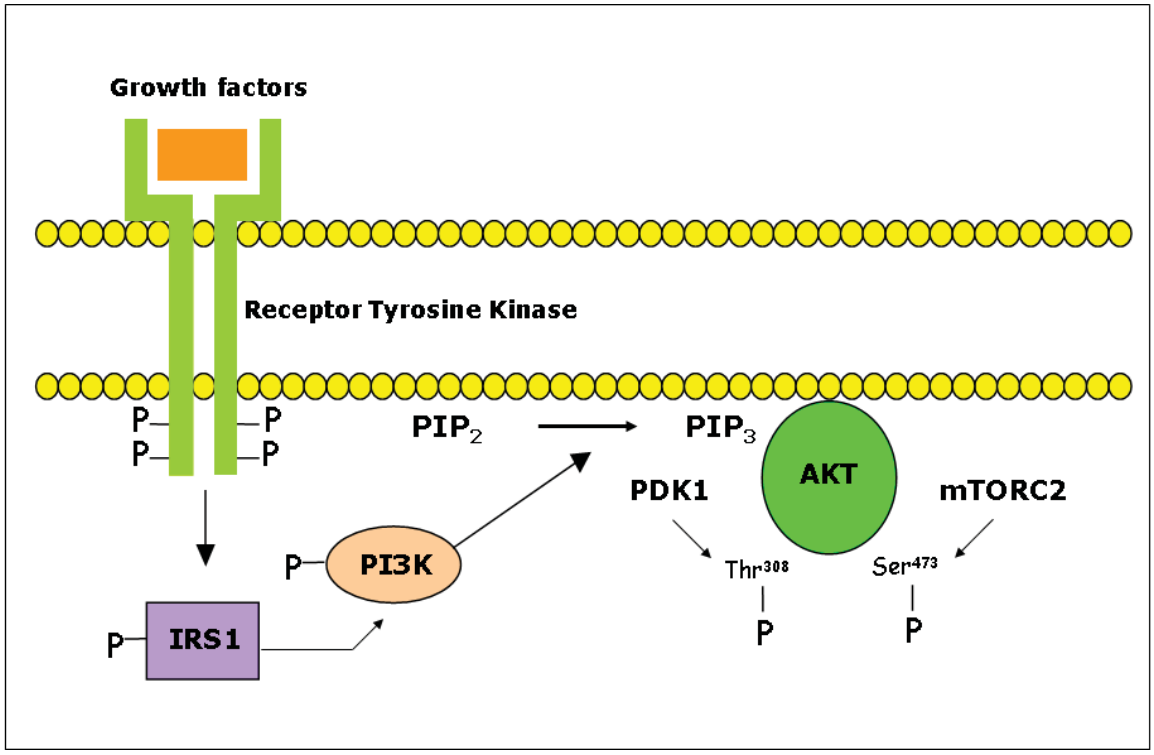

Figure 3 A schematic diagram of the activation of Akt by growth factors. IRS1= insulin receptor substrate 1; mTORC2= mammalian target of rapamycin complex 2; P= phosphorylation; PDK1= 3-phosphoinositide dependent kinase 1; PI3K= phosphatidylinositol 3-kinase; PIP_2= phosphatidylinositol-4,5-bisphosphate; PIP_3= phosphatidylinositol-3,4,5-trisphosphate.

Activation of the PI3K-Akt signalling pathway is now recognized as one of the most critical pathways in the regulation of cell survival, and numerous downstream targets of Akt involved in cardioprotection have been identified (Fig. 4). Akt has been reported to have direct effects on the apoptosis pathway by inactivating the pro-apoptotic proteins caspase-9 (Csp9) (Cardone et al. 1998) and bcl2-antagonist of cell death (Bad) (Datta et al. 1997). Akt is also able to regulate cell survival through transcriptional factors that are responsible for both pro- and anti-apoptotic genes, including Forkhead (Burgering & Medema 2003), nuclear factor κB (NF-κB) (Kane et al. 1999), cyclic AMP response element binding protein (CREB) (Du & Montminy 1998) and p70s6 kinase (Jonassen et al. 2001). Furthermore, Akt is linked to vascular function and angiogenesis through the activation of endothelial nitric oxide synthase (eNOS) (Dimmeler & Zeiher 2000), and it inhibits GSK-3β, a key player in Akt signalling (Cross et al. 1995) which will be described later. In addition, insulin-induced activation of Akt is involved in regulation of glucose metabolism by inducing translocation of glucose transporters (GLUTs) to the plasma membrane and inducing glycolysis via phosphorylation and activation of phosphofructokinase 2 (PFK2) (Hue et al. 2002).

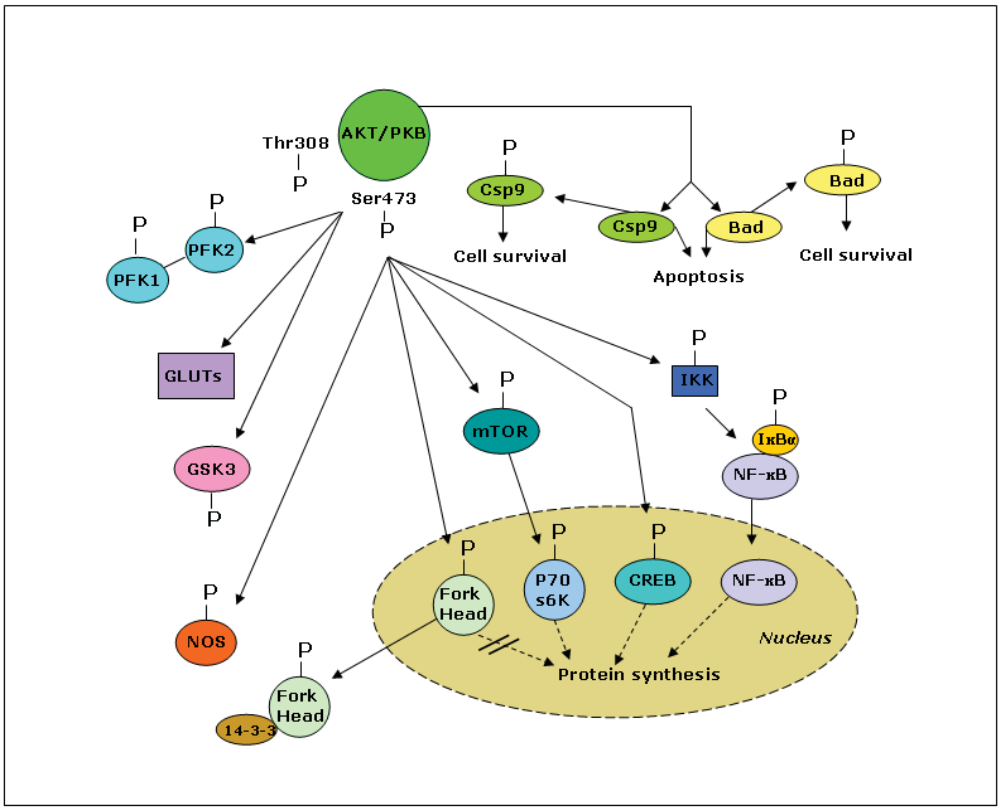

Figure 4 Schematic illustration of downstream effectors of Akt. BAD= bcl2-antagonist of cell death; CREB= cyclic AMP response element binding protein; Csp9= caspase 9; GLUT= glucose transporter; GSK-3= glycogen synthase kinase 3; IKK= IκB kinase, IκBα= inhibitory subunit of NF-κB alpha; mTOR= mammalian target of rapamycin; NF-κB= nuclear factor κB; NOS= nitric oxide synthase; P= phosphorylation; PFK= phosphofructokinase; p70s6K= p70s6 kinase (Modified from The San Diego Biotech Journal Oct/Nov 2001; www.biotechjournal.com).

Inhibition of PI3K has been shown to block the protective effect of IPC on functional recovery (Tong et al. 2000) and attenuate the infarct sparing effect of IPC (Mocanu et al. 2002). Jonassen et al. (2001) showed that the cardioprotective effect of insulin therapy at reperfusion was mediated by activation of the PI3K-Akt pathway. Furthermore, Hausenloy et al. (2004) reported that IPC resulted in phosphorylation of the PI3K-Akt pathway during reperfusion after a prolonged ischemic episode, and that PI3K and Akt were essential for IPC-induced protection.

mTOR

Mammalian target of rapamycin (mTOR) is an atypical serine/threonine protein kinase, belonging to the phosphatidylinositol kinase-related kinase (PIKK) family (Fingar & Blenis 2004). Various extracellular and intracellular signals are integrated through mTOR, including growth factors, nutrients, energy and stress (Tsang et al. 2007). mTOR forms at least two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Fig. 5). mTORC1 is responsible for sensing nutrient signals (Kim et al. 2002), whereas mTORC2 is involved in the

organization of actin (Schmidt et al. 1996). In addition, mTORC2 has been identified as the Ser[473] kinase for Akt (Sarbassov et al. 2005). Growth factor-/insulin-induced mTOR activation is one of the best characterized, and it is mediated by activation of PI3K and Akt. The link between Akt and mTOR is phosphorylation and inhibition of the tuberous sclerosis protein complex (TSC), which is a heterodimer consisting of TSC1 and TSC2 (Tee et al. 2002). TSC2 acts as a GTPase-activating protein (GAP) for the small G-protein Rheb (Ras-homolog enriched in brain) (Manning & Cantley 2003). In a GTP-bound state, Rheb will activate mTOR (Wang & Proud 2006). In mammals, mTOR is best known to regulate growth through activation of the ribosomal protein S6 kinases (S6Ks) and the eukaryotic translation initiation factor 4E-binding proteins (4EBP1) (Hay & Sonenberg 2004).

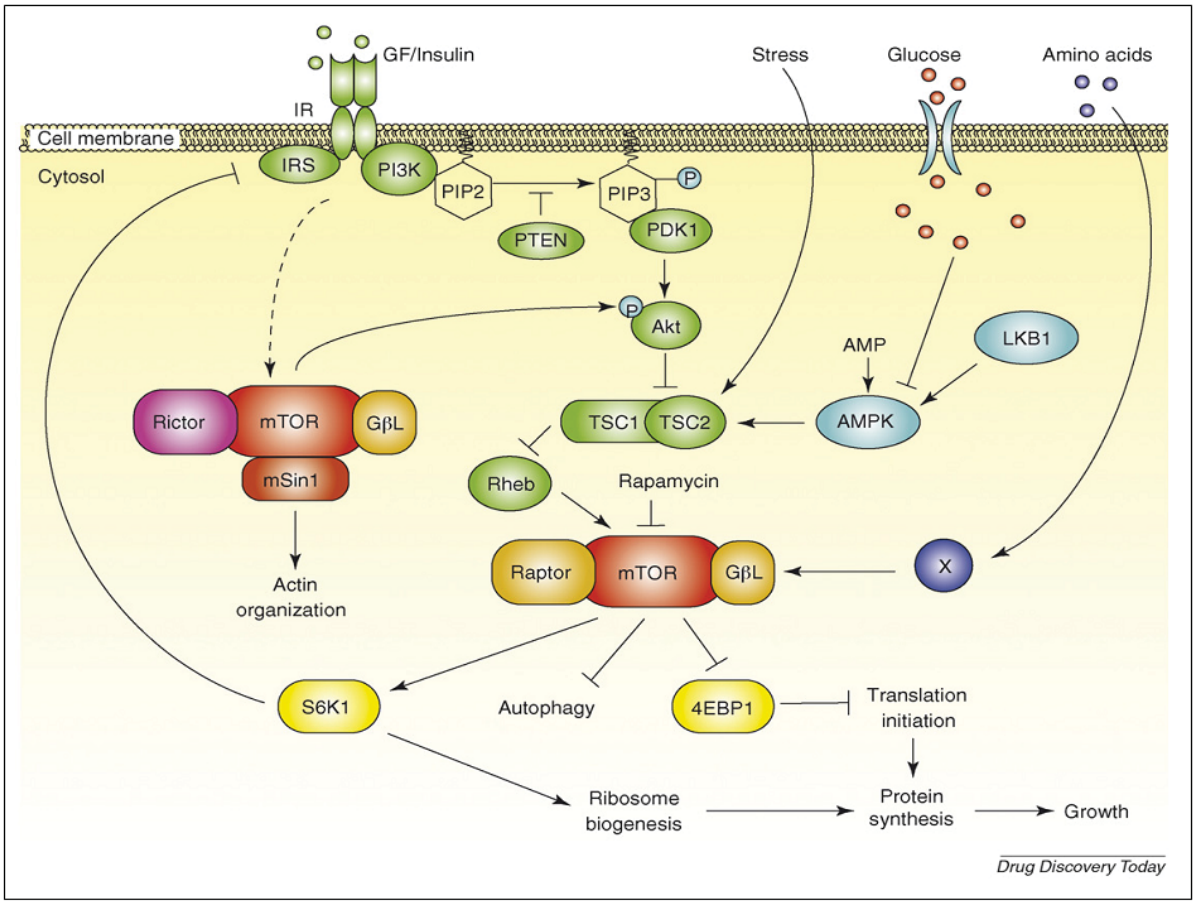

Figure 5 The signalling network of mTOR. Insulin activates the PI3K-Akt pathway as previously described which leads to activation of the raptor-mTOR complex. AMPK= AMP-activated protein kinase; 4EBP1= 4E-binding protein 1; GβL= G-protein β subunit like; GF= growth factor; mTOR= mammalian target of rapamycin; PTEN= phosphatase and tensin homolog deleted on chromosome 10; Raptor= regulatory associated protein of mTOR; Rheb= ras-homolog enriched in brain; Rictor= rapamycin insensitive companion of mTOR; TSC= tuberous sclerosis protein complex; s6K1= protein s6 kinase 1. Protein X represents an unknown mediator. Arrows and bars represent activation and inhibition, respectively. How the rictor-mTOR complex is regulated is currently unknown. Dashed lines indicate interactions that are likely not direct (Copied from Tsang et al. 2007).

Blocking of the mTOR pathway by rapamycin has previously been shown to abolish cardioprotection of delayed ischemic preconditioning in intact rabbit hearts (Kis et al. 2003), opioid-induced cardioprotection at reperfusion in intact rat hearts (Gross et al. 2004), and insulin-induced cardioprotection at reperfusion in isolated rat hearts (Jonassen et al. 2001). Hausenloy et al. (2004) showed that administrating rapamycin at reperfusion blocked the protection exerted by IPC.

STAT3

Signal transducers and activators of transcription (STATs) are a family of regulatory proteins, originally characterized as transcription factors involved in interferon signalling (Ihle 1996). At present, seven different STAT family members which are structurally and functionally related have been characterized; STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Most of the data on STAT activity in the heart involves STAT1 and STAT3. STAT1 has been shown to be detrimental for cell survival (Stephanou et al. 2000; Barry et al. 2007), whereas STAT3 is protective in the ischemic myocardium (Xuan et al. 2001; Hattori et al. 2001; Smith et al. 2004). In the present work only STAT3 was investigated.

The STATs are part of the Janus activated kinase (JAK) pathway, also called the JAK-STAT pathway. The JAKs are cytoplasmic tyrosine kinases which consist of four members: JAK1, JAK2, JAK3 and Tyrosine kinase 2 (TYK2) (Sandberg et al. 2004). JAKs have been reported to be activated by a diversity of receptors, including receptor tyrosine kinases (Shuai et al. 1993; Zong et al. 2000) and G-protein-coupled receptors (Pelletier et al. 2003; Ferrand et al. 2005). Binding of a ligand to an extracellular receptor activates a cytosolic JAK which will phosphorylate tyrosine residues (Tyr^{705}) on STAT3. The phosphorylated STAT3 proteins dimerize and translocate to the nucleus where they bind to specific DNA sequences in the promoters of genes and will stimulate transcription (Darnell 1997) (Fig. 6). STAT3 can also undergo phosphorylation at Ser^{727}, and although the precise role of serine phosphorylation remains elusive, it seems to be necessary for full transcriptional activity of STAT proteins in many instances (Shen et al. 2005).

The JAK-STAT pathway has been reported to play a role in multiple processes within the heart, including hypertrophy (Kunishada et al. 1998), apoptosis (Mascareno et al. 2005), angiotensin signalling (Pan et al. 1997) and ischemia-reperfusion (I/R) injury (Negoro et al. 2000; Bolli et al. 2001; Mascareno et al. 2001; Hattori et al. 2001; Xuan et al. 2001; Smith et al. 2004; Gross et al. 2006).

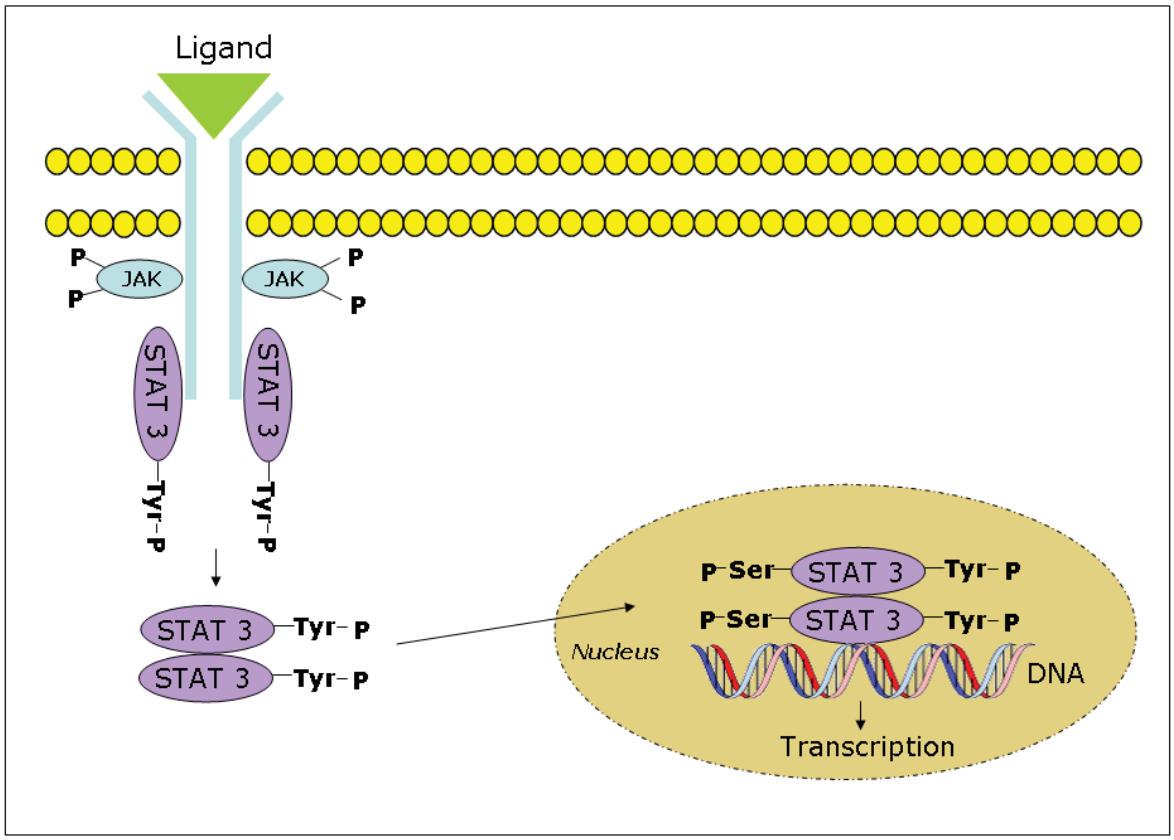

Figure 6 JAK-STAT signalling. JAK= janus activated kinase; P= phosphorylation; STAT= signal transducer and activator of transcription.

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Involvement of the JAK-STAT pathway in conferring cardioprotection has been demonstrated in both early (Negoro et al. 2000) and late preconditioning (Xuan et al. 2001). Mouse hearts in which STAT3 has been depleted can not be preconditioned (Smith et al. 2004). To achieve maximal protection in IPC, the JAK-STAT pathway needs to be activated both during the IPC stimulus (Hattori et al. 2001) and at the early phase of reperfusion (Lecour et al. 2005). Insulin is also capable of activating the JAK-STAT pathway. Insulin has been reported to induce activation of JAK2 in NIH 3T3 cells (Gual et al. 1998), and in liver, heart, adipose tissue and skeletal muscle in the intact rat (Saad et al. 1996). Following insulin-stimulated activation of JAK2, STAT3 and STAT5 were shown to be phosphorylated and thereby activated in rat aorta (Zecchin et al. 2005).

GSK-3β

GSK was originally identified for its role in the control of glycogen metabolism (Embi et al. 1980). Two highly homologous forms of mammalian GSK-3 have been described, GSK-3α and GSK-3β, both of which are ubiquitously expressed in mammalian tissue (Woodgett 1990). GSK-3α is required for amyloid production (Pilcher 2003), whereas active GSK-3β is a central protein in many cellular signalling pathways as it phosphorylates and inactivates a number of substrates like metabolic and signalling proteins, structural proteins and transcription factors (Eldar-Finkelman & Krebs 1997; Embi et al. 1980; Ginger et al. 2000; van Noort et al. 2002). GSK-3β can be phosphorylated and thereby inactivated via several signalling transduction pathways (as illustrated in Fig. 2), for example the PI3K-Akt pathway activated by insulin (Cross et al. 1995), the MAPK pathway activated by phorbol esters (Shaw & Cohen 1999), and the p70s6 kinase pathway activated by amino acids (Armstrong et al. 2001). Inhibition of GSK-3β results from phosphorylation at Ser^{9} (Plyte et al. 1992).

In addition to being involved in regulation of metabolism which may be an important component in cardioprotection (Lopaschuck 1998; Apstein 2000); inhibition of GSK-3β has also been reported to reduce apoptosis (Pap & Cooper 1998), reduce infarct size (Tong et al. 2002; Gross et al. 2004) and improve recovery of postischemic function (Tong et al. 2002). Inhibition of GSK-3β prior to ischemia or at onset of reperfusion was reported to reduce infarct size in the rat heart (Gross et al. 2004). Juhaszova et al. (2004) showed that cardiomyocytes from mice expressing a mutant GSK-3β, which is unable to be phosphorylated and thereby can not be inhibited by upstream kinases, could not be protected by IPC or insulin administration; and they suggested that signals from different cyto-protective signalling pathways may converge at the level of GSK-3β, and that inhibition of this kinase promotes cell survival by limiting MPT induction. Although many studies report that GSK-3β inhibition can induce cardioprotection, depending on the context, it can also enhance apoptosis and result in hypertrophy (Murpy & Steenbergen 2005).

ROS

Oxygen derived free radicals, also called reactive oxygen species (ROS) are a family of highly reactive molecules formed by stepwise, enzymatic, one-electron reductions of molecular oxygen, yielding superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) (Fig. 7). During aerobic respiration molecular oxygen acts as a terminal electron acceptor in the mitochondria, enabling production of ATP. 98% of O_2 in the myocardium is reduced to water by

tetravalent reduction in the mitochondrial electron transport chain without any production of free radical intermediates, whereas the remaining 2% proceeds by a univalent pathway in which free radicals are produced (Ballinger 2005). Under normal physiological conditions, the superoxide formed by cardiomyocyte mitochondria is reduced to hydrogen peroxide, which is broken down to water by catalase and glutathione peroxidase, thereby permitting a safe disposal of ROS (Fig. 7). In addition to the mitochondria there are many potential sources of free radicals in the myocardium, including NADPH oxidase, xanthine oxidase, cycloxygenase and cytochrome P450 reductases (Seddon et al. 2007).

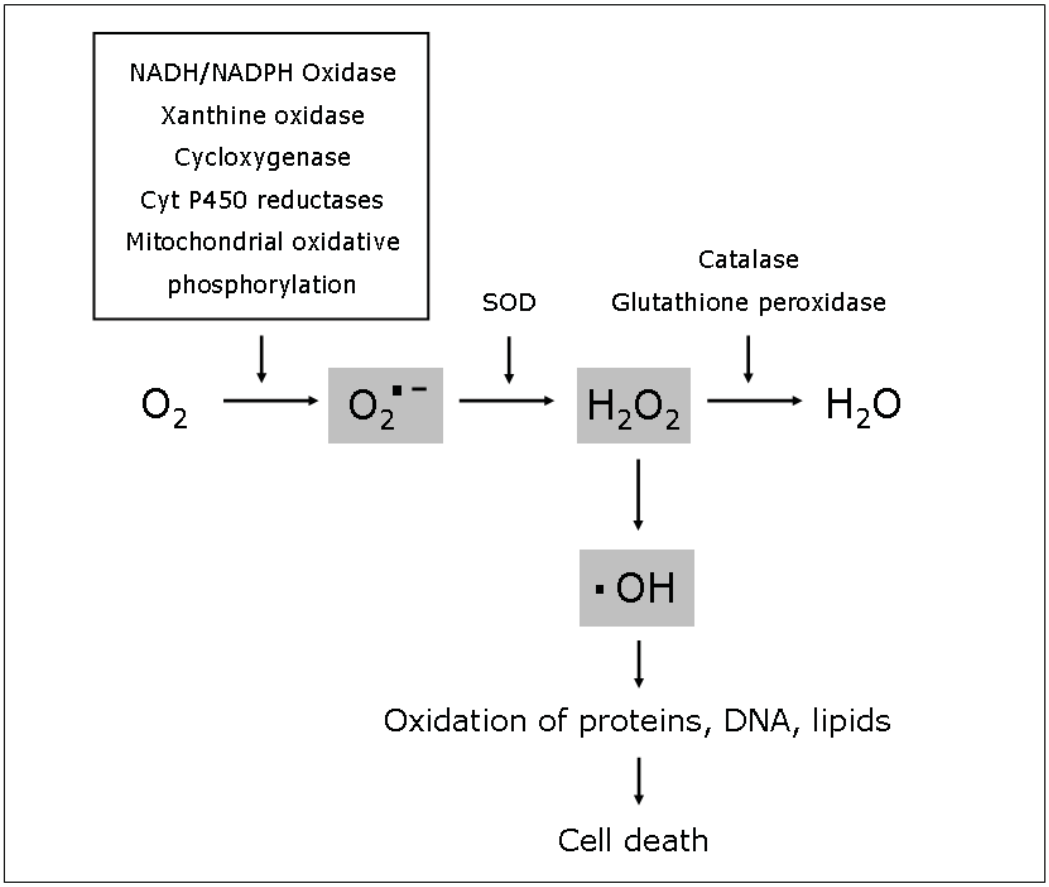

Figure 7 Sources of ROS generated endogenously by cardiovascular cells. SOD= superoxide dismutase (Adapted from Yoshizumi et al. 2001).

During ischemia, the excessive production of ROS results in H_2O_2-derived hydroxyl radical formation (Becker 2004) which may cause direct damage to the cell membrane and proteins and deregulate lipid peroxidation (Grune et al. 1997). Many reports have supported the notion that ROS in the myocardium is one of the principal mechanisms contributing to the pathogenesis of I/R injury (Weisfeldt et al. 1988; Park & Lucchesi 1999; Ambrosio & Tritto 1999). However; already 20 years ago it was suggested that oxygen radicals could be involved in preconditioning

(Kinsman et al. 1988), and thereby contribute to induce protection in the heart. Baines et al. (1997) showed that oxygen radicals could mimic the protective effect of IPC, and that administration of a ROS scavenger blocked IPC-induced cardioprotection. A second messenger role for ROS has later been confirmed in IPC (Das et al. 1999) and pharmacological preconditioning (Sovershaev et al. 2006). Hegstad et al. (1997) reported that treatment with low concentrations of hydrogen peroxide at reperfusion after 30 min of global ischemia improved post-ischemic recovery of function in isolated rat hearts. Already in 1979 it was shown that insulin could elicit the generation of H_2O_2 in adipocytes (May & de Haën 1979). A role for small amounts of ROS in facilitating the normal signal transduction by insulin has been suggested (Goldstein et al. 2005).

Further details on the involvement of Akt, mTOR, STAT3, GSK-3β and ROS in signalling pathways activated by IPC and insulin will be elaborated in the discussion section.

Aims of study

The main focus of the present work was to elaborate the common signalling proteins activated by IPC and insulin therapy leading to cardioprotection.

Specific aims of the individual studies were as follows:

1. To confirm a role for mTOR in cardioprotection by either ischemic preconditioning or insulin at reperfusion, and to test whether insulin pre-treatment involved cardioprotection through similar mechanisms.
2. To test the putative Akt inhibitor HIMO.
3. To investigate whether activation of the JAK-STAT pathway is an alternative protective pathway for insulin-induced cardioprotection.
4. To investigate if GSK-3β is a downstream target of JAK-STAT signalling.
5. To examine if pre-treatment with insulin induces generation of cardioprotective ROS.

Methodological considerations

Langendorff perfusions

The Langendorff perfusion technique, first described in 1895, is a well established model for non-working hearts (Langendorff 1895). The technique was used for infarct studies (paper I-III) and obtaining tissue samples for Western blot analysis (paper I and II) in this thesis. In addition to constitute a low-cost, technically simple and easy reproducible method, isolated Langendorff perfused hearts have the advantage that external variables may be readily standardised with respect to temperature, pH, pressure, ion concentrations, energy substrates and administration of drugs. Also neuro-hormonal and metabolic interference are eliminated; however this can be both advantageous and disadvantageous as direct cardiac effects can be studied, but results do not necessarily apply to the *in vivo* situation. Also one must keep in mind that species specificity may apply to the reported mechanisms and any extrapolation to the human heart must be done cautiously.

Experimental protocols

IPC and pharmacological pre-treatment

A variety of protocols for IPC have been used in different experimental studies. Typically, 1-4 brief (2-5 min) cycles of ischemia and reperfusion are used, dependent on the species examined. IPC in paper I and III in this thesis were conferred by three times 5 min of global ischemia and reperfusion prior to the main ischemic insult. This preconditioning protocol for rats is well established in our laboratory, and has previously been shown to give significant reductions in infarct size (Bugge & Ytrehus 1995; 1996; Munch-Ellingsen et al. 2000). Pre-treatment with insulin given in three cycles of 5 min treatment separated by 5 min of washout (paper I and III) was designed to match the IPC protocol. However we observed that pre-treating hearts with an insulin-concentration of 0.3 or 1 mU/ml did not confer cardioprotection, and we had to increase the dose to 5 mU/ml in order to see a reduction in infarct size. In paper I, 50 mU/ml of insulin was administered, whereas 5 mU/ml was used in the experiments in paper III. A comparison of the two different concentrations showed no significant differences in infarct size, cardiac flow or heart rate (Table 1, InsPC).

The pre-treatment protocol with the GSK3β-inhibitor SB21673 given 10 min prior to the index ischemia (paper III) was based on previous reports (Tong et al. 2002; Gross et al. 2004).

Insulin therapy at reperfusion

The protocol for insulin therapy at reperfusion was based upon a previous study reporting that insulin must be present at onset of reperfusion in order to offer cardioprotection in the isolated perfused rat heart (Jonassen et al. 2001). Insulin-doses of 0.3, 1 and 5.0 mU/ml was shown to be effective in terms of reducing infarct size in the isolated heart preparation (Jonassen et al. 2001). In paper I an insulin-dose of 3.0 mU/ml was administered upon reperfusion of the isolated rat heart, whereas in paper II 0.3 mU/ml of insulin was chosen for both the isolated rat heart and the isolated cardiomycytes. A comparison of the two different concentrations showed no significant differences in infarct size, cardiac flow or heart rate (Table 1, Ins_R). Insulin-administration was started 1 min prior to reperfusion and continued throughout the 2 hrs of reperfusion.

Table 1 Impact of different concentrations of insulin on infarct size, coronary flow and heart rate.

Experimental groups	**Infarct size (% of AAR)**	**Coronary flow (ml/min)**		**Heart rate (beats/min)**	
InsPC		**20' stab**	**29' IP**	**20' stab**	**29' IP**
50 mU/ml	16.0 ± 2.9	15.9 ± 1.0	12.1 ± 1.0	290.2 ± 19.5	260.5 ± 13.7
5.0 mU/ml	16.2 ± 2.1	15.0 ± 0.9	13.5 ± 1.3	286.1 ± 14.4	286.4 ± 26.2
Ins_R		**20' stab**	**15' rep**	**20' stab**	**15' rep**
3.0 mU/ml	22.6 ± 2.3	16.0 ± 0.7	11.1 ± 1.0	297.8 ± 14.3	276.1 ± 13.1
0.3 mU/ml	17.9 ± 2.9	15.3 ± 0.7	12.4 ± 1.2	273.5 ± 6.5	245.1 ± 13.3

Values are mean ± SEM; InsPC= pretreatment with insulin; Ins_R= insulin at reperfusion; AAR= area at risk; 20' stab= 20 min of stabilisation; 29' IP= 29th minute of insulin pre-treatment; 15' rep= 15 min of reperfusion.

In relation to clinical use of insulin, it can be difficult to convert mU/ml to human circulating concentrations after cardioprotective insulin infusions. A current study in the USA, Immediate Metabolic Myocardial Enhancement During Initial Assessment and Treatment in Emergency care (IMMEDIATE) has administered doses of about 5 U/hr to patients with acute myocardial infarction (available at: www.clinicaltrials.gov/show/NCT00091507). Another current study (INTENSIVE) uses about 2.5 U/hr (Nesto et al. 2008). As the average human has a blood volume of about 7% of total body weight, for a 70 kg person the blood volume is about 5 litres.

Assuming complete vascular distribution of the infused insulin, 2.5 U/hr would go into 5 litres or about 0.5 U/litre or 0.5 mU/ml. Infusion at 5 U/hr would give 1.0 mU/ml in humans. The real concentrations would be lower to allow for adhesion of insulin to the tissue receptors.

Infarct size as end point

To assess protection from IPC and insulin therapy, infarct size expressed as percentage of the risk zone was used as end point in all three papers included in this thesis. Infarct size was assessed by staining viable tissue with triphenyl-tetrazoliumchloride (TTC). Tetrazoliuim salts react with NADH and dehydrogenase enzymes staining the viable tissue red due to formation of formazan. Thus the red colour indicates active mitochondrial respiration. Dead cells lose their content of intracellular enzymes and NADH during reperfusion due to defect membranes and do not stain (Examples of TTC-stained hearts shown in Fig. 8). TTC-staining is a well recognized and widely used method in infarct studies; however an underestimation of the extent of necrosis might be made as the method is too coarse to detect small scattered areas of necrosis (Vivaldi et al. 1985).

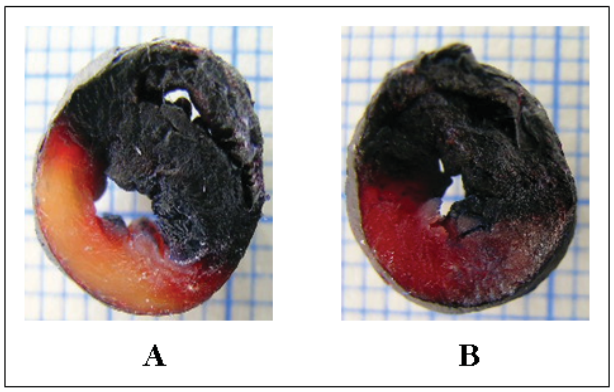

Figure 8 Examples of TTC-stained heart slices from A) a control heart and B) an IPC heart. The blue colour demarcates the area not at risk of infarction. In the area at risk, the stained area represents viable cells whereas the unstained tissue represents necrotic cells. Infarct size was expressed as percentage of risk zone. The volume of the risk zone did not differ between the experimental groups.

The rat heart does not develop collaterals, and the size of the area at risk is therefore a major determinant of infarct size. In Table 2 rat weight, heart weight, ventricular volumes and risk volumes from all three papers in the present thesis are displayed. When comparing risk volume to total ventricular volume, no significant difference between the groups was observed.

Table 2 Rat weight, heart weight, heart volume and risk volume in all experimental groups (unpublished data).

Experimental groups	n	Rat weight (g)	Heart weight (g)	Heart volume (mm^3)	Risk volume (mm^3)
Paper I					
Ctr	18	318 ± 7.7	1.70 ± 0.05	565 ± 19.8	331 ± 17.9
Ctr+HIMO	8	310 ± 5.4	1.78 ± 0.07	550 ± 27.6	321 ± 27.3
IPC	9	318 ± 12.7	1.74 ± 0.07	595 ± 22.6	332 ± 15.5
IPC+HIMO	7	323 ± 16.0	1.85 ± 0.07	532 ± 30.1	306 ± 33.8
IPC+Rapa	7	320 ± 9.8	1.85 ± 0.09	578 ± 30.6	322 ± 21.5
InsPC	7	326 ± 17.3	1.98 ± 0.11	564 ± 39.2	378 ± 37.5
InsPC+HIMO	5	392 ± 4.9	2.03 ± 0.11	617 ± 23.7	328 ± 19.4
InsPC+Rapa	6	300 ± 4.0	1.88 ± 0.11	529 ± 34.1	242 ± 25.7
Ins_R	10	312 ± 9.2	1.69 ± 0.05	547 ± 25.3	331 ± 23.9
Ins_R+HIMO	6	300 ± 5.2	1.77 ± 0.07	634 ± 40.0	361 ± 30.9
Ins_R+Rapa	6	310 ± 4.5	1.85 ± 0.18	635 ± 30.1	368 ± 18.3
Paper II					
Ctr	6	327 ± 6.7	1.78 ± 0.04	626 ± 18.2	336 ± 10.3
Ctr+AG	4	305 ± 11.9	1.69 ± 0.08	607 ± 25.0	324 ± 17.7
Ins_R	6	333 ± 6.7	1.77 ± 0.05	658 ± 22.3	338 ± 37.2
Ins_R+AG	6	349 ± 5.9	1.81 ± 0.05	635 ± 35.0	334 ± 19.5
Paper III					
Ctr	11	300 ± 11.1	1.88 ± 0.07	708 ± 39.7	378 ± 23.4
IPC	8	340 ± 14.5	1.80 ± 0.02	622 ± 28.6	312 ± 24.2
IPC+AG	10	376 ± 7.8	2.01 ± 0.06	695 ± 25.0	400 ± 23.0
IPC+MPG	5	282 ± 9.2	1.53 ± 0.16	512 ± 20.3	294 ± 15.7
InsPC	5	364 ± 19.4	2.20 ± 0.10	655 ± 52.7	417 ± 49.3
InsPC+AG	6	342 ± 7.5	1.84 ± 0.03	700 ± 55.0	418 ± 29.4
InsPC+MPG	4	290 ± 5.8	1.68 ± 0.12	547 ± 53.2	303 ± 20.6
SBPC	5	400 ± 4.9	2.16 ± 0.06	878 ± 26.3	479 ± 26.9
SBPC+AG	6	293 ± 8.4	1.73 ± 0.03	720 ± 57.3	393 ± 36.6
MPG	3	300 ± 0.0	1.52 ± 0.03	544 ± 46.3	334 ± 21.1

Values are mean ± SEM; n= number of rats; Ctr= control; HIMO= 1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2-O-methyl-3-O-octadecylcarbonate] (Akt inhibitor); IPC= ischemic preconditioning; Rapa= rapamycin (mTOR inhibitor); InsPC= insulin pre-treatment; Ins_R= insulin at reperfusion; AG= Tyrphostin AG490 (JAK-STAT inhibitor); MPG= N-2-mercaptoproprionyl glycine (ROS scavenger); SB= SB216763 (GSK-3β inhibitor).

The end point in all three studies in the present thesis was infarct size. However, functional data were also recorded and used to confirm an adequate drop in coronary flow (CF) and left ventricular developed pressure (LVDP) during ischemia. No significant differences in baseline function between the groups in any of the papers were observed; neither did any of the treatments cause deviations in CF or LVDP. During regional ischemia the decline in pressure and flow was similar between the groups and recovery of function was only partial upon reperfusion. Functional parameters based on experiments in paper I are shown in Table 3 as an example.

Table 3 Functional parameters based on experimental groups from paper I (unpublished data).

Experimental groups	n	Baseline	25th min of RI	30th min of rep	120th min of rep
CF (ml/min)					
Ctr	18	14.6 ± 2.0	8.4 ± 1.9	11.6 ± 2.4	8.8 ± 2.1
Ctr+HIMO	8	13.8 ± 1.9	7.3 ± 2.2	10.8 ± 3.2	7.7 ± 2.6
IPC	9	14.9 ± 3.0	7.6 ± 3.3	10.4 ± 3.3	7.8 ± 3.2
IPC+HIMO	7	14.9 ± 3.1	7.9 ± 1.6	10.9 ± 1.8	8.1 ± 1.4
IPC+Rapa	7	15.9 ± 1.0	7.2 ± 1.4	9.2 ± 2.6	7.9 ± 1.4
InsPC	7	14.6 ± 2.7	8.1 ± 2.2	10.5 ± 1.9	9.8 ± 3.8
InsPC+HIMO	5	13.0 ± 2.0	8.2 ± 1.8	9.7 ± 1.6	6.4 ± 0.8
InsPC+Rapa	6	16.4 ± 2.2	8.1 ± 2.4	9.9 ± 3.2	8.7 ± 2.5
Ins_R	10	16.0 ± 2.3	8.1 ± 2.6	10.4 ± 3.3	7.8 ± 3.3
Ins_R+HIMO	6	14.7 ± 1.7	8.7 ± 2.2	10.4 ± 3.0	7.6 ± 2.4
Ins_R+Rapa	6	16.3 ± 1.9	9.6 ± 0.9	10.8 ± 2.4	8.8 ± 2.7
LVDP (% from baseline)					
Ctr	18	100	61.0 ± 3.0	68.2 ± 2.5	56.3 ± 5.0
Ctr+HIMO	8	100	55.7 ± 3.6	58.1 ± 6.3	49.6 ± 4.7
IPC	9	100	52.6 ± 3.8	65.8 ± 4.4	51.6 ± 5.1
IPC+HIMO	7	100	52.6 ± 6.3	75.8 ± 8.7	61.6 ± 7.1
IPC+Rapa	7	100	52.0 ± 4.0	60.6 ± 4.6	51.3 ± 3.9
InsPC	7	100	68.1 ± 9.9	73.5 ± 4.5	62.7 ± 3.3
InsPC+HIMO	5	100	56.1 ± 7.5	65.2 ± 6.1	45.5 ± 3.1
InsPC+Rapa	6	100	68.1 ± 10.8	71.5 ± 10.0	56.5 ± 6.7
Ins_R	10	100	50.4 ± 3.8	65.1 ± 3.4	52.5 ± 4.7
Ins_R+HIMO	6	100	55.4 ± 4.5	71.9 ± 8.1	56.6 ± 6.6
Ins_R+Rapa	6	100	59.9 ± 5.0	73.5 ± 9.3	60.9 ± 8.7

Values are mean ± SEM; n= number of rats; CF= coronary flow; LVDP= left ventricular developed pressure; Ctr= control; IPC= ischemic preconditioning; Rapa= rapamycin; HIMO= 1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2-O-methyl-3-O-octadecylcarbonate]; InsPC= insulin pre-treatment; Ins_R= insulin at reperfusion; RI= regional ischemia; rep= reperfusion.

Western blot analysis

Relative changes in the phosphorylation of a specific protein are commonly measured by the Western blot technique by forming the ratio between densitometric values of bands containing the phosphorylated form of a specific protein and the total amount of the given protein. It is generally assumed that this analysis provides an accurate determination of relative changes in phosphorylation status of a specific protein, given that a linear relation between increasing amounts of phosphorylated protein exist. However, the use of densitometric analysis for quantification of protein has been criticized. Pitre et al. (2007) reported that densitometric ratios differ substantially from actual ratios of known protein amounts even in the presence of a linear relationship, and they suggested that the use of purified protein standards to plot a standard densitometry curve should be used to avoid this problem, a method seldom used in the literature. Also it is important to be aware that phosphorylation levels do not necessarily reflect the activity

of an enzyme; an inhibitor or an activator may change activity even though it may or may not change phosphorylation.

Western blot analysis from whole heart preparations in the present work showed a great deal of variability. In paper I, baseline samples in which the hearts were not subjected to ischemia-reperfusion were freeze clamped, and hence there was no separation of area at risk from area not at risk. In paper II the samples were dissected to separate the risk area from area not at risk without Evans blue-dying. Separating the non-ischemic zone from the ischemic risk zone without any dye was based on experience after observing the ease of repeatability with our infarct size experiments. However, it can not be excluded that the observed variations in our results were due to variable preparation of the area at risk. The balloon used for pressure measurements during infarct size experiments was not used when collecting samples for Western blots, as introducing the balloon may influence phosphorylation of proteins. Furthermore, since exposing the heart to ischemia and reperfusion by itself will induce phosphorylation of proteins, and the whole heart preparation represents a variety of different cells, we used the HL-1 cell line to investigate basal levels of protein phosphorylation. Cells represent a much "cleaner" model for investigating cellular signalling events, as it represents a homogenous cell population.

Cell cultures

The HL-1 cell line was used for Western blot analysis (paper I) and to investigate insulin-induced ROS production (paper III). This is a cell line established from AT-1 cardiac myocytes, which are atrial cardiac muscle cells that can only be maintained as a subcutaneous tumor lineage in syngeneic mice, and myocytes must be prepared from these tumors as primary cell cultures (Delcarpio et al. 1991). In contrast to freshly isolated cardiac myocytes which can only be kept in culture for a limited amount of time, this cell line has been reported as being capable of indefinite passaging in culture, as well as recovering from frozen stocks, retaining a differentiated cardiac myocyte phenotype and maintaining contractile activity (Claycomb et al. 1998). HL-1 cells have been used extensively for studies of different aspects of cardiac biology, including hypoxia (Cormier-Regard et al. 1998), cellular signalling (Dhanasekaran et al. 2008) and apoptosis (Marinovic et al. 2008). In contrast to the isolated heart model, using cell culture models eliminates the problems with non-homogenous cell populations. The HL-1 cells are atrial cells and may have unique features not present in ventricular cells, but even if they may exhibit a structural phenotype other than normal cardiomyocytes, they represent a simple and easy

reproducible system in the quest to understand the different cellular and molecular mechanisms in the heart.

In paper II isolated cardiac myocytes from wild type and STAT3 deficient mice were used for short term culture (1-2 days). As knocking out the STAT3 gene results in embryonic lethality (Takeda et al. 1997), conditional knockouts of STAT3 using the Cre-LoxP system have been generated. Conditional knockouts allow the gene of interest to be removed from a single organ or a subset of tissues, making it possible to define the function of a particular gene in the physiology of a specific organ or tissue. For instance STAT3-deficient macrophages have shown that STAT3 plays an important role in IL-10 signalling and down-regulation of immune response (Takeda et al. 1999); in skin loss of STAT3 resulted in compromised wound healing (Sano et al. 1999); and in cardiomyocytes deficiency of STAT3 abolished the capacity to activate ischemic and pharmacological preconditioning (Smith et al. 2004) and resulted in higher sensitivity to inflammation, cardiac fibrosis, as well as heart failure with advanced age (Jacoby et al. 2003). The possibility to knock out specific target genes is an excellent model for unraveling effects and importance of the given genes, however knocking out one gene could have unknown effects on other genes. We observed that the total gain of isolated cardiomyocytes was less from mice with cardiac specific STAT3 deficiency.

Quantitative analysis of ROS production by confocal microscopy

Confocal microscopy was used in paper III to investigate ROS production in HL-1 cells. Cells were loaded with dihydroethidium (DHE, 10 μM). DHE is cell permeable and after entering the cells it is oxidized by oxygen-derived free radicals (mainly superoxide radical) and converted to a reversible ethidium-like compound which causes a red-shift in the electromagnetic light spectrum in a proportional manner (Kevin et al. 2003; Zhao et al. 2003). The advantage of using DHE is that certain physiological variables like pH changes, Ca^{2+} levels and phosphate concentrations have no effect on the ethidium fluorescent signal, and DHE does not autooxidate to ethidium during incubation at room temperature in a darkened room for up to 2 hrs (Supinski et al. 1999). In a previous study from our lab, 10 μM of DHE was used to detect ROS in isolated heart sections (Sovershaev et al. 2006), and this concentration was therefore chosen in the present experiments. A suitable field of cells for imaging was selected and the fluorescent signal was captured from the same field at different time-points. The fluorescence intensity was quantified using the whole image area after subtraction of the background. This way of analysing the data is

only semi-quantitative. Should one field contain more cells than the other, it could be falsely interpreted as an increase in total ROS-production.

Pharmacological agents

The use of pharmacological agents in delineating signalling transduction pathways are valuable tools. However, most drugs exhibit non-specific effects, and one must be cautious about the concentration of the administered drug.

HIMO

1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2-O-methyl-3-O-octadecylcarbonate] (HIMO) is a modified phosphatidylinositol (PI) analogue reported as a putative inhibitor of both Akt and PI3K (Hu et al. 2000) and was used in paper I. Up to a concentration of 20 μM HIMO does not significantly affect PI3K activity, and IC_{50} for PI3K inhibition is 80 μM (Martelli et al. 2003). This implies that at a concentration of 20 μM, as we have used in paper I, PI3K activity should not be affected, and approximately 73% of Akt activity should be inhibited (Martelli et al. 2003).

Rapamycin

Rapamycin is an immunosuppressant macrolide antibiotic secreted by bacteria discovered in Rapa Nui in the South Pacific Ocean. In addition to its ability to prevent rejection of transplanted organs by suppressing the immune system, it has anti-cancer activity, and has currently reached clinically applications in drug-eluting stents. Rapamycin inhibits mTOR/p70s6 kinase by forming a complex with an intracellular receptor FK506-binding protein (FKBP12) which then binds to mTOR (Wullschleger et al. 2006). At a concentration of 1 μM, 10-20 fold higher concentration than that required to inhibit mTOR in cell-based assays, rapamycin has been shown to selectively inhibit mTOR activity without affecting other protein kinases (Davies et al. 2000). Studies have shown that inhibiting the mTOR/p70s6k-pathway by rapamycin abolishes the cardioprotective effect of IPC (Kis et al. 2003; Hausenloy et al. 2005) and insulin therapy at reperfusion (Jonassen et al. 2001). However, cardioprotective effects of rapamycin have also been reported (Khan et al. 2006).

AG490

Tyrphostin AG490 has been described as a potent and specific JAK2-inhibitor, and has been shown to inhibit the JAK2-STAT3/5 pathway in an ATP competitive manner (Levitzki & Mishani 2006), having no effect on the kinase activity of other protein tyrosine kinases (Meydan et al. 1996; De Vos et al. 2000). At a dose of 50 μM, Negoro et al. (2000) reported that AG490 reduced phosphorylation of STAT3 through inhibition of JAK2, while AG490 had no direct effect on either the mitogen activated protein kinase (MAPK) family or PI3K signalling. The dose of 5 μM used in the present work was based on previously published data by collaboration partners in paper II (Lecour et al. 2005).

SB216763

SB216763 is a maleimide derivative (Martinez et al. 2002) reported to selectively inhibit GSK3 in an ATP-competitive manner, and to exhibit minimal activity against 24 other protein kinases, including Akt and PDK1 (Coghlan et al. 2000). In the heart, SB216763 has been shown to reduce ischemia-reperfusion injury when added before (Tong et al. 2002) and after ischemia (Gross et al. 2004).

MPG

N-2-mercaptoproprionyl glycine is reported as a very selective scavenger of hydroxyl radical and peroxynitrite which does not react with H_2O_2 or superoxide (Bolli et al. 1989; Nadtochiy et al. 2007). MPG has been reported to block the protection induced by a single cycle of IPC, but not four cycles of IPC (Baines et al. 1997). Furthermore, the infarct-reducing effect of pharmacological preconditioning by bradykinin and opioids can be abolished by MPG (Cohen et al. 2001), and administration of MPG upon reperfusion after the main ischemic insult also eradicated IPC-induced cardioprotection (Hausenloy et al. 2007). Previous results from our lab showed that MPG, when used in conjunction with pharmacological preconditioning, needs to be washed out before induction of regional ischemia or it will be cardioprotective by itself (Sovershaev et al. 2006). We therefore stopped MPG administration 1 min prior to RI.

Summary of results

Paper I

The aim of this study was to examine the cardioprotective potential against ischemia-reperfusion injury by administrating insulin before ischemia or upon reperfusion, and to confirm a role for mTOR in cardioprotection by ischemic preconditioning or insulin therapy in the isolated rat heart. The putative, novel Akt inhibitor HIMO was also tested. Pre-treatment with insulin was just as effective in reducing infarct size as IPC and insulin therapy upon reperfusion. Although HIMO blocked cardioprotection in all three models tested, it could not be confirmed that this was due to inhibition of Akt phosphorylation, rather it seemed like the compound was an unspecific kinase inhibitor. The mTOR inhibitor rapamycin abolished cardioprotection induced by IPC or insulin therapy, indicating that mTOR is a common signalling protein playing an essential role in IPC- and insulin-induced cardioprotection against ischemia and reperfusion.

Paper II

A role for the JAK-STAT pathway in ischemic and pharmacological preconditioning has been indicated. This study was initiated in order to investigate the possible involvement of the JAK-STAT pathway in mediating the acute cardioprotective effect of insulin administered at reperfusion. Two different models were used: Langendorff perfused rat hearts exposed to 30 min of regional ischemia followed by 2 hrs of reperfusion and mouse cardiac myocytes exposed to 26 hrs of anoxia and 2 hrs of reperfusion. In both models, insulin was administered at onset of reperfusion. In the Langendorff perfused rat hearts, a reduction in infarct size was observed when insulin was present at reperfusion, and the JAK-STAT inhibitor AG490 abolished the insulin-induced protection. Insulin also increased cardiac myocyte survival in wild type mice, but not in cardiac deficient STAT3 myocytes. In isolated rat hearts a tendency towards insulin-induced phosphorylation of STAT3 at Tyr^{705} was shown, and AG490 attenuated the phosphorylation. AG490 also abrogated the insulin-induced phosphorylation of Akt at Ser^{473}. In cardiac deficient STAT3 myocytes, insulin failed to phosphorylate Akt. It is suggested that STAT3 phosphorylation and activation of Akt are closely associated in the cardioprotective signalling pathway activated by insulin treatment at reperfusion.

Paper III

Having demonstrated that insulin therapy at reperfusion involved activation of the JAK-STAT pathway (paper II), we wanted to examine the significance of the JAK-STAT pathway in cardioprotection induced by pre-treatment with insulin, and to investigate if cardioprotective GSK-3β blockade occured via JAK-STAT signalling. We also wanted to examine whether insulin-induced cardioprotection involved ROS. In isolated rat hearts, infarct size reduction by pre-treatment with insulin was abolished by the JAK-STAT inhibitor AG490 and the ROS-scavenger MPG, suggesting that JAK-STAT and ROS are important for insulin-induced cardioprotection. The HL-1 cell model was used in order to prove that stimulation with insulin lead to production of ROS. AG490 also abolished the infarct sparing effect of IPC, confirming a role for JAK-STAT in IPC-induced cardioprotection. MPG could not attenuate the protection offered by IPC; however, this was probably a result of the preconditioning protocol consisting of three cycles of global ischemia and reperfusion, overcoming the threshold for preconditioning. The GSK-3β inhibitor SB216763 reduced infarct size, and AG490 could not abolish this cardioprotection, indicating that GSK-3β is a downstream target of JAK-STAT.

Discussion

After more than 20 years since the phenomenon of IPC was described for the first time, intensive research is still dedicated to elucidate the mechanisms behind the IPC-induced cardioprotection. Many agents capable of mimicking the IPC-induced protection have been investigated, and the signalling pathways activated by different treatments have been and are still being unravelled. Even if IPC is one of the most powerful interventions in cardioprotection, it is not easy to apply clinically as most patients do not arrive in the emergency room until after ischemia has occurred. Reperfusion is essential to save the ischemic myocardium; however, further injury may be induced by reperfusion. Pharmacological agents applied at the time of reperfusion have the ability to reduce infarct size by activation of pro-survival signalling pathways. Recently, emerging evidence indicates that with preconditioning the mechanisms resulting in infarct size reduction occur after the heart is reperfused. Moreover, IPC and PC-mimetic agents as well as agents given at reperfusion, with a few exceptions, all seem to activate the cardioprotective RISK pathway.

The main focus of this thesis has been to elaborate the common signalling proteins activated by IPC or insulin therapy leading to cardioprotection. Insulin administered at onset of reperfusion after ischemia was previously shown to reduce infarct size in isolated rabbit (Baines et al. 1999) and rat hearts (Jonassen et al. 2001), and increase cell viability in neonatal ventricular myocytes (Jonassen et al. 2000). We therefore sought to investigate whether pre-treatment with insulin could offer cardioprotection to the same extent as IPC and insulin therapy at reperfusion, and if the same signalling mechanisms were involved. Two previous studies have reported an infarct reducing effect of insulin when administrating insulin prior to the main ischemic insult. The first study performed in pigs (Vogt et al. 1997) showed that a 60 min infusion of insulin lead to significant reduction in infarct size. The other study was performed in rabbits, and it was shown that a 5 min infusion of insulin followed by a 10 min washout period prior to the main ischemic insult was as protective as IPC (Baines et al. 1999). In the present thesis, IPC was mimicked by three cycles of insulin infusion followed by reperfusion prior to the index ischemia in the rat heart, and infarct size reduction was similar to hearts subjected to three cycles of global ischemia and reperfusion prior to the prolonged ischemic episode (paper I and III). Furthermore, the cardioprotective effect of insulin administration at reperfusion was similar to InsPC and IPC (paper I and II).

Previous studies have shown that both IPC and insulin induce cardioprotection via activation of the PI3K-Akt signalling pathway. IPC has been reported to induce phosphorylation of Akt in rat hearts after both two (Mocanu et al. 2002) and four preconditioning cycles (Tong et al. 2000), as well as at the time of reperfusion following two preconditioning cycles and prolonged ischemia (Hausenloy et al. 2004). In all these three studies, blocking PI3K attenuated phosphorylation of Akt, as well as abolished the infarct sparing effect of IPC. In the present thesis we observed increased phosphorylation of Akt by insulin in pre-ischemic hearts and HL-1 cells (paper I), as well as in post-ischemic hearts and isolated cardiac myocytes from wild type mice (paper II). Phosphorylation of Akt as well as reduction in infarct size after insulin-administration at reperfusion have previously been reported (Jonassen et al. 2001). The present work demonstrate that IPC, pre-treatment with insulin and insulin administration at reperfusion reduce infarct size in isolated rat hearts (paper I, II and III), and that insulin therapy at reoxygenation following simulated ischemia in wild type cardiac myocytes increase cell viability (paper II), suggesting a causal relationship between phosphorylation of Akt and cardioprotection. This was further supported by the results from cardiac deficient STAT3 mice (paper II), where insulin failed to induce phosphorylation of Akt and failed to rescue the cells at reoxygenation after simulated ischemia. However, phosphorylation of a protein may be an unreliable indicator of how significant the role played by the protein is; it does not necessarily give information about the activity of the given protein.

A good way to test whether activation of Akt is required for cardioprotection would be to use a specific Akt-inhibitor. Abolishment of insulin-induced cytoprotection at reperfusion by the putative Akt-inhibitor HIMO after 6 hrs of simulated ischemia in human derived Girardi cells was previously reported (Jonassen et al. 2004). In paper I we therefore tested HIMO in the isolated rat heart. HIMO eradicated the cardioprotective effect of both IPC and insulin treatment, indicating that activation of Akt is necessary with respect to reducing infarct size after prolonged ischemia. However, Western blot analysis of whole heart preparations revealed inconsistent results regarding the effect of HIMO on phosphorylation of Akt, and interestingly, in baseline hearts receiving insulin+HIMO, phosphorylation of Akt at Ser^{473} was significantly increased compared to both controls and hearts receiving insulin. Due to these surprising data, we went on to test the compound in HL-1 cells where dose-response experiments range 20 to 100 μM demonstrated that HIMO did not inhibit phosphorylation of Akt at Ser^{473} at concentrations below 80 μM. This was in contrast to a previous report stating that at a concentration of 20 μM, the dose used in paper I, 73% of Akt activity should be inhibited

(Martelli et al. 2003). However, there are some differences in the study design between Martelli et al. (2003) and the present work. Firstly, they incubated the cells with the inhibitor for more than 12 hrs, whereas in our experiments the blocker was present for 30 min at maximum. Secondly the cell models were completely different from ours, as Martelli et al. (2003) used apoptotic resistant cells derived from a human acute promyelocytic leukaemia. Davidson et al. (2006) used the HIMO-compound (10 μM, named SH-6 in their studies) in insulin-stimulated ventricular myocytes and showed that insulin induced prolonged resistance to MPT, an effect eliminated by HIMO. We can speculate that opening of the mPTP could be the mechanism by which HIMO abolished the cardioprotective effect of insulin in paper I. However, Davidson et al. (2006) also showed that insulin-stimulated phosphorylation of Akt was inhibited by the compound. It is difficult to explain the discrepancies between Davidsons' studies and ours; however, dose related effects and different cell models may be a cause.

In order to explain the loss of insulin-induced cardioprotection by HIMO, we sought to find other putative targets for the compound. Western blot tests from HL-1 cells revealed that HIMO probably acted as an unspecific protein kinase inhibitor as it abolished insulin-induced phosphorylation of a number of targets involved in cardioprotection, including PKCε and p70s6k, explaining the loss of cardioprotection by insulin when HIMO was present. Interestingly phosphorylation of GSK-3β was not abolished by HIMO. This observation was also made in Western blots from whole heart preparations (n=2, data not shown). In the study by Davidson et al. (2006) mentioned above, several mechanisms by which insulin-induced Akt phosphorylation could mediate inhibition of mPTP opening were proposed, one of these being inhibition of GSK-3β. Our data contradict that theory, as HIMO abolished insulin-induced cardioprotection without affecting phosphorylation and inhibition of GSK-3β. If insulin-induced mPTP-inhibition is abolished by HIMO, it must be by some other mechanism than GSK-3β inhibition.

p70s6k was one of the targets found to be inhibited by HIMO. It has previously been reported that the cardioprotection offered by insulin therapy at reperfusion (Jonassen et al. 2001) and the second window of protection following IPC (Kis et al. 2003) is inhibited by rapamycin, an mTOR inhibitor. In line with these studies, the results of the present thesis confirmed a role for mTOR in IPC and insulin therapy as rapamycin abolished the infarct sparing effects of IPC and insulin administration (paper I). p70s6k is a transcription factor, but the acute effect in cardioprotection can not be ascribed to increased transcription, as the time frame is too short. However, a possible anti-apoptotic effect of p70s6kinase has previously been reported (Jonassen

et al. 2000), and furthermore, mTOR/p70s6k was shown to inhibit GSK-3β and thereby prevent MPT induction leading to cell survival (Juhaszova et al. 2004). However, in paper I we observed attenuation of insulin-induced p70s6k-phosphorylation by HIMO, but not phosphorylation of GSK-3β. One can speculate that activated Akt phosphorylates p70s6k and GSK-3β in parallel, implying that inhibition of GSK-3β is not important for the cardioprotection offered by insulin whereas activation of p70s6k is crucial for reducing infarct size. Alternatively, HIMO inhibits downstream targets of GSK-3β, possibly the mPTP or other components of the mPTP. Further studies are needed to elucidate the signalling proteins affected by HIMO and to identify putative cardioprotective targets of p70s6k.

The involvement of STAT3 in cardioprotection by IPC has been widely documented. STAT3 has been shown to be important in both classic (Negoro et al. 2000) and delayed preconditioning (Xuan et al. 2001), and conditional knock out of STAT3 in the heart has proven that STAT3 is needed for IPC as these hearts can not be preconditioned (Smith et al. 2004). Also pharmacological preconditioning with TNFα (Lecour et al. 2005) and opioids (Gross et al. 2006) have been reported to activate the JAK-STAT pathway. The present work indicates that activation of the JAK-STAT pathway is necessary for cardioprotection as the infarct sparing effect of both reperfusion therapy with insulin (paper II) and pre-treatment with insulin (paper III) was abolished by the JAK-STAT inhibitor, and insulin failed to rescue cardiomyocytes deficient of STAT3 upon ischemic reperfusion (paper II). In accordance with previous studies, we also confirmed that IPC is dependent of the JAK-STAT pathway in order to reduce infarct size after ischemia (paper III). In paper II we showed that insulin administered at reperfusion lead to phosphorylation of Akt in isolated rat hearts, and that the JAK-STAT inhibitor AG490 abolished this phosphorylation. This suggest that JAK-STAT is activated upstream of Akt in the signalling cascade activated by insulin. Further support to this was added by the results in isolated cardiac myocytes from STAT3 deficient mice in which insulin failed to induce phosphorylation of Akt. Other studies have also suggested a role for JAK-STAT upstream of Akt. Granulocyte colony stimulating factor (G-CSF) was reported to activate JAK-STAT, PI3 kinase and Akt; and the JAK2 inhibitor AG490 abrogated G-CSF induced phosphorylation of JAK2, STAT3, Akt; whereas the PI3K inhibitor LY294002 suppressed G-CSF induced phosphorylation of Akt, but not JAK2 or STAT3, suggesting that JAK-STAT is upstream of Akt (Ueda et al. 2006). Furthermore, Gross et al. (2006) showed that opioid-induced phosphorylation of JAK2 was necessary for phosphorylation of Akt and STAT3, and it was suggested that STAT3 needs to be

phosphorylated in order to activate PI3K, thereby placing PI3K in parallel with or downstream of JAK2.

In paper II we could only observe a trend in insulin-induced STAT3 phosphorylation, so we can not firmly conclude that STAT3 is activated by insulin. However, the results showing that AG490 abrogates the cardioprotective effect of insulin in both paper II and III indicates that the JAK-STAT pathway is important for insulin-induced cardioprotection. There are several possible explanations for our results. First of all, AG490 might not be a specific inhibitor although previous studies have shown that it has no effect on the kinase activity of other protein tyrosine kinases (Meydan et al. 1996; De Vos et al. 2000). Another possibility is that AG490 inhibits JAK2, without affecting STAT3, so that the abolishment of the infarct-sparing effect of insulin seen by use of AG490 is only due to the inhibition of JAK2 and the targets of the insulin signalling pathway activated by JAK2 stimulation. Previous data have shown that following insulin stimulation, JAK2 interacts with the insulin receptor and IRS-1 forming stable complexes in the heart as well as in the liver, adipose tissue and skeletal muscle (Saad et al. 1996). We can therefore speculate that interaction between JAK2 and the insulin receptor is necessary for insulin-induced cardioprotection, and that AG490 inhibits insulin signalling via STAT3 independent mechanisms. Our Western blot analysis of phosphorylated Akt showed that AG490 inhibited insulin-induced phosphorylation of Ser^{473} (paper II), further supporting the hypothesis of involvement of JAK2 in the insulin signaling cascade. However, not being able to rescue cardiomycytes deficient of STAT3 by insulin strongly suggests that also STAT3 is important in the insulin signalling pathway.

The lack of significant phosphorylation of STAT3 in perfused rat hearts stimulated with insulin at reperfusion could be due to a transient phosphorylation of STAT3 so that we have missed the peak of phosphorylation after 15 min of perfusion. However, we also tried to perform Western blots after 5 min of perfusion (data not shown), and there was no significant insulin-induced phosphorylation of STAT3 at that time point. In non-ischemic hearts perfused for 10 min with insulin ± AG490, there was an increase of phospho-STAT3 (Fig. 9). This could indicate that the time-point at which samples are collected is of outmost importance. Still, one can not ignore the possibility that differences exist between the non-ischemic heart and hearts which have been subjected to ischemia and reperfusion. Ischemia by itself might lead to activation or inhibition of specific pathways. Also, since phosphorylation of kinases will depend on availability of ATP, unspesific variability in the results could be introduced. Moreover, at the European Section

meeting of the ISHR in Athens 2008, Pedretti et al. (2008) reported that STAT3 was differently distributed in the embryonic heart under basal conditions, and the level of STAT3 tyrosine phosphorylation was higher in atria compared to ventricles. In paper II, we collected samples from the ventricular area at risk after 15 min of reperfusion and pulverized them under liquid nitrogen to ensure homogeneity of the samples; however different cell populations may be present in the different samples even if the method is standardized. The Western blots presented in Fig. 9 are from the whole heart preparation, so this represents a larger cell population than the samples collected from area at risk. It is possible that STAT3 occurs in specific subcellular compartments of the cell; a phenomenon just recently described for protein kinase G (PKG) (Piggott et al. 2006), and not unlikely to be the case for many other protein kinases involved in cardioprotection.

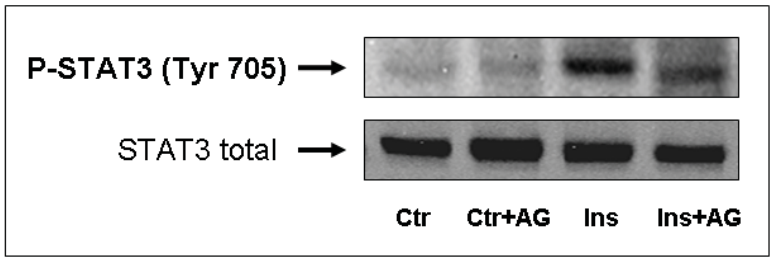

Figure 9 Representative Western blots of baseline hearts subjected to perfusion with Krebs buffer or insulin ± AG490 for 10 min and subsequently freeze clamped. (Fuglesteg BN, unpublished data).

Tyrosine phosphorylation of STAT3 is known to induce dimerization, nuclear transport and transactivation of STAT-responsive genes (Levy & Darnell 2002). It is possible that since we only looked at the cytosolic fractions in the present work, most of phospho-STAT3 had translocated to the nucleus. We suggest that STAT3 is tyrosine-phosphorylated, translocates to the nucleus where it initiates protein transcription and then moves back out to the cytoplasm where it performs yet unknown actions associated with acute cardioprotection. It is therefore possible that the trend we see of insulin-induced phosphorylation of STAT3 at 15 min is the start of re-localization of STAT3 from the nucleus back to the cytoplasm. Recent data from Boengler et al. (2008) presented at the ISHR in Athens, suggest that STAT3 is present in the matrix of cardiomyocyte mitochondria, and that there is a possible interaction of STAT3 with mitochondrial connexin 43 yielding cardioprotection. This could explain how activation of STAT3 can induce acute cardioprotection independent of its regulatory control of transcription. Alternatively, STAT3 is translocated both to the nucleus and to the mitochondria.

In the present thesis, only phosphorylation of the tyrosine residue was investigated. However, both tyrosine and serine phosphorylation of STAT3 are required for maximal activation of transcription (Wen et al. 1995). Interestingly, mTOR has been reported as an activator of STAT3, mediating serine phosphorylation of the peptide (Yokogami et al. 2000). This could indicate that STAT3 is tyrosine phosphorylated upstream of Akt, and serine phosphorylated by mTOR downstream of Akt. Serine phosphorylation of STAT3 independent of tyrosine phosphorylation has also been reported (Chung et al. 1997), suggesting that independent signalling pathways can converge on STAT3, and furthermore, phosphorylation of the serine residue was found to negatively modulate its tyrosine phosphorylation (Chung et al. 1997; Jain et al. 1998).

ROS have been reported as activators of the JAK-STAT pathway. In a study of Rat-1 fibroblasts it was shown that 5 min exposure to H_2O_2 caused a rapid activation of STAT3 activity independent of new protein synthesis (Simon et al. 1998). Interestingly, a biphasic induction was described, with a decrease in activity observed at 15 min and a return to peak levels by 30 min. Furthermore, antioxidants inhibited the H_2O_2-induced activation of STATs. In paper III we confirmed the results from paper I showing that IPC or pre-treatment with insulin conferred cardioprotection. Co-administration of AG490 abolished the cardioprotective effect of both treatments, implying a role for JAK-STAT in the signalling cascade activated by IPC and InsPC. Furthermore, the ROS-scavenger MPG was able to abolish cardioprotection offered by insulin therapy, implying a role for ROS in insulin-induced cardioprotection. In light of the study by Simon et al. (1998), it would be tempting to speculate that insulin-induced ROS production is cardioprotective by activation of the JAK-STAT pathway. HL-1 cells loaded with the ROS indicator DHE and images captured by confocal microscopy confirmed that insulin induced ROS-production (Fig. 10).

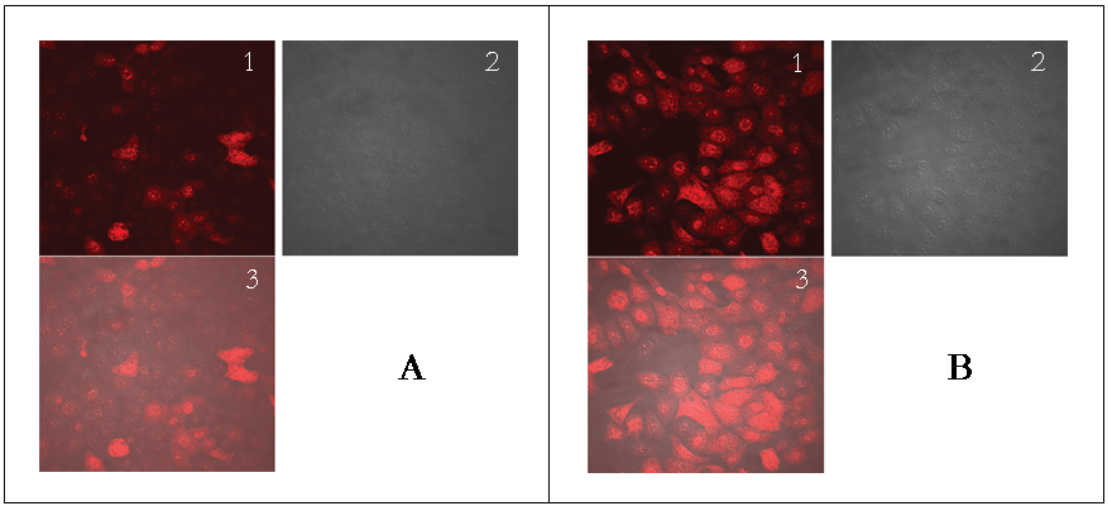

Figure 10 Representative images of HL-1 cells loaded with the ROS-indicator DHE in A) untreated control cells and B) cells treated with insulin for 10 min followed by 5 min washout of insulin. 1= confocal image; 2= transmitted light; 3= confocal and transmitted light combined (Fuglesteg BN, unpublished data).

Interestingly Juhaszova et al. (2004) associated cell protection exhibiting a memory with increased ROS production due to triggered mitochondrial swelling. Insulin was categorized as a "nonswelling" agent not acting via ROS that would act poorly in terms of the ability to precondition. In data not shown we tested whether 15 min washout of insulin prior to the main ischemic insult would confer cardioprotection. Infarct size was increased from 20.0% in hearts (n=3) where insulin was not washed out prior to the index ischemia to 35.7% (n=2) in hearts with 15 min of insulin washout (data not shown). In accordance with the data from Juhaszova et al. (2004) it seems like insulin is a cardioprotective agent without the ability to induce cardiac memory, however in contradiction to Juhaszova et al. (2004), our data suggest that insulin does act via increased ROS-production. Further investigations regarding the protein kinases activated in response to insulin-induced ROS generation in the myocardium is warranted. However, previous studies have indicated that targets upstream of PI3K, like the phosphatase and tensin homolog (PTEN) (Seo et al. 2004) and protein-tyrosine phosphatases (PTPases) (Mahadev et al. 2001) are important in insulin-induced ROS production.

When treating the hearts with insulin upon reperfusion, a concentration of 0.3 mU/ml was sufficient to induce cardioprotection, whereas a concentration of 5 mU/ml was needed to protect the heart when adding insulin as a pre-treatment agent. In light of the recent reports on activation of the RISK pathway at reperfusion after an IPC-protocol (Hausenloy et al. 2005), we speculate that a higher dose of insulin at pre-treatment is needed in order to set the heart in the protected state at reperfusion.

MPG was not able to abolish the cardioprotection offered by three cycles of IPC in the present thesis. This was not surprising though, as MPG has been reported to block the protection induced by a single cycle of IPC, but not four cycles of IPC (Baines et al. 1997). This has previously been explained by the concept of a "preconditioning threshold" implying that the triggers (adenosine, bradykinin, opioids and ROS) released from an ischemic heart are ineffective alone, but when added together a threshold for protection can be reached (Cohen et al. 2000). The use of three preconditioning cycles can therefore lead to an accumulation of the other triggers of IPC in sufficient amounts to reach a threshold for protection, independent of ROS. Recent data imply that ROS trigger preconditioning during the early reperfusion phase of IPC and hydroxyl radicals were suggested as the species responsible for triggering protection (Dost et al. 2008). DHE detects dismutation of superoxide ion, and according to Dost et al. (2008), oxidation of DHE is not performed by the same pool of ROS which triggers preconditioning. This would imply that the insulin induced ROS-production seen in HL-1 cells in paper III is not involved in the triggering phase of cardioprotection. And yet MPG abolished insulin-induced reduction of infarct size, strongly suggesting that insulin-induced ROS is needed for cardioprotection. It is important to keep in mind that with regards to ROS, everything is mixed in the cell. ROS production starts with superoxide and ends up with hydroxyl radical as illustrated in Fig. 7. It is therefore difficult to conclude that a certain ROS-species is responsible for triggering protection.

Paper III also shows that pre-treatment with the GSK-3β inhibitor SB216763 is capable to reduce infarct size, but AG-490 did not abolish the cardioprotection, implying that GSK-3β is a downstream target of JAK-STAT. This is in accordance with data from Gross et al. (2006) which showed that opioid-induced cardioprotection via JAK-STAT could not be blocked by AG490, concluding that GSK-3β was downstream of JAK2. However, it is also possible that the inhibition of GSK-3β occurs via JAK-STAT independent pathways.

In order to sum up the findings and speculations in the present thesis, proposed mechanisms of insulin-induced cardioprotection is illustrated in Fig. 11. There are unresolved issues with regards to the question whether insulin and IPC share universal signalling events. We do not know whether insulin acts via PKC and K_{ATP}. Insulin has been reported to increase PKC activity in adipocyte plasma membranes (Egan et al. 1990) and in cultured fetal chick neurons (Heidenreich et al. 1990). Baines et al. (1999) reported that myocardial protection by insulin in the isolated rabbit heart was not dependent on PKC or K_{ATP} channels; however, this may be species

dependent. Downey suggested orally at the ISHR in Athens 2008 that PKC activated by ROS during the trigger phase of IPC needs to be reactivated during the reperfusion phase in order to protect the heart. We do not know if insulin-induced ROS production and the ROS species produced by insulin stimulation is the same ROS which act as a trigger in IPC. Data from the literature suggest that upon insulin stimulation in adipocytes, NADPH oxidase is activated yielding superoxide which generates H_2O_2 (Mahadev et al. 2004), whereas the source of ROS in IPC is thought to be the mitochondria (Vanden Hoek et al. 1998; Forbes et al. 2001). Moreover, we do not know which protein kinases that are activated by insulin-induced ROS production. Based on the study by Davidson et al. (2006) showing that insulin induced prolonged resistance to mPTP, it is tempting to speculate that this effect is due to ROS.

Increasing evidence suggest involvement of gap junctions and connexin 43 in IPC (Schwanke et al. 2002; Sundset et al. 2007). Since both IPC and insulin therapy have been shown to involve the JAK-STAT pathway in the present work, and a possible interaction of STAT3 with mitochondrial connexin 43 yielding cardioprotection has been reported (Boengler et al. 2008), reduced dephosphorylation of connexin 43 by insulin could be one of the mechanisms by which insulin induce cardioprotection. Moreover, IPC has been shown to delay the detrimental rise in intracellular Ca^{2+} at reperfusion after 25 min of global ischemia (Wang et al. 2001). Insulin has recently also been reported to induce protection against reoxygenation-induced Ca^{2+} overload via activation of the RISK pathway (Abdallah et al. 2006). Improved Ca^{2+} handling is therefore another common cardioprotective mechanism shared by IPC and insulin therapy. Continuation of investigations regarding the underlying cardioprotective mechanisms induced by insulin is important as using insulin therapy for AMI could be of great clinical potential.

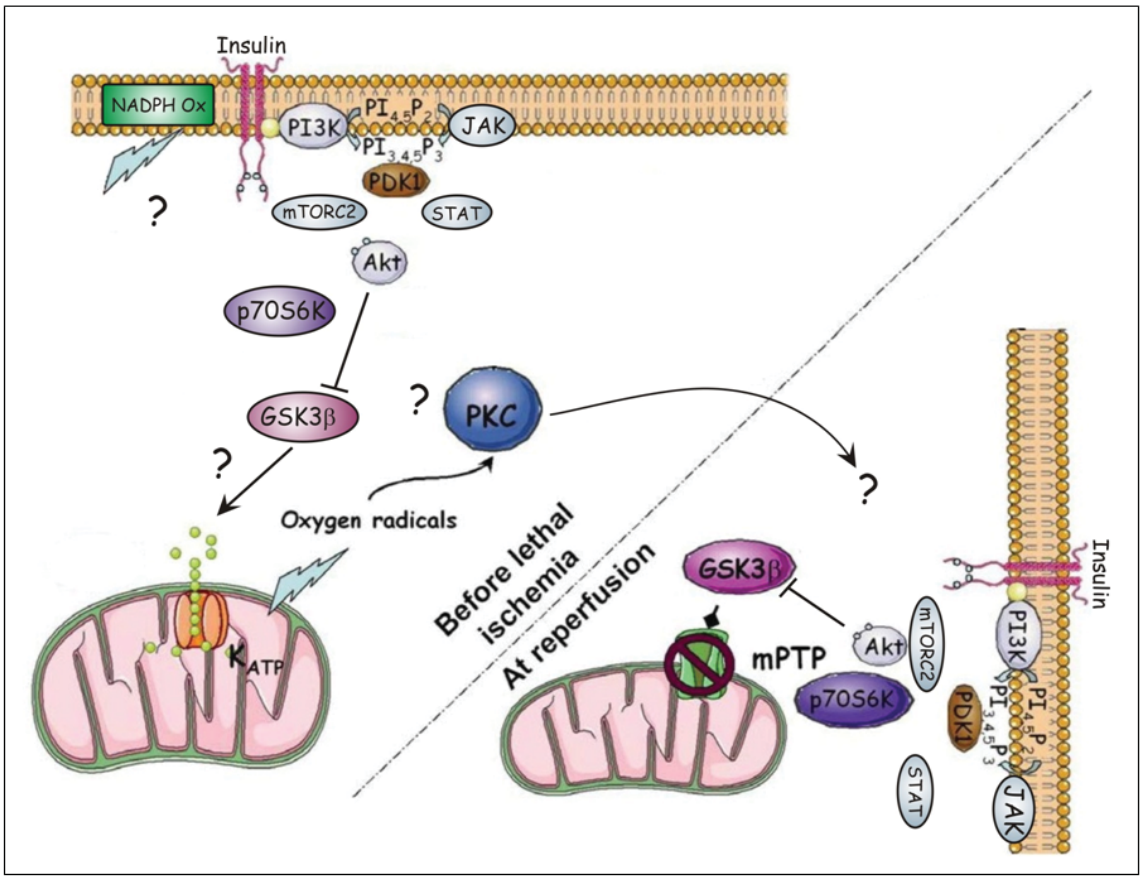

Figure 11 Proposed mechanisms of insulin signalling resulting in cardioprotection examined in the present thesis. GSK-3β= glycogen synthase kinase-3β; JAK= janus activated kinase; K_{ATP}= potassium dependent ATP channel; mPTP= mitochondrial permeability transition pore mTORC2= mammalian target of rapamycin complex 2; NADPH Ox= NADPH oxidase; PDK1= 3-phosphoinositide dependent kinase; PI3K= phosphatidylinositol 3-kinase; $PI3,4,5P_3$= phosphatidylinositol-3,4,5-trisphosphate; $PI4,5P_2$= phosphatidylinositol-4,5-bisphosphate; PKC= protein kinase C; p70s6K= p70s6 kinase; STAT= signal transducer and activator of transcription. (Modified from Tissier et al. 2008).

Concluding remarks

The following conclusions may be drawn from the present work:

1. Pre-treatment with insulin reduces infarct size to the same extent as IPC or insulin therapy at reperfusion and mTOR is part of the cellular signalling cascade conferring cardioprotection by all three treatments.
2. The putative Akt inhibitor HIMO is a non-specific kinase inhibitor able to abolish IPC- or insulin-induced cardioprotection.
3. Cardioprotection induced by insulin and IPC occurs through activation of the JAK-STAT pathway.
4. GSK-3β may be a downstream signalling target of the JAK-STAT pathway.
5. Insulin-induced cardioprotection is dependent on production of ROS.

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Paper I

Paper II

Paper III


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