Postconditioning the isolated perfused rat heart: the role of kinases and phosphatases

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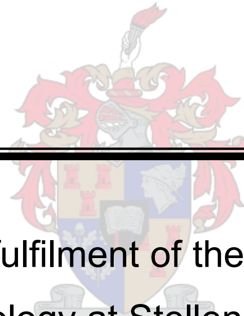

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:

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Abstract

It has recently been observed that the application of multiple short cycles of reperfusion and ischaemia, at the onset of reperfusion, elicits cardioprotection against injury due to prior sustained ischaemia. This phenomenon has been termed “postconditioning” (postC) and is of special interest due to its clinical applicability. Although much work has been done to delineate the mechanism of protection, there is still controversy regarding the precise algorithm of postC, the importance of the reperfusion injury salvage kinases (RISK), as well as uncertainty about the possible role of p38 MAPK and the protein phosphatases in postC cardioprotection.

The aims of this study were therefore:

I. To develop and characterise a cardioprotective postC protocol in the *ex vivo* rat heart, using both the retrogradely perfused and working heart models.
II. To characterise the profiles of PKB/Akt, ERK p42/p44 and p38 MAPK associated with the postC intervention.
III. To investigate the possible role of the serine/threonine protein phosphatases type 1 and type 2A (PP1 and PP2A) in the mechanism of postC.

Hearts from male Wistar rats were perfused in both the retrograde Langendorff (at a perfusion pressure of 100 cmH_2O and diastolic pressure set between 1 and 10 mmHg) and working heart models (preload: 15 cmH_20 and afterload: 100 cmH_20). Several different postC protocols were tested for their cardioprotective effect, as analysed by infarct size (IFS; determined by triphenyltetrazolium chloride (TTC) staining) and functional recovery. Experimental parameters tested were the number of cycles (3,4 or 6), the duration of the cycles (10, 15, 20 or 30 seconds), the method of application (regional or global) and temperature during the intervention (36.5 or 37 °C). Different sustained ischaemic insults were also utilised: 35 minutes regional (RI) or 20, 25, 30 and 35 minutes global ischaemia (GI).

Hearts treated with a cardioprotective postC intervention or standard reperfusion after sustained ischaemia, were freeze-clamped at 10 and 30 minutes reperfusion in both perfusion models. Tissue samples were then analyzed using Western blotting, probing for total and phosphorylated PKB/Akt, ERK p42/p44 and p38 MAPK. The contribution of

PKB/Akt and ERK p42/p44 activation to cardioprotection was also investigated by administration of inhibitors (A6730 and PD098059 respectively) in the final 5 minutes of ischaemia and the first 10 minutes of reperfusion, in the presence and absence of the postC intervention. The effect of these inhibitors were analyzed in terms of IFS and kinase profiles.

The possible role of the phosphatases in postC was investigated by observing the effect of cantharidin (a PP1 and PP2A inhibitor) treatment directly before sustained ischaemia (PreCanth) or in reperfusion (PostCanth), in the presence and absence of postC, on IFS and kinase profiles.

A postC protocol of 6x10 seconds global reperfusion / ischaemia, at 37°C, was found to give the best and most consistent reduction in infarct size in both the Langendorff (IFS in NonPostC: 47.99±3.31% vs postC: 27.81±2.49%; $p<0.0001$) and working heart (IFS in NonPostC: 35.81±3.67% vs postC: 17.74±2.73%, $p<0.001$) models. It could however only improve functional recovery in the Langendorff model (after 30 minutes GI: rate pressure product (RPP) recovery: NonPostC = 12.27±2.63% vs postC = 24.61±2.53%, $p<0.05$; and after 35 minutes GI: left ventricular developed pressure (LVDP) recovery: NonPostC = 28.40±7.02% vs postC = 48.49±3.14%, $p<0.05$). This protection was associated with increased PKB/Akt (NonPostC: 0.88±0.26 AU (arbitrary unit) vs postC: 1.65±0.06 AU; $p<0.05$) and ERK p42 (NonPostC: 2.03±0.2 AU vs postC: 3.13±0.19 AU; $p<0.05$) phosphorylation. Inhibition of PKB/Akt activation with A6730 (2.5 µM) abrogated the infarct sparing effect of postC.

Administration of cantharidin, either before of after ischaemia, in the absence of postC, conferred an infarct sparing effect (IFS in PreCanth: 15.42±1.80%, PostCanth: 21.60±2.79%; $p<0.05$) associated with an increase in the phosphorylation of MAPK p38 (administration before ischaemia: NonCanth: 1.52±0.26 AU vs PreCanth: 2.49±0.17 AU, $p<0.05$; and administration after ischaemia: NonCanth: 5.64±1.17 AU vs PostCanth: 10.69±1.29 AU, $p<0.05$) and ERK p42 (when administered in reperfusion; NonCanth: 2.24±0.21 AU vs PostCanth: 3.34±0.37 AU; $p<0.05$). Cantharidin treatment combined with the postC intervention did not elicit an additive infarct sparing effect (postC: 17.74±2.72%, PreCanth-postC: 13.30±3.46% and PostCanth-postC: 15.39±2.67%).

In conclusion: a postC protocol of 6x10 seconds global ischaemia / reperfusion, at 37°C, confers the best infarct sparing effect in both the Langendorff and working rat heart models. This protection is associated with ERK p42 and PKB/Akt phosphorylation, although only PKB/Akt is necessary for cardioprotection. We could not find evidence for PP1 and PP2A involvement in postC, although inhibition of these phosphatases *per se* does elicit an infarct sparing effect. The latter observation suggests that phosphatase activation during ischaemia / reperfusion is potentially harmful.

Opsomming

Dit is onlangs waargeneem dat toediening van meervoudige siklusse herperfusie / iskemie, met die aanvang van herperfusie, die hart teen iskemie / herperfusie beskadiging beskerm. Hierdie verskynsel, bekend as postkondisionering (postC), geniet tans baie aandag vanweë die kliniese toepaslikheid van die ingreep. Ten spyte van intensiewe navorsing om die betrokke meganisme van beskerming vas te stel, is daar steeds kontroversie oor die presiese algoritme van die ingreep, asook die betrokkenheid van die sogenaamde iskemie herperfusie oorlewings kinases (RISK). Daar bestaan ook onsekerheid oor die rol van die stres-kinase, p38 MAPK, asook die proteïen fosfatases in die meganisme van beskerming teen iskemiese besering.

Hierdie studie het dus drie doelstellings gehad:

I. Ontwikkeling van 'n postC protokol wat beskerming ontlok in die rothart *ex vivo*, deur gebruik te maak van beide die retrograad geperfuseerde ballon model, asook die werkhart model.
II. Analiese van die profiele van die kinases PKB/Akt, ERK p42/p44 en p38 MAPK tydens herperfusie van postC en kontrole (NonPostC) harte.
III. Ondersoek na die moontlike rol van die serien / treonien proteïen fosfatases tipe 1 en tipe 2A (PP1 en PP2A) in die meganisme van postC beskerming.

Harte van manlike Wistar rotte is geperfuseer in beide die retrograad geperfuseerde ballon (d.i. die Langendorff) model (teen 'n konstante perfusie druk van 100 cmH_20 en 'n diastoliese druk gestel tussen 1 en 10 mmHg), asook die werkhart model (teen 'n voorbelading van 15 cmH_20 en 'n nabelading van 100 cmH_20). Verskeie moontlike postC protokolle is getoets vir hul vermoë om kardiobeskerming te ontlok, in terme van funksionele herstel en infarktgrootte (IFS), soos bepaal deur trifenieltetrazolium chloried (TTC) kleuring. Die eksperimentele veranderlikes tydens die postC protokol wat ondersoek is, sluit in: die aantal siklusse (3, 4 of 6), die duur van die siklusse (10, 15, 20 of 30 sekondes), die wyse van postC toediening (streeks of globaal) en laastens die temperatuur tydens die ingreep (36.5 of 37 °C). Daar is ook gebruik gemaak van verskillende periodes iskemie: 35 minute streeks iskemie (RI), asook 20, 25, 30 en 35 minute globale iskemie (GI).

Na 10 of 30 minute herperfusie is harte wat blootgestel is aan ‘n kardiobeskermende postC ingreep of gewone standaard herperfusie na iskemie, in beide perfusie modelle, gevriesklamp. Die weefsel proteïen-inhoud is verder geanaliseer deur van die Western blot tegniek gebruik te maak vir bepaling van die totale en fosforileerde vlakke van PKB/Akt, ERK p42/p44 en p38 MAPK. Die funksionele belang van PKB/Akt en ERK p42/p44 is verder ondersoek deur die effek van ‘n geskikte inhibitor (onderskeidelik A6730 en PD098059, toegedien tydens die laaste 5 minute van iskemie en die eerste 10 minute van herperfusie), in die teenwoordigheid en afwesigheid van die postC ingreep, op infarktgrootte en kinase aktiwiteit te monitor.

Die moontlike rol van proteïen fosfatases in postC is ondersoek deur die effek van cantharidin (‘n PP1 en PP2A inhibitor) op infarktgrootte en kinase profiele te ondersoek. Cantharidin is óf onmiddelik voor iskemie óf tydens herperfusie toegedien, in die aan – en afwesigheid van die postC ingreep.

Daar is bevind dat ‘n postC protokol van 6x10 sekondes globale iskemie / herperfusie, teen 37°C, die mees effektiewe en konstante verlaging in infarktgrootte teweeg gebring het in beide die ballon model (IFS in NonPostC: 47.99±3.31% vs postC: 27.81±2.49%; $p<0.0001$), asook die werkhart (IFS in NonPostC: 35.81±3.67% vs postC: 17.74±2.73%, $p<0.001$). Funksionele herstel kon egter slegs ontlok word in die ballon model (na 30 minute GI: tempo druk produk (RPP) herstel: NonPostC = 12.27±2.63% vs postC = 24.61±2.53%, $p<0.05$; en na 35 minute GI: linker ventrikulêre ontwikkelde druk (LVDP) herstel: NonPostC = 28.40±7.02% vs postC = 48.49±3.14%, $p<0.05$). Die infarkt-besparende effek van postC was geassosieer met ‘n toename in die fosforilasie van beide PKB/Akt (NonPostC: 0.88±0.26 AU (arbitrêre eenhede) vs postC: 1.65±0.06 AU; $p<0.05$) en ERK p42 (NonPostC: 2.03±0.2 AU vs postC: 3.13±0.19 AU; $p<0.05$). Inhibisie van PKB/Akt met A6730 (2.5 μM) het die infarkt-besparende effek van postC opgehef.

Inhibisie van PP1 en PP2A opsigself, deur toediening van cantharidin óf voor óf na iskemie (in die afwesigheid van postC), het ‘n infarkt-besparende effek ontlok (IFS in PreCanth: 15.42±1.80%, PostCanth: 21.60±2.79%; $p<0.05$). Hierdie kardiobeskerming was geassosieer met ‘n toename in die fosforilasie van beide p38 MAPK (met toediening voor iskemie: NonCanth: 1.52±0.26 AU vs PreCanth: 2.49±0.17 AU, $p<0.05$; en toediening na iskemie: NonCanth: 5.64±1.17 AU vs PostCanth: 10.69±1.29 AU, $p<0.05$), asook ERK p42, indien cantharidin toegedien is tydens herperfusie (NonCanth: 2.24±0.21 AU vs

PostCanth: 3.34±0.37 AU; $p<0.05$). Kombinasie van cantharidin behandeling met postC toediening kon egter nie ‘n kumulatiewe infarkt-besparende effek uitlok nie (postC: 17.74±2.72%, PreCanth-postC: 13.30±3.46% en PostCanth-postC: 15.39±2.67%).

In samevatting: ‘n PostC protokol van 6x10 sekondes globale iskemie / herperfusie, teen 37°C, ontlok die mees effektiewe infarkt-besparende effek in beide die ballon, sowel as die werkhart modelle. Alhoewel hierdie beskerming geassosieer is met ‘n toename in die fosforilasie van beide PKB/Akt en ERK p42/p44 tydens herperfusie, is dit slegs PKB/Akt wat van funksionele belang is in die meganisme van kardiobeskerming. Ons kon geen bewyse vind vir die betrokkenheid van PP1 en PP2A in postC beskerming nie, alhoewel inhibisie van hierdie fosfatases opsigself infarkt-besparend is. Laasgenoemde waarneming toon dat fosfatase aktivering tydens iskemie / herperfusie skadelike gevolge mag hê.

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Abbreviations

1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one	:	ODQ
5' -(*N*-ethylcarboxamido) adenosine	:	NECA
8-p-(sulfophenyl) theophylline	:	SPT
Adenine nucleotide translocase	:	ANT
Adenosine triphosphate	:	ATP
Arbitrary unit	:	AU
Area at risk	:	AAR
Area at risk	:	AR
ATP – sensitive pottasium channel	:	K_{ATP} – channel
Cardiac output	:	CO
Cardiovascular disease	:	CVD
c-Jun NHP_2 terminal kinase	:	JNK
Constitutive NOS	:	cNOS
Coronary artery bypass grafting	:	CABG
Coronary flow	:	CF
Coronary heart disease	:	CHD
Creatine kinase	:	CK
Cyclic guanosine-monophosphate	:	cGMP
Cyclooxygenase – 2	:	COX-2
Cyclophilin-D	:	CypD
Deoxyribonucleic acid	:	DNA
Dimethyl sulfoxide	:	DMSO
electrophoresis	:	SDS-PAGE
Endothelial NOS	:	eNOS
Extracellular signal – regulated kinase	:	ERK
Global ischaemia	:	GI
Glycogen synthase kinase - 3β	:	GSK-3β
Guanylyl cyclase	:	GC
Heart rate	:	HR
Heat shock protein 27	:	HSP27
Hypoxic preconditioning	:	HP
Inducible NOS	:	iNOS

Infarct size	:	IFS
Inorganic phosphates	:	P_i
Ischaemia / reperfusion injury	:	IRI
Ischaemic preconditioning	:	IPC
Lactate dehydrogenase	:	LDH
Lactate dehydrogenase	:	LDH
Left ventricular developed pressure	:	LVDP
Malondialdehyde	:	MDA
MAPK / ERK kinase	:	MEK
MAPK kinase 1	:	MAPKK1
Membrane attack complex	:	MAC
Mitochondrial ATP-dependent potassium channel	:	mK_{ATP} – channel
Mitochondrial permeability transition pore	:	mPTP
Mitogen Activated Protein Kinase p38	:	p38 MAPK
Mitogen Activated Protein Kinase	:	MAPK
Myocardial infarct	:	MI
Myocardial infarction	:	MI
Myosin light chains	:	MLC
Nitrcic oxide synthase	:	NOS
Nitric oxide	:	NO
Nitric oxide	:	NO
Okadaic acid	:	OA
Phospatidylinositol 3-kinase	:	PI3-kinase
Phospholamban	:	PLB
Polyvinylidene fluoride	:	PVDF
Postconditioning	:	PostC
Protein kinase C	:	PKC
Protein phospatase type 1	:	PP1
Protein phosphatase type 2A	:	PP2A
Protien kinase B	:	PKB/Akt
Reactive nitrogen species	:	RNS
Reactive oxygen species	:	ROS
Regional ischaemia	:	RI
Reperfusion Injury Salvage Kinases	:	RISK
Sarcoplasmic reticulum Ca^{2+} ATPase	:	SERCA

Sarcoplasmic reticulum	:	SR
Sarcoplasmic reticulum	:	SR
Second	:	sec
Signal transducer and activator of transcription 3	:	STAT3
Sodium dodecyl sulfate – polyacrylamide gel	:	SDS-PAGE
Standard error of the means	:	SEM
Tissue factor	:	TF
Triphenyltetrazolium chloride	:	TTC
Tris - buffered saline	:	TBS
Tropinin inhibitor	:	TnI
Troponin I	:	TnI
Tumour necrosis factor – alpha	:	TNF – α
Voltage – dependent anion channel	:	VDAC
Wistar-Ottawa-Karlsburg-W-rats	:	WOKW

Chapter 1: Literature overview

“I praise you because I am fearfully and
wonderfully made;
your works are wonderful,
I know that full well.”

Psalm 139:14
The Bible

Chapter 1: Literature overview

1.1. Ischaemia and reperfusion: an introduction

1.1.1. Ischaemic heart disease

One of the leading causes of death, in especially the developed world, is ischaemic heart disease. According to the projections of Murray & Lopez (1997), heart disease is set to remain a major contributor to mortality in years to come. This was confirmed by follow-up projections done by Mathers & Loncar, published in 2006. Ischaemic heart disease is usually not found in isolation, but is often a single facet of the so-called metabolic syndrome (Bonora *et al.*, 2003; Caglayan *et al.,* 2005). The metabolic syndrome is also in itself a growing cause of morbidity and mortality in the developed world (Smith, 2007), as well as in South Africa (Seedat, 1998; StatsSa, 2005).

Various conditions, especially abdominal obesity and atherosclerosis, are associated with the development of coronary heart disease (CHD) and cardiovascular disease (CVD) (AACE/ACE, 1998; Kim *et al.* 2000; Eckel & Krauss, 2007). The basic series of events leading to a cardiac ischaemic event originates in the vasculature, and usually involves atherogenesis. This is the formation of plaques consisting of calcium crystals, smooth muscle cells, macrophages, fatty streaks and inflammatory mediators (Ross, 1999; Guyton & Hall, 2000). For reviews on the development and nature of atherosclerosis see Ross (1999), Opie (2004) and Scott (2004). In brief: Such a lesion can either be stable or unstable. In the case of an unstable plaque, shear stress can lead to the disruption of the plaque, leading to the expulsion of pro-thrombotic factors into the blood. Pieces of the plaque structure can also enter the circulation. The net effect is the presence of solid particles, or thrombi, in the bloodstream that will eventually lodge in a smaller artery, capillary or vein. Even if the latter does not happen, the plaque itself can grow in size to such an extent that it decreases the vascular lumen diameter. If an occlusion of atherosclerotic origin occurs in the coronary arteries, the myocardium "downstream" of the occlusion is left without sufficient oxygen supply to meet metabolic demand, leading to compromised myocardial viability and function.

Oxygen deprivation will lead to several metabolic changes in the cardiomyocytes. Primarily, adenosine triphosphate (ATP) levels decrease, leading to a switch from aerobic to anaerobic metabolism, and the accumulation of intracellular protons – with a corresponding decrease in intracellular pH. The decrease in blood supply also leads to a reduction in the rate of removal of metabolic products – leading to the accumulation of potentially toxic substances. If the hypoxic condition prevails, ATP levels will become insufficient for the maintenance of ion-pump activity, causing a disturbance of normal ion homeostasis (Opie, 2004). The combination of these detrimental intracellular changes, eventually leads to cell death. It should be mentioned that it has been shown that apoptosis also contributes to cell death in myocardial ischaemia. The precise contribution of necrosis versus apoptosis to cell death in ischaemia is still controversial. Apoptosis in ischaemia and reperfusion will be discussed in more detail later in the text.

It therefore stands to reason that the best treatment for such a state of ischaemia is to re-establish perfusion of the affected tissue as soon as possible, i.e. transport of nutrients and oxygen to the affected tissue and of metabolites away from it. Reperfusion is a clinical reality in the setting of thrombolysis, coronary angioplasty, coronary bypass grafting and transplantation. The efficacy of reperfusion depends on the duration of ischaemia and the degree of tissue injury. In general, the shorter the time of ischaemia, the more tissue can be salvaged by reperfusion (Jennings & Reimer, 1983).

1.1.2. Lethal reperfusion injury

Unfortunately, reperfusion also has a sinister side, since it can itself induce further injury, i.e. reperfusion injury. This refers to reperfusion-associated events that cause the death of cells, only reversibly injured at the end of ischaemia (Piper & García-Dorado, 1999; Park & Lucchesi, 1999; Yellon & Hausenloy, 2007). This concept is paradoxical, since reperfusion is the only way to salvage reversibly damaged tissue. Without reperfusion all the ischaemic tissue will eventually be lost. However, ischaemia and reperfusion injury do go hand in hand, and in the literature they are often singularly referred to as ischaemia / reperfusion injury (IRI).

Some of these possible mechanisms are, briefly, as follows:

- **Calcium overload**. This is probably one of the most well known causes of tissue damage in the setting of ischaemia / reperfusion. In this regard calcium has been implicated with the phenomenon of stunning (Kusuoka *et al.*, 1987). This refers to the

transient decrease in contractile activity in the first hours of reperfusion (Braunwald & Kloner, 1982; Gross *et al.*, 1999). For a review on the alterations in calcium homeostasis during reperfusion, see Gross *et al.* (1999). Obviously various calcium transporters have been implicated: In the sarcolemma the L-type calcium channel has been implicated in an increase in calcium influx into the cell (Przyklenk *et al.*, 1989). In the SR both the Ca^{2+}-ATPase (responsible for calcium uptake) and ryanodine receptors (involved in calcium release) could contribute to disturbances in Ca^{2+} ion homeostasis (Smart *et al.*, 1997; Valdivia *et al.*, 1997; Gross *et al.*, 1999). Another possibility is that the ischaemia-induced reduction in ATP levels leads to a decrease in Na^{+}-K^{+} -ATPase activity, causing an increase in intracellular sodium. This in turn reverses the action of the Na^{+} / Ca^{2+} exchanger, causing sodium export in exchange for calcium import – eventually leading to excess intracellular calcium levels. In this respect, Inserte *et al.* (2002) found that the administration of a Na^{+} / Ca^{2+} exchange inhibitor (KB-R7943) during reperfusion or re-energization leads to a decrease in intracellular Ca^{2+} levels, associated with a reduction in hypercontracture and infarct size. Calcium overload in itself can also lead to mitochondrial damage (Crompton & Costi, 1988).

- **Free radical generation**. A free radical can be defined as an atom, or molecule, with an unpaired electron in its outer orbital (Park & Lucchesi, 1999). These chemical species are therefore highly reactive, as reflected in a very short half-life, which means they can be very harmful in biological systems. Various studies have shown that free radicals are generated at the very onset of myocardial reperfusion. These free radicals include the reactive oxygen species (ROS), namely superoxide anions, hydrogen peroxide and hydroxyl radicals, as well as the reactive nitrogen specie (RNS) peroxynitrite (which is formed when nitric oxide (NO) reacts with superoxide) (Zweier & Hassan Talukder, 2006).

  For a review on the possible sources of free radicals and their effects in the ischaemia-reperfused myocardium, see Zweier & Hassan Talukder (2006). Free radicals could damage the cell by peroxidation of membrane lipids, damaging and denaturating proteins (including ion channels and enzymes) and also damaging DNA. Free radicals could therefore significantly contribute to cell damage and death via swelling, as well as calcium overload due to damaged ion pumps. It is therefore not surprising that ROS have been implicated in the phenomenon of stunning (Bolli *et al.*, 1989). Reactive

oxygen species could also contribute to mitochondrial dysfunction by contributing to the opening of the mitochondrial permeability transition pore (mPTP), which then leads to a dysregulation of the mitochondrial membrane potential, compromising ATP production in the mitochondria (for a review on the role of the mPTP in reperfusion, see Halestrap *et al.*, 2004). In this regard Akao *et al.* (2003) found that oxidative stress in ventricular myocytes was associated with changes in mitochondrial structure, eventually leading to the opening of the mPTP, which in turn initiated cell death. Free radical generation could also be important in the human heart, as illustrated by Ferrari *et al.* (1990). They found that there is an increase in oxidative stress in human myocardium at reperfusion, which, in turn, is dependent on the duration of ischaemia.

It should however be mentioned that the importance of free radical generation in reperfusion injury is not universally accepted, especially in the light of the failure of administered free radical scavengers to prevent reperfusion-associated injury in some studies (Richard *et al.*, 1988; Piper & Garciá-Dorado, 1999; Park & Luchessi, 1999).

- **Inflammatory processes.** Since ischaemia and reperfusion causes tissue damage, it is not surprising that inflammatory processes are also involved in ischaemia / reperfusion. These inflammatory processes can be detrimental through various mechanisms. Neutrophils are recruited to the area of damage in the myocardium – as would be the case in any damaged tissue. Dreyer *et al.* (1999) for example found that, in canine hearts, there was an increase in neutrophile accumulation during reperfusion, especially in the subendocardium. In the heart neutrophils potentially contribute to damage by generating free radicals and / or various cytotoxic agents. Various studies have also shown that inhibition or depletion of neutrophils lead to a decrease in tissue injury associated with ischaemia / reperfusion (Romson *et al.,* 1983*;* Kin *et al.*, 2006). The increase in neutrophils in the coronary system can also lead to the formation of "plugs" in the coronary capillaries. These plugs then contribute to the phenomenon of no-reflow (Engler *et al.*, 1983). This is a form of reperfusion injury in which the opening of a blocked coronary artery does not lead to the perfusion of the tissue distal to the initial blockage (Rezkalla & Kloner, 2002). For an extensive review on the possible role of neutrophils in ischaemia-reperfusion injury, see Jordan *et al.* (1999).

The complement system has also been implicated in ischaemia / reperfusion (for a short review see Park & Luchessi, 1999). The complement system is a complicated

extracellular system of up to thirty proteins, that can interact via a classical (beginning with protein C1) -, or a alternative pathway (initiated with protein C3). After activation various proteins bind to each other in a sequential manner, in the process contributing to chemotaxis, opsonization and eventually severe cell damage and death via the formation of the C5b-9 membrane attack complex (MAC) (Guyton & Hall, 2000). In fact, Hill & Ward (1971) already found C3-cleavage fragments in myocardial tissue due to coronary ligation in 1970. They also implicated these fragments in the chemotaxis of neutrophils to the injured area. More recently, Yasojima *et al.* (1998) found that components of the classical complement pathway are expressed in injured heart tissue and could contribute to myocardial injury after the initiation of reperfusion. Buerke *et al.* (2006) did a proteomic study of complement in the setting of ischaemia-reperfusion, in the presence of a specific C1s inhibitor. They found that inhibition of C1s was associated with a decrease in both tissue damage, as well as C5b-9 deposition in myocardial tissue. It therefore seems that the complement system is activated during ischaemia (as evident by an increase in the presence of MAC complexes on myocardial tissue), and its activity is increased by reperfusion (Mathey *et al.*, 1994; Parks & Lucchesi, 1999).

- **Sudden wash-out of metabolites.** Anaerobic metabolism, occuring during ischaemia, leads to the accumulation of both carbon dioxide (CO_2) and hydrogen ions (H^+), with an associated reduction in pH (Opie, 2004). On reperfusion, the H^+ concentration in the interstitial space could normalize, before normalization of intracellular pH. The resulting H^+ concentration gradient drives a speedy decrease in intracellular H^+ concentration, which could be detrimental for two reasons: 1.) Slight, transient acidosis could be protective against Ca^{2+}-dependent contracture (Eisner *et al.*, 1989). During reperfusion, this protective factor is however quickly removed. 2.) The transport of H^+ out of the cell occurs, amongst others, via a Na^+ / H^+ exchanger. This causes an increase in intracellular Na^+, which in turn drives the Na^+ / Ca^{2+} exchanger in a direction that leads to Ca^{2+} overload. In this context, Inserte *et al.* (1997) found that reoxygenation-oedema was decreased in the presence of a Na^+ / H^+ exchange inhibitor (HOE642), as well as when bicarbonate (HCO_3^-)-dependent Na^+ transport into the cell was prevented. Wang *et al.* (2007) also found that combined treatment with a Na^+ / H^+ exchange inhibitor (cariporide) and a β-blocker, prior to ischaemia, improved mitochondrial function and decreased infarct size in reperfusion. They speculate that the protection of

mitochondrial function is attributable to stabilisation of the mPTP, which then prevents calcium overload of the mitochondria.

Other osmotic active metabolites also accumulate during ischaemia. Just as in the case of the H^+ ions, interstitial metabolites could be washed out of the extracellular space before the accumulated intracellular metabolites. This leads to the generation of an osmotic gradient, driving an influx of fluid into the cells (Tranum-Jensen *et al.*, 1981). This influx then contributes to cell stress, and combined with other reperfusion stressors, could lead to the disruption of the cell membrane. In this regard Ruiz-Meana *et al.* (1995) found that hypercontracture, together with osmotic cellular swelling, in mechanically fragile myocardiocytes (due to metabolic inhibition), lead to sarcolemmal disruption. Askenasy *et al.* (2001) however found that the osmotic-gradient across the membrane decreases after ischaemia / reperfusion. They speculate that this could be due to the free movement of osmolytes out of the cells, since they also showed that membrane permeability increases with the duration of ischaemia. This increase of sarcolemmal permeability due to ischaemia, or ischaemia with reperfusion, was also observed by Koba *et al.* (1995).

1.1.3. Necrosis and apoptosis in ischaemia / reperfusion

Both necrosis, as well as apoptosis, have been implicated in ischaemia / reperfusion injury. Initially it was thought that necrosis was the only form of cell death due to ischaemia / reperfusion. Freude *et al.* (2000) reported that after 90 minutes global ischaemia, up to 92% of cell death was due to necrosis. Necrosis refers to cell-death due to loss of ATP, which then leads to loss of cell membrane integrity and spillage of the cellular content (including lactate dehydrogenase (LDH), which can be measured to assess necrotic death) into the interstitial space.

It has however been reported that a degree of cell death in the injured myocardium can be attributed to apoptosis. In contrast to the above, Anversa *et al.* (1998) observed that up to 86% of cell death in a large infarct, following coronary occlusion, could be attributed to apoptosis. Apoptosis is an energy dependent, and ordered phenomenon in which a cell dies without comprimising membrane integrity. Instead the cell is ‘divided’ into small membrane vesicles, after enzymatic digestion of its chromosomal DNA into internucleosomal fragments. Apoptosis can be identified by microscopic identification of

the apoptotic bodies, the identification of DNA fragments that form a 'DNA ladder' in an agarose gel, or quantification of the presence of proteases typical of the apoptotic process, such as caspase-3 (Edinger & Thompson, 2004; Eefting *et al.*, 2004).

The relative contribution of necrosis and apoptosis to cell death in ischaemia / reperfusion is however still very much a matter of debate.

The precise timing of apoptosis in the injury-process is also controversial. In one study (Fliss & Gattinger, 1996) it was found that apoptosis was initiated and executed during ischaemia, while Gottlieb *et al.* (1994) reported that apoptotic death only occurs during reperfusion. Others (Freude *et al.*, 2000) proposed that apoptosis could be initiated during ischaemia, but only executed during reperfusion. This is indeed a possibility, since apoptosis is an ATP-dependent process. Otani *et al.* (2006) found that mechanical stress early in reperfusion could also influence the mode of death. Specifically, an increase in mechanical stress favours oncosis (necrosis due to oedema), even if the cells initially seem to be heading for apoptosis.

Differences in the distribution of apoptosis have also been reported in whole tissue. Scarabelli *et al.* (2001) found that apoptosis in reperfusion (as measured by DNA damage, using the TUNEL method) initially occurs in the endothelial cells of small bloodvessels, followed by the endothelium of larger coronary vessels after 5-60 minutes of reperfusion. This process then expands concentrically from these vessels to neighbouring cardiomyocytes.

For a brief review on apoptosis, necrosis and programmed necrosis see Edinger & Thompson (2004). For a review on apoptosis, specifically in reperfusion, see Eefting *et al.* (2004).

1.1.4. Summary

In summary, suffice it to say that: ischaemia in heart tissue leads to progressive cellular damage and death, especially necrotic death. Early reperfusion is necessary to limit cell death and salvage reversibly damaged tissue. The process of reperfusion itself however, also damages cells through necrosis and apoptosis. It is therefore of clinical importance to find methods of reperfusion that can limit reperfusion injury, and in that way limit the overall extent of damage due to an ischaemia / reperfusion-incident.

1.2. Clinical reality

In the clinical setting, there are primarily four situations in which ischaemia / reperfusion injury (IRI) is a complicating factor:

- Myocardial infarction (MI).
- Coronary artery bypass grafting (CABG).
- Cardiac surgery, necessitating cardiopulmonary bypass.
- Heart transplant.

In this text MI will be used as an example to briefly describe the clinical setting and treatment of IRI.

1.2.1. Currently accepted treatment of myocardial infarction

When a patient presents with a myocardial infarction, treatment simply entails the rapid revasculerization of the affected tissue. Currently, the emphasis is on keeping the time of ischaemia as short as possible, by instituting rapid reperfusion (Cannon, 2001). In 1983 Jennings *et al.* already demonstrated that the period of ischaemia is the most important factor determining the measure of myocardial damage.

There are two ways to reperfuse ischaemic tissue:

1. Thrombolytic treatment. The use of thrombolytic drugs to lyse the obstructing blood clot in the coronary circulation ushered in the era of reperfusion.
2. Percutaneous coronary intervention (PCI). PCI refers to coronary angioplasty, i.e. the use of an inflatable balloon or a stent, delivered by a catheter to the blocked coronary artery. Since this intervention requires a hospital with a catherization laboratory, the application thereof is limited.

For a brief review on the guidelines for the application of these two methods see Ting (2006).

Together with these reperfusion interventions, it is also standard practice to administer certain drugs, especially to help maintain reperfusion:

- Glycoprotein IIb/IIIa receptor blockers, such as eptifibatide and abciximab. These drugs are only used in the setting of PCI, since they increase the risk of bleeding when used alongside thrombolysis therapy (Ting, 2006).

- Aspirin (Amin, 2005). Ting (2006) reports that apirin must be used, if possible, together with thrombolytic therapy. The antiplatelet function of aspirin is necessary to counter platelet activation due to thrombolytic treatment.
- Heparin (Amin, 2005).
- Beta-blockers (ß-blockers): Advantageous effects associated with ß-blocker treatment could possibly be attributed to its anti-arrythmic and anti-tachyarrythmic effects, as well as a decrease in oxygen demand (by decreasing heart rate and contractility) (Amin, 2005; Kloner & Rezkalla, 2004).

1.3. Natural infarct-sparing mechanisms

In the laboratory two major natural phenomena have been described that can significantly lessen infarct-related injury. They can be described as "natural" in that both were initially described without the use of any drugs. These phenomena are:

- Ischaemic preconditioning (IPC)
- Postconditioning (postC).

1.3.1. Ischaemic Preconditioning: an overview

In 1986, Murry and colleagues made the surprising discovery that multiple brief episodes of ischaemia applied before a sustained ischaemic insult, did not contribute to ischaemic injury, but rather induced an increased tolerance against ischaemia. They termed this phenomenon ischaemic preconditioning (IPC) and the observation catapulted ischaemia / reperfusion research in a new direction. Experimentally, the stimulus for cardioprotection is elicited by one or more brief ischaemic episodes, which is then followed by a brief period of reperfusion before the sustained ischaemic insult (against which it protects). This period between stimulus and actual protection implies the presence of myocardial 'memory', one of the most unique attributes of IPC (Yellon & Downey, 2003; Bolli, 2007).

Initial research focussed on elucidating the optimal protocol to elicit IPC protection. Some researchers found that a single brief ischaemic episode elicited the same degree of protection as multiple cycles (Li *et al.*, 1990; Iliodromitis *et al.*, 1997) – implying that there is a threshold ischaemic stimulus required for protection (Crisostomo *et al.*, 2006). Once this threshold is reached, protection is then elicited in an all-or-nothing manner. The degree of protection is then set, whereafter a further ischaemic stimulus could itself even become detrimental (Iliodromitis *et al.*, 1997). The opposite has however also been

reported: that IPC protection is graded, and an increase in the ischaemic stimulus (such as number of cycles) augment cardioprotection (Volovsek *et al.*, 1992; Lawson *et al.*, 1993; Schulz *et al.*, 1998). This protection will however also reach a plateau. These variable observations might reflect differences in species and experimental setup. It should be noted that IPC has been shown in all species tested, even man (Yellon *et al.*, 1993). Its cardioprotective efficacy has been illustrated in the context of infarct size reduction (Murry *et al.*, 1986), functional recovery improvement (Cave & Hearse, 1992) and in some cases, reduction in reperfusion arrythmias (Shiki & Hearse, 1987).

In an attempt to understand the mechanisms behind IPC cardioprotection, its mechanism has been conceptualised as a "trigger" – "mediator" – "end-effector" pathway, in which the trigger occurs during the IPC stimulus before sustained ischaemia, while the mediators and end-effectors come into play after the onset of sustained ischaemia (Yellon & Downey, 2003). Various possible molecular role-players have been implicated in this framework, although it is not always clear where these molecules exert their effect. It is outside the scope of this text to elaborate on the precise role of the different molecules, let it therefore suffice to say that the most prominent role-players that have been identified are:

- Adenosine, which probably acts as a ligand trigger for IPC (Liu *et al.*, 1991; Crisostomo, *et al.* 2006).
- Protein kinase C, as well as protein kinase A, both of which probably act as mediators during sustained ischaemia (Yang, *et al.* 1997; Yellon & Downey, 2003).
- Free radicals and reactive oxygen species (Tritto, *et al.* 1997).
- The mitochondrial ATP-dependent potassium channel (mK^{+}_{ATP} – channel), although its precise role is still to be determined (Pain *et al.*, 2000; Yellon & Downey, 2003).
- Signalling kinases, such as the mitogen activated protein kinases (MAPKs) and protein kinase B (PKB/Akt) (Hausenloy & Yellon, 2006) .
- The mitochondrial permeability transition pore has increasingly been implicated (Halestrap *et al.,* 2007; Hausenloy & Yellon, 2007).

It is especially the latter two role-players that have complicated the framework of the IPC mechanism, since they exert some of their effects in reperfusion. Both the MAPK, extracellular signal-regulated kinase p42/p44 (ERK p42/p44) (Fryer *et al.*, 2001), and phospatidylinositol 3-kinase (PI3-kinase) – PKB/Akt (Tong *et al.*, 2000; Uchiyama *et al.*, 2004) have been implicated as important triggers during the IPC protocol itself (prior to sustained ischaemia), but recently activation of these kinases in reperfusion has also been

shown to be important in IPC cardioproptection (Hausenloy *et al.*, 2005). Hausenloy *et al.* (2004) also demonstrated "cross-talk" between these two survival kinase pathways (PI3-kinase – PKB/Akt and Raf – MEK1/2 (MAPK/ERK kinase) – ERK p42/p44). They found that inhibition of the one pathway led to an increase in the activation of the other, suggesting that they could compensate for each other. However, activation of both pathways is necessary to elicit optimal IPC protection.

The mitochondrial permeability transition pore (mPTP) also seems to play a role both before and after sustained ischaemia. Hausenloy *et al.* (2004) found that transient, low-conductance opening of the pore during the IPC protocol is necessary to mediate cardioprotection (although this conclusion has been challenged by Halestrap *et al.*, 2007). On the other side of ischaemia, Javadov and coworkers (2003) showed that IPC inhibits the opening of the mPTP during reperfusion, through indirect mechanisms (i.e. possibly by changing the intracellular milieu so that it does not favour mPTP opening, for example by decreasing calcium load and / or reactive oxygen species production).

It is also important to note that IPC can induce an acute protected state (lasting for 1-2 hours), as well as a "second window" of protection approximately 24 hours after the initial IPC stimulus and lasting for 2 to 3 days (Kuzuya *et al.*, 1993; Yellon & Downey, 2003; Bolli, 2007). This late phase of protection probably utilises the same signal transduction components as the early phase, but with different end-effectors. The early phase of protection recruits posttranslational changes in cellular molecules, while the late phase utilises synthesis of new proteins to exert its effect. Two such proteins that have received a lot of attention is NOS (nitric oxide synthase) and COX (cyclooxygenase) – 2, although other proteins such as heat shock and anti-oxidant proteins are probably also involved (Yellon & Downey, 2003; Bolli, 2007).

As mentioned earlier, even human myocardium has been preconditioned – which opens the door for the possibility of applying IPC in the clinical setting. Small scale studies have already been done in the settings of:

- Percutaneous coronary intervention (PTCI); although it could be argued that this study, by Deutsch and colleagues (1990), did not really investigate IPC in a true clinical situation.

- Coronary artery bypass grafting (CABG). In the studies done by Yellon *et al.* (1993) and Teoh *et al.* (2002) two cycles of 3 minutes ischaemia and 2 minutes reperfusion were applied after the institution of cardiopulmonary bypass.
- Studies also showed that IPC can be used as a safe and advantageous adjunct to cold blood cardioplegia (Illes & Swoyer, 1998; Li *et al.*, 1999).

The successful translation of IPC to the clinical setting has however not met expectations. It seems this could be due to the risks of applying repetitive ischaemia – specifically the risk of particulate emboli being dislodged by the process (Vaage *et al.,* 2000). It could also be that IPC is already induced in patients – either due to the inherent inflammatory response in sick patients, or possibly the cardiopulmonary bypass process in itself (in the setting of cardiac surgery), which might induce preconditioning (Valen & Vaage, 2005), or angina prior to a cardiovascular incident (Kloner *et al.*, 1995). There are also questions about the ability of IPC to confer cardioprotection in patients who have other pathologies, such as diabetes (Ishihara *et al.*, 2001), or in aged patients (Wu *et al.*, 2001; Pasupathy & Homer-Vanniasinkam, 2005).

Despite these clinical limitations, there is still a large amount of research being done on IPC. The primary goal is to elucidate the mechanism of protection and identify possible pharmacological interventions that could be used safely and easily as adjunct to current ischaemia / reperfusion therapies. Some of the pharmacological agents that have shown potential are:

- ß-adrenergic stimulation. Schwarz *et al* (1999) reports that although ß-adrenergic stimulation shows experimental promise, it could also lead to a increase in oxygen demand – which could have adverse effects in ischaemia / reperfusion injury (IRI).
- Adenosine. Clinical evidence that adenosine limits cell death, is conflicting (Kloner & Rezkalla, 2004).
- Adenosine triphosphate-sensitive potassium channel (K_{ATP}-channel) openers. Although these drugs show good potential, Schwarz *et al.* (1999) report that their side-effects could also be problematic.
- Nitroglycerin (a nitric oxide (NO) donor) has shown benefit in the setting of delayed preconditioning (Leesar *et al.*, 2001).
- Volatile anesthetics. Research has shown cardioprotective effects for these anesthetics (for a review see Kloner & Rezkalla, 2006). A possible explanation is

that these drugs generate small amounts of free radicals, which in turn act to trigger IPC.

Another major drawback of IPC is the fact that it must be applied before ischaemia, which is impossible in the setting of MI. A truly clinical relevant cardioprotective intervention would be one that is applicable at the onset of reperfusion.

1.3.2. Postconditioning: an introduction

In 2003, Zhao and coworkers demonstrated the cardioprotective effects of very brief cycles of reperfusion and ischaemia at the very onset of reperfusion, after sustained ischaemia, in the canine heart. This intervention became known as postconditioning (Crisostomo *et al.* 2006, Tsang *et al.* 2005, Vinten-Johansen *et al.* 2005). In fact, it has been reported that postconditioning is just as effective in reducing infarct size as IPC. (Zhao *et al.*, 2003).

As is the case with IPC, the application of repetitive episodes of ischaemia and reperfusion seems risky in the ischaemic heart disease patient. The focus of research is therefore on deciphering the mechanisms behind postC, with the goal of identifying possible pharmacological mimetics of postC.

The concept of applying an intervention during reperfusion, in an attempt to minimise myocardial injury is not a new one. The existence of reperfusion interventions that can minimise damage is also argued to be proof of the existence of reperfusion injury. Examples of reperfusion interventions are: pressure-controlled initial reperfusion (Selimoglu *et al.*, 2007), initial hypoxic reperfusion (Serviddio *et al.*, 2005), altering the content of the reperfusate and initial low flow reperfusion (Hori *et al.* 1991; Sato *et al.*, 1997; and Schlensak *et al.,* 1999). A large amount of research has also been done on postconditioning. For a review on the possible clinical implications of postC, see Kloner & Rezkalla (2006). Some of the drugs that have shown potential as postC mimetics, are as follows:

- Adenosine. Involvement of adenosine has been suggested by the finding that adenosine receptor antagonists can inhibit the protective effects of postC (Kin *et al.*, 2005). Jin and coworkers (2007) also demonstrated the effective utilisation of adenosine administration in reperfusion, as an adjunct to cold-blood cardioplegia.

- K_{ATP}-channel openers. It has been reported that blocking the K_{ATP}-channel abolishes postC cardioprotection (Yang *et al.*; 2004), implying a role for these channels in the mechanism of postC.
- Volatile anesthetics, such as isoflurane, have also been implicated as postC-mimetics (Chiari *et al.*, 2005).

Although various mechanisms have been implicated in postC (to be discussed later in this text), it seems that pharmacological reperfusion-based treatment is still lacking.

1.4. Postconditioning

As already mentioned, a large body of research points to the possibility of actually intervening in ischaemia / reperfusion injury at the very onset of reperfusion. Reperfusion is also clinically the most realistic window of treatment opportunity. Despite evidence for the possible advantages of reperfusion-intervention, the very existence of reperfusion injury, and hence the value of reperfusion-intervention, has for a long time been controversial (Schaper & Schaper, 1997; Tsang *et al.* 2005).

Nevertheless, Zhao and colleagues (2003), inspired by the potent cardioprotective effects of ischaemic preconditioning and motivated by the clinical relevance of a reperfusion-intervention, combined the two concepts. Using an open-chest canine model of coronary occlusion and reperfusion, they showed that three cycles of 30 seconds reperfusion and 30 seconds ischaemia, at the onset of reperfusion, could confer cardioprotection comparable to the protection associated with ischaemic preconditioning. This intervention became known as postconditioning. Postconditioning can therefore be defined as the application of multiple brief cycles of reperfusion / ischaemia at the very onset of reperfusion, after sustained ischaemia (Zhao & Vinten-Johansen, 2006). Since the first report of the possibility of conditioning a heart *after* the ischaemic period, many research efforts – spanning from basic laboratory science to clinical research – have been directed at this phenomenon.

1.4.1. Postconditioning in the laboratory

1.4.1.1. The postconditioning algorithm

When considering the precise algorithm that should be employed to elicit postconditioning-mediated cardioprotection, there are three variables that have been reported to be of importance.

1. The time lapse between the onset of reperfusion and the initiation of the postconditioning cycles. Several workers reported a loss of cardioprotection when administration of the postC intervention was delayed by one minute (Kin *et al.* (2004) using a rat model and Downey & Cohen (2005) in a rabbit model). In contrast, Bopassa *et al*. (2006) employed a protocol in which they allowed one minute reflow before initiating postconditioning. Despite this delay their postC protocol elicited functional recovery. Yang *et al.* (2004) even found that postC only lost its protective effect when delayed for as much as 10 minutes in the rabbit heart. In general most studies apply the postconditioning protocol at the very onset of reperfusion, without any significant delay.
2. The number of cycles applied.
3. The duration of each cycle.

The latter two factors seem to be species-related. Vinten-Johansen *et al.* (2005) reported in their review that, in general, the smaller the species the shorter the periods of reperfusion and ischaemia should be. It also appears that the efficacy of the protocol is dependent on the duration of the cycles, but less sensitive to the number of cycles (although this was not observed in all experimental setups). The precise optimal algorithm for postC is still a subject of investigation, but it seems to be species-dependent. Some of these species-dependent variations and findings concerning postC will be discussed in the following section.

1.4.1.2. Postconditioning: success, limitations and experimental variations

The canine heart

Postconditioning was first described in the *in vivo* canine heart, with an infarct sparing effect comparable to IPC (Zhao *et al.*, 2003). In this model a postC protocol, of 3 cycles of 30 seconds (3 x 30 sec) reperfusion and ischaemia, was also associated with a decrease in neutrophil accumulation in the area at risk (AAR), preserved coronary endothelial

function and a reduction in reactive oxygen species generation and oxidative damage. Although the precise interaction between these observations (cause vs effect) is still unknown, this early study clearly demonstrated the advantageous effects of postC. These positive results were confirmed by a follow-up study (Halkos *et al.,* 2004) which investigated the possibility that postC and IPC, if administered together, could have additive cardioprotective effects in the same *in vivo* canine model. Just as in the first study, they found that the cardioprotective effects of postC is associated with a decrease in superoxide anion generation and free radical-mediated damage (as indicated by malondialdehyde (MDA) levels, a marker of lipid peroxidation). The degree of protection elicited by postC was, however not potentiated by co-administration of a IPC protocol. The cardioprotective potential of postC was further tested by Couvreur and colleagues (2006), who investigated the ability of postC to protect against stunning (after only 10 minutes regional ischaemia) in conscious dogs. Various protocols (4 x 15, 30 or 60 second reperfusion / ischaemia) could however not protect against stunning. Mykytenko *et al*. (2007), following the same postC protocol as Zhao *et al*. (2003) and Halkos *et al.* (2004), found that postC cardioprotection in the canine heart (as measured by IFS and plasma creatine kinase (CK) levels) persisted after 24 hours of reperfusion (compared to control hearts, in which they observed continual injury development as reperfusion continued). Fujita *et al*. (2007), also using the *in vivo* canine heart model, followed a completely different protocol to investigate the association between transient acidosis and postC in reperfusion: They applied a 90 minute period of regional ischaemia (contrary to the 60 minutes used by others), followed by a postC protocol of 4 x 60 seconds reperfusion / ischaemia. Despite these differences they could also illustrate a postC-mediated decrease in infarct size. In view of all these studies, it seems that the canine heart can readily be protected against infarct development by applying a postC protocol.

The rabbit heart

The next species to be postconditioned was the rabbit. The positive outcomes found in the initial canine-study of Zhao *et al.* (2003) could also be replicated in the *in vivo* rabbit heart. Yang *et al.* (2004) reported a 43% decrease in infarct size, comparable to the infarct sparing effects of IPC, with a postC protocol of either 4 or 6 cycles of 30 seconds reperfusion / ischaemia. Interestingly, they demonstrated an additive effect when both IPC and postC was applied – in contrast to the findings of Halkos *et al.* (2004) in the canine heart. The same group went on to demonstrate postconditioning in the isolated rabbit heart (Yang *et al*., 2005). This illustrated that, at least a measure of the observed protection was

due to intrinsic mechanisms of the heart – independent of blood borne factors. Interesting to note, they found that a postC protocol of 6 x 10 seconds reperfusion / ischaemia protected the isolated heart model much better than 4 x 30 seconds reperfusion / ischaemia – which was sufficient in the *in vivo* model. Another group also managed to postcondition the isolated rabbit heart model (Darling *et al.*, 2005), applying a 4 x 30 seconds postC protocol. In their model postC had an infarct sparing effect, although it could not elicit a significant improvement in function.

Differences in the postC protocol between *in vivo* an *ex vivo* models are to be expected (Yang *et al.*, 2005; discussed above), but further studies in the *in vivo* rabbit model illustrated slight differences in the optimal protocol. Iliodromitis *et al.* (2006) tried both 4 x 30 sec, as well as 6 x 10 sec protocols. In their model only the 6 x 10 sec protocol conferred cardioprotection comparable to an IPC intervention. Chiari *et al.* (2005) found that a 3 x 20 sec postC protocol could elicit an infarct sparing effect, whereas 3 x 10 sec reperfusion / ischaemia could not. Couvreur and colleagues (2006), as well as Philipp *et al.* (2006), succesfully applied a 4 x 30 second reperfusion / ischaemia postC protocol. Argaud *et al.* (2005) however, could elicit a longterm (72 hours reperfusion) postC-mediated infarct sparing effect with a 4 x 60 second protocol. Despite these slight differences in protocols applied by the different researchers, it seems that generally in the *in vivo* rabbit heart, a protocol of 4 cycles of 30 seconds reperfusion / ischaemia should confer cardioprotection.

The murine heart

The phenomenon of postC has also been investigated in probably the most used laboratory animals, namely rats and mice.

It seems that most of the studies done in mice employed the same postC protocol of 3 x 10 seconds reperfusion / ischaemia to induce a reduction in IFS (Heusch *et al.*, 2006; Lim *et al.*, 2007; Boengler *et al.*, 2007). Interesting to note that Lim and colleagues (2007) found the 3 x 10 second reperfusion / ischaemia protocol more efficient in decreasing infarct size, than a 6 x 10 second algorithm (although both decreased IFS significantly in comparison to controls). It is therefore interesting that Kin *et al.* (2005) reported that a 6 x 10 second protocol was associated with an improved post-ischemic systolic and diastolic function, in the first minutes of reperfusion, while a 3 x 10 second protocol was ineffective. They therefore concluded that this protocol could protect against myocardial stunning

(contrary to the findings of Couvreur *et al*. (2006) in the canine and rabbit heart). Confirming these results, Morrison *et al.* (2007) also applied a 6 x 10 sec protocol in their *ex vivo* preparation, which also elicited an increase in functional recovery, as well as a reduction in cardiac troponin I (TnI; a marker of cell damage) release.

The murine model of postC however, does not escape the variability of the phenomenon: Gomez *et al*. (2007) applied a 3 times 60 second postC protocol after 60 minutes of coronary ligation (other studies employed between 20 and 30 minutes of ischaemia), which conferred an infarct sparing effect 24 hours into reperfusion. Tsutsumi and coworkers (2007) demonstrated an infarct sparing effect elicited by a postC protocol of 3 x 20 sec reperfusion / ischaemia, while Yang and colleagues (2006), using an *in vivo* balloon occlusion model, successfully postconditioned their mice with a 3 cycles of only 5 second occlusion / reperfusion protocol.

The rat heart

The rat has been extensively used in postC research, despite variable outcomes reported. The first researchers to attempt postconditioning the rat heart were Kin and coworkers (2004), who found in an *in vivo* model that a postC protocol of 3 or 6 x 10 seconds applied immediately at the onset of reperfusion, led to a decrease in infarct size, creatine kinase (CK) activity, neutrophil accumulation in the AAR, as well as a decrease in oxidative-related damage (as measured by plasma MDA activity), and superoxide anion generation. It is interesting to note that the authors mentioned that the postC-related infarct sparing effects observed were not as robust as in the dog or rabbit, and it was also notably weaker than the infarct sparing effect of IPC in the rat heart. The authors speculated that these differences could be due to species-related differences in xanthine oxidase activity and anti-oxidative mechanisms. Since this initial study by Kin and colleagues, other researchers have also illustrated the cardioprotective effect of this postC protocol. Wang *et al.* (2007) recently reported that a 6 x 10 second protocol, imparted functional recovery in their isolated rat heart model. In the *ex vivo* isolated rat heart, Penna *et al.* (2006) employed a basic, what they termed “classical”, postC protocol of 5 times 10 second reperfusion / ischaemia. They also used a modified protocol in which the reperfusion periods became progressively longer (15, 20, 25 and 30 seconds), while the ischaemic episodes were shortened (20, 15, 10 and 5 seconds). They found that both protocols imparted the same measure of infarct sparing effect. Tang *et al.* (2006) did a interesting study in which they used an implanted balloon occluder to investigate postC and IPC in

conscious rats. Applying a 6 x 30 sec protocol, they could not demonstrate any cardioprotection. They did however find that both 6 x 10 sec and 20 x 10 sec protocols were infarct sparing after 30 minutes of coronary occlusion, but not after 45 minutes of ischaemia. Increasing the number of cycles (60 x 10 seconds) however reversed the protective effect of postC. It is noteworthy that they noted that IPC (12 x 2 minutes occlusion / reperfusion) could also protect against infarct development after 45 minutes of ischaemia, while late IPC (applied 24 hours before occlusion) even protected after 60 minutes ischaemia. These observations confirm those of Kin *et al.* (2004) that in the rat, postC does not seem to be as robust in its cardioprotective effect as IPC.

Application of longer reperfusion / ischaemia cycles (30 seconds) may also be effective in eliciting protection, contrary to the observations of Tang *et al.* (2006). Manintveld *et al.* (2007) found that 3 x 30 second cycles reperfusion / ischaemia applied after 45 or 60 minutes coronary occlusion, in an *in vivo* model, reduced infarct size. In their study postC (3 x 30 second, 3 x 5 second and 3 x 15 second cycles of reperfusion / ischaemia) could not confer cardioprotection after 90 or 120 minutes ischaemia, and surprisingly, significantly aggravated infarct size when applied after 30 or 15 minutes of ischaemia. These latter observations are contrary to expectation, but (as the authors argue) it does illustrate that the duration of sustained ischaemia could also determine the efficacy of a postC protocol. Intriguingly, Tillack *et al.* (2006) successfully employed a 3 x 30 sec protocol to decrease infarct size after 30 minutes regional ischaemia, in their *in vivo* model. This difference in outcome, between these two similar experimental setups, still remain to be explained. A 3 x 30 second postC protocol was also applied by Bopassa *et al.* (2006), after an initial one minute period of reperfusion, following 30 minutes global ischaemia. In their *ex vivo* model this protocol was associated with an increase in functional recovery after 60 minutes of reperfusion. Although they did not measure infarct size, their postC protocol decreased the levels of markers of myocardial necrosis (lactate dehydrogenase (LDH), creatine kinase and TnI) in the coronary effluent, during 60 minutes reperfusion.

One of the most unique postC protocols was applied in one of the first papers on the phenomenon. Galagudza *et al.* (2004) applied a postC intervention of two minutes global ischaemia after 15 minutes of reperfusion, in hearts that developed persistent ventricular fibrillation. This was done in an isolated heart model, with a 30 minute regional ischaemic insult. They found that this protocol possessed strong antiarrythmic effects. It could

however, be argued that their protocol was per definition, not a true postconditioning protocol.

In light of the above discussion, it is clear that postconditioning of the rat heart is not as straight forward or reproducible as seems to be the case in some of the other animal species. This is especially highlighted by two recent studies. Dow & Kloner (2007) attempted to postcondition the *in vivo* rat heart after either 30 or 45 minutes of regional ischaemia. They applied various protocols: 4 x 10 second, 4 x 20 second, 8 x 30 second and 20 x 10 second cycles of reperfusion / ischaemia. None of these protocols could reduce infarct size. This is despite the successful application of IPC, as well as previous findings in their lab that postC does reduce ventricular arrythmias. One possible explanation for these findings may lie in the fact that they used female rats. Crisostoma *et al.* (2006) found that although the female rat heart can be postconditioned (postC protocol: 6 x 10 second cycles), this protection is dependent on the degree of ischemic injury. In their *ex vivo* model, male hearts were postconditioned after 20 and 25 minutes of global ischaemia, while female hearts could only be protected after 20 minutes and not 25 minutes of ischaemia. It should however be noted that Crisostoma and colleagues used functional recovery as end-point, while the effect of gender on infarct size and postconditioning has not yet been investigated. Kaljusta *et al.* (2007) also experienced problems postconditioning the rat heart. In their study they investigated both rats and mice, both *in vivo* and *ex vivo*, with the goal of developing a robust postC protocol. Although they could illustrate cardioprotection in mice, only in one laboratory (of two) could they elicit cardioprotection in the *in vivo* rat model (with a protocol of 3 x 10 second reperfusion / ischaemia after 30 minutes of regional ischaemia). In the isolated rat heart they investigated various protocols: 3 x 10 second, 3 x 30 second or 2 x 60 second cycles of reperfusion / ischaemia following 30 minutes of global ischaemia; while after 40 minutes regional ischaemia they applied a 3 x 10 second, as well as a 6 x 10 second cycle protocol. They could however, not induce an infarct sparing effect with any of these protocols.

It is clear from the above that some researchers experience problems in obtaining cardioprotection with postconditioning. Up until the present time, we do not yet have an explanation for these variable outcomes.

The porcine heart

Despite the irregular results found in the rat heart, the postC experiments in the pig heart seemed to cause the most concern. The first paper published on postC in the swine heart did not report success: Schwartz & Lagranha (2005) applied a protocol of 3 x 30 second reperfusion / ischaemia in their *in vivo* pig heart model of regional ischaemia (30 minutes of coronary occlusion). This protocol could however not limit infarct size, although IPC could confer cardioprotection in this model. Interestingly, both interventions of IPC and ineffectual postC were associated with an increased phosphorylation of the survival kinases: ERK p42/p44 and PKB/Akt. These findings opened the door for the possibility that the ability to be postconditioned might in fact really be very species dependent. Iliodromitis *et al.* (2006) subsequently evaluated the efficacy of the protocol applied by Schwartz & Lagranha (2005). They compared a 4 x 30 second cycle with a 8 x 30 second cycle protocol, applied after 60 minutes of coronary ligation in the *in vivo* model. The latter protocol (8 x 30 sec) elicited an infarct sparing effect. The authors speculated that the total time of postC intervention (four minutes vs eight minutes) might explain these differences in protocol efficacy. According to this explanation, in the longer protocol the heart is exposed for a more substantial time to the postC protective trigger(s). However, these findings are in contrast to the protocol of 3 x 30 second cycles that Jiang *et al.* (2005) applied in closed-chest pigs (they induced and manipulated the ischaemic episodes using an angioplasty balloon), which did elict an infarct sparing effect.

Two recently published research projects also reported successful postconditioning of the swine heart, despite application of different protocols. Skyschally *et al.* (2007) found that a postC protocol of 6 x 20 seconds (total time: four minutes) significantly decreased infarct size, following 90 minutes of regional low-flow ischaemia. Zhao *et al.* (2007) applied a postC protocol of 6 x 10 second (total time: two minutes) cycles of reperfusion / ischaemia after 3 hours of regional ischaemia. They found that this protocol reduced infarct size, as well as the area of so-called no-reflow, combined with an increase in coronary flow and ventricular function. Unfortunately these studies did not reveal the reason why in the initial study the swine heart failed to show successful postconditioning. The fact that both studies utilised a shorter total time of intervention than the 8 minutes reported by Iliodromitis *et al.* (2005), raises doubt about the explanation proposed by these workers. If the findings made by Manintveld *et al.* (2007), concerning the possibility that a too short ischaemic duration could render postC non-cardioprotective, can be extrapolated to pigs, it might also be that Schwartz & Lagranha (2006) applied a suboptimal period of regional ischaemia

(i.e. too short) to successfully elicit postC protection. It should be noted that all the studies that have shown cardioprotective postC in swine, applied a longer index ischaemia period than Schwartz & Lagranha (2006), in the first study on this species.

In summary

Despite the problems experienced and the differences in protocols, these studies done in the different animal models clearly confirm the existence of the cardioprotective postconditioning phenomenon. It is however also clear that postconditioning is difficult to induce, primarily because of the variability and seeming unreproducibility in the efficacy of the reported protocols. There are five variables that may influence the efficacy of postconditioning: 1) The animal species being studied; 2) the time lapse between the end of sustained ischaemia and the onset of postconditioning; 3) duration of the reperfusion / ischaemia cycles; 4) the number of cycles might be important as well (the latter two determine the total time of postC intervention), contrary to the initial observations made by Vinten-Johansen *et al.* (2005); and 5) the duration of sustained ischaemia. This variable might not be as straightforward as one might think, as illustrated by the research done by Manintveld *et al.* (2007).

In a recent review, Vincent-Johansen *et al.* (2007) speculate that the observed species differences may be due to differences in the rate and degree of ischaemia / reperfusion injury development in different animal species. These parameters are determined by factors such as myocardial metabolism, endogenous anti-oxidant defences and the role of inflammatory cells during reperfusion.

In cell culture

It is worthy to note that a successful postC protocol has also been developed for cell cultures. This was first described in neonatal rat cardiomyocytes by Sun *et al.* (2005), and entailed a postC protocol of 3 cycles of 5 minutes alternating normoxia and hypoxia at the onset of a 6 hour reoxygenation period, after a 3 hour hypoxic period. This protocol was associated with decreases in superoxide production, tumour necrosis factor-alpha (TNF-α) release, as well as cellular markers of apoptosis (such as caspases and DNA fragmentation). Exactly the same protocol was also applied in H9c2 cardiac muscle cells (Zhao *et al.*, 2006), the only difference being that the hypoxic episode was longer (8 hours vs 3 hours) and the reoxygenation period was shorter (3 hours vs 6 hours). These authors also reported a postC associated reduction in apoptosis, as well as an increase in

mitochondrial preservation. A different protocol was applied by Wang and colleagues (2006) in an isolated adult rat cardiomyocyte model. Following a hypoxic episode of 2 hours, at the onset of 3 hours reoxygenation, they exposed the cells to 2 x 5 minutes normoxia / hypoxia. Similiarly to the other studies on postC in cell culture, Wang *et al.* (2006) reported a postC-associated reduction in apoptosis, increase in cell viability and a decrease in free radical (specifically peroxynitrite ($ONOO^-$)) generation.

The application of postC in cell culture introduces an ideal model for research into the cellular and molecular changes associated with postC.

However, the ultimate goal of postconditioning is to apply it in humans, in the clinical setting. Surprisingly, despite the difficulties experienced in eliciting postconditioning in animal models, a considerable amount of research has already been done in humans, as will be described in the next section.

1.4.1.3. Postconditioning the human heart

In 2005 Laskey published a pilot study in which he investigated the effects of a "preconditioning-like" intervention in reperfusion. This study focused on patients presenting with an acute myocardial infarct (AMI), receiving percutaneous coronary intervention (PCI). The conditioning intervention entailed two 90 second balloon inflations in the stenotic artery, divided by 3 to 5 minutes of reperfusion. In the control group only a single 90 second inflation was performed at the same time as the second inflation in the conditioned group. It should be noted that flow greater than TIMI grade 0–1 was established by minimum intervention in the infarct-related artery before the intervention. All patients experienced relief of angina, a decrease in stenosis to less than 10 % and coronary flow greater than TIMI grade 2. In this study Laskey (2005) found that the preconditioning-like stimulus was associated with favourable changes in electrocardiographic and coronary hemodynamic markers. Although it is questionable if this study really applied a true postC intervention, it certainly illustrated the potential for postconditioning in humans. This potential for postC protection was also illustrated by a retrospective analysis of patients who had received angioplasty after presenting with myocardial infarction (Darling *et al.*, 2007). It was found that 4 or more balloon inflations at reperfusion were associated with less peak creatine kinase release, than when between 1 and 3 inflations were applied. Oddly enough, despite this positive finding, patients receiving 4 or more balloon inflations had a longer sojourn in hospital.

Two studies done on human tissue also suggested that human tissue could be postconditioned. Loukogeorgakis *et al.* (2006) investigated human endothelium *in vivo* by applying a 20 minute ischemic insult on the forearm of test subjects. In their model this insult confers a transient decrease in flow-mediated dilation of the brachial artery (which is the reversible injury end-point monitored). Two postC protocols were applied on the ischemic arm: a 3 x 10 second cycle and a 3 x 30 second cycle protocol, in the absence and presence of a minute delay before the postC intervention. They found that both protocols protected the endothelium, provided they were applied within the first minute of postC. Due to the model used, this study could however not adress the efficacy of postC in a more rigorous ischaemia scenario – such as myocardial infarction, in which prolonged ischaemia leads to irreversible cell damage and death. A study by Sivaraman *et al.* (2007) applied a more potent ischaemic insult of 90 minutes hypoxia (paced at 3 Hz) on isolated human atrial trabeculae. After 120 minutes simulated reperfusion (exposure to oxygenated buffer while paced at 1 Hz) residual contractile function was measured as end-point. They investigated both a 4 x 30 second cycle, as well as a 4 x 60 second cycle postC protocol. Only the latter (4 x 60 sec) protocol conferred protection, comparable to a preconditioning stimulus (4 minutes hypoxia at a pacing rate of 3 Hz, followed by 16 minutes of simulated reperfusion at 1 Hz pacing).

Three studies have been reported that purposefully investigated postC in humans in the clinical setting. Staat *et al.* (2005) applied a postC protocol of 4 cycles of 1 minute reperfusion / ischaemia at the onset of reflow, after angioplasty. This was achieved by inflating and deflating the angioplasty balloon upstream of the implanted stent (to avoid damaging the stent, as well as to prevent thrombi embolization). This intervention decreased infarct size (as measured by the area under the creatine kinase curve) after 72 hours of reperfusion, illustrating the feasibility and cardioprotective ability of postC in the human heart. The question whether postC permanently protects tissue, or just delays damage, was answered by Yang *et al.* (2006). They applied a postC protocol of 3 x 30 seconds reperfusion / ischaemia by deflating and inflating the angioplasty balloon. They confirmed the reduction in infarct size observed by Staat *et al.* (2005), but by using nuclear imaging they also observed a sustained decrease in infarct size after 7 days of reperfusion. Applying a similar protocol as Yang *et al.* (2006), of 3 x 30 seconds angioplasty balloon deflation and inflation, Ma and coworkers (2006) found that postC was associated with a decrease in blood levels of MDA and CK – illustrating a decrease in free

radical mediated cell injury. They also reported an increase in microcirculation reperfusion, peripheral artery endothelial function (using a technique similar to the one used by Loukogeorgakis *et al.* (2006)) and left ventricular wall motion (measured 8 weeks after PCI).

It therefore seems that postconditioning does indeed have potential as a reperfusion intervention in human ischaemia / reperfusion, such as coronary angioplasty following a myocardial infarct.

1.4.2. Possible mechanisms of postconditioning

When postconditioning was initially described by Zhao *et al.* (2003) they speculated that it was unlikely that the mechanisms (and timing of these mechanisms) recruited by postC are the same, as those at work in IPC. This made sense, due to the radical differences in the timing of application of these interventions (IPC before sustained ischaemia and postC after sustained ischaemia). Since then there have been studies supporting this line of thought. For example Schwartz & Lagranha (2006) found a similar activation of the salvage kinases in both postC and IPC, although only IPC conferred protection. Heusch *et al.* (2006) also demonstrated that postC is independent of connexin 43, a molecule that is known to be important for IPC cardioprotection. There have however, also been several studies showing common mechanisms for postC and IPC. For a review on the common signalling pathway recruited by both postC and IPC in reperfusion, see Hausenloy and Yellon (2007). This section will briefly focus on some of the possible mechanisms that have been implicated in postconditioned cardioprotection.

1.4.2.1. Attenuation of the inflammatory response

PostC was first described in an *in vivo* setting, and Zhao *et al*. (2003) also initially explained this phenomenon in this context. They observed that postC was associated with a decrease in tissue edema, and neutrophil accumulation in the area at risk, as well as a preserved coronary artery endothelial function. The latter two observations could explain the postC-related decrease in no-reflow area, seen by Zhao *et al.* (2007) in mini-swine, since neutrophil capillary plugging and edema can contribute to the “no-reflow” phenomenon (Rezkalla & Kloner, 2002). Interesting to note that postC protection against “no-reflow” was lost in hypercholesterolemic conditions (Zhao *et al*., 2007). Halkos *et al.* (2004) confirmed that postC was associated with a decrease in tissue edema in the ischemic epicardium. In the open chest rat model Kin *et al.* (2004) also reported a

decrease in neutrophil accumulation in the area at risk, associated with postC. Mykytenko *et al.* (2007) found in the *in vivo* canine model, that this reduction in neutrophil accumulation (together with a decrease in IFS) was maintained in postC hearts, even after 24 hours of reperfusion (compared to control hearts). There was however still an increase in IFS and neutrophil accumulation between 3 and 24 hours of reperfusion, even in the postC hearts. This illustrates that postC does not seem to have an effect on reperfusion / injury events that occur later in reperfusion. Associated with this theme of reduced inflammation, Sun and colleagues (2006) reported a decrease in TNF-α associated with postC, in neonatal cardiomyocyte lysates.

1.4.2.2. Free radical generation

As previously mentioned, reperfusion is associated with a burst of free radicals that can be harmful to the myocardial tissue (Zweier & Hassan Talukder, 2006). Interestingly, reactive oxygen species (ROS) have been implicated in the triggering and mediation of both IPC (Baines *et al.*, 1997 & Vanden Hoek *et al.*, 1998) and postC. In this regard; Penna *et al.* (2006) found that the administration of a ROS scavenger during the postC protocol in the isolated rat heart, abrogated the infarct sparing effect of postC – implicating a trigger role for ROS in postC. This triggering role of ROS in postC was also more recently confirmed in an *in vivo* murine model, in which administration of a ROS scavenger just before (but not after) a postC intervention blocked its infarct sparing effect (Tsutsumi *et al.*, 2007).

It is however, generally accepted that large amounts of free radicals contribute to cell damage and death in ischaemia / reperfusion (Zweier & Hassan Talukder, 2006 & Valko *et al.*, 2007). It is therefore not surprising that *in vivo* studies (Zhao *et al.*, 2003; Kin *et al.,* 2004) have found that postC is related to a reduction in both ROS (specifically the superoxide anion) and plasma MDA (a marker of free radical mediated membrane lipid peroxidation). Mykytenko *et al.* (2007), in their study on the longterm effects of postC, also found that postC was associated with a decrease in superoxide generation after 24 hours of reperfusion. Interestingly, Halkos *et al.* (2004) found that both IPC and postC is associated with a reduction in ROS generation during reperfusion. In their study on the association between the duration of sustained ischaemia, postC and IFS, Manintveld *et al.* (2007) found that postC reduction in infarct size (after 60 minutes of regional ischaemia) was associated with a reduction in superoxide generation. Unsuccessful postC implimentation (after 15 minutes ischaemia) was however associated with increased infarct size development and superoxide production (compared to control).

Studies in cell culture have also shown an important role for ROS reduction, associated with postC. In this regard Sun *et al.* (2005), found that in neonatal rat cardiomyocytes, postC was associated with a decrease in cell death, reduction in ROS generation, lipid peroxidation and cytosolic, as well as mitochondrial Ca^{2+}-levels. They speculate that these phenomena are linked. The same researchers confirmed these early results by finding a decrease in superoxide generation in a postconditioned cell preparation (Sun *et al.*, 2006). Reactive oxygen species could contribute to an increased influx of Ca^{2+} into the cell via three routes: 1) increased Ca^{2+} influx through damaged cell membranes (Bagchi *et al.*, 1997); 2) increased Na^+ / H^+-exchanger activity, which in turn drives the Na^+ / Ca^{2+}-exchanger to pump Ca^{2+}-ions into the cell (Rothstein *et al.,* 2002) and; 3) inactivation of the sarcoplasmic reticulum Ca^{2+} - transporting ATPase (Lounsbury *et al.,* 2000), which then leads to inhibition of sarcoplasmic reticulum Ca^{2+} uptake.

These studies in cell preparations, isolated hearts and *in vivo* models demonstrate the significance of free radical activity in the phenomenon of postC. It seems probable that, as is the case with IPC, postC protects the heart against deleteriously high levels of reactive species, although itself is triggered by a slight exposure to these same reactive molecules.

It is noteworthy that many of these studies were conducted in the absence of blood borne agents and cells. The fact that both *in vivo*, as well as blood-free preparations, show postC cardioprotection illustrates that postC functions through mechanisms both intrinsic and extrinsic to cardiomyocytes.

1.4.2.3. Triggering postconditioning – the delayed washout of metabolites

Convincing evidence has been produced for a role for the so-called Reperfusion Injury Salvage Kinase (RISK) pathway in postC. The fundamental question is though: How are these signalling pathways activated? The delayed washout of triggering metabolites could be prominent in this regard.

Penna and coworkers (2007) found that postC protection is dependent on the availability of the bradykinin B_2 receptor, since they could mimick postC-protection by the intermittent (5 cycles of 10 seconds) administration of bradykinin to the isolated rat heart preparation. Just as with postC, ROS scavengers administered during the bradykinin-cycles, inhibited

this protective effect. The beneficial effect of bradykinin in reperfusion was also shown by Yang *et al.* (2004) and Lim *et al.* (2007).

Besides bradykinin, opioids have also been implicated in postC. The administration of non-selective opioid antagonists in reperfusion abrogated the infarct sparing effect of postC, in an open-chest rat model (Kin *et al.*, 2005). Wang and coworkers (2007) specifically identified the κ-opioid receptor as being involved in the mechanism of postC. Stimulation of the δ-opioid receptor in the first minutes of reperfusion (as a pharmacological postC intervention) has also been shown to elict cardioprotection in the *in vivo* murine model (Tsutsumi *et al.*, 2007), suggesting a possible role for this receptor in postC as well.

It is however especially endogenous adenosine which has received the most attention as possible trigger for the cellular postC-related signalling cascade. In one of many studies on the protective effects of adenosine *per se* in reperfusion (for a review see Donato & Gelpi, 2003), Yang *et al.* (2004) suggested a role for adenosine, when they found cardioprotection associated with the administration of NECA (5'-(*N*-ethylcarboxamido) adenosine), a A_1/A_2 receptor agonist, in reperfusion. Similarly, AMP 579, another A_2-receptor agonist, also elicited cardioprotection when given during reperfusion (Xu *et al.*, 2001).

In 2005 Yang *et al.* made the connection between postC and adenosine receptor stimulation, when they found that blockade of this receptor (using a nonselective adenosine receptor blocker, 8-p-(sulfophenyl) theophylline (SPT)) impaired the ability of postC to limit infarct size. It appears that an increase in the retention time of extracellular adenosine during the postC protocol, early in reperfusion, may be of significance (Kin *et al.*, 2005). They also found that the stimulation of both the A_{2A} and A_{3A} receptors are necessary for postC cardioprotection. Using the same pharmacological inhibitor approach as Kin *et al.* (2005), Philipp *et al.* (2006) also demonstrated the importance of adenosine receptor stimulation in postC, with the difference that they found the A_{2b} receptor to be involved (and not A_{2A}). Two recent studies, using a knock-out mouse model, however favour the A_{2A} receptor as being the important one in the mechanism of postconditioning (Yang *et al.*, 2006; Morrison *et al.*, 2007). It is therefore clear that the cardioprotection elicited by postC is dependent on the binding of endogenous adenosine to adenosine receptors (specifically the A_{2A} receptors) in reperfusion.

1.4.2.4. The role of protein kinase C

Penna *et al.* (2006) found that the inhibition of protein kinase C (PKC) during reperfusion (even after the postC protocol itself) was associated with a loss in postC-related cardioprotection. Utilising pharmacological inhibition, Philipp *et al.* (2006) made the interesting observation that blockade of the adenosine A_{2A}-receptor (by administering an A_{2A} antagonist just before reperfusion) also abrogated the cardioprotective effects of a PKC activator (administered in reperfusion), indicating that PKC is upstream from adenosine stimulation in the signalling pathway, and participates in the activation of adenosine-mediated protective mechanisms. Fantinelli & Mosca (2006) linked PKC with the free radical reduction associated with postC, since they observed a loss in postC cardioprotection (in terms of functional recovery and reduced lipid peroxidation), when PKC was inhibited in their isolated spontaneously hypertensive rat heart model.

1.4.2.5. Nitric oxide and guanylyl cyclase activity

Nitric oxide synthase (NOS) activity has also been implicated in postconditioning (Yang *et al.*, 2004 & Manintveld *et al.*, 2007). It seems that NOS is of importance in the protective pathways activated by adenosine and bradykinin, during reperfusion (Yang *et al.*, 2004). Zhao *et al.* (2007) measured constitutive NOS (cNOS) and inducible NOS (iNOS) activity in swine myocardial tissue after 2 hours of reperfusion and found that postC was associated with an increase in cNOS and a decrease in iNOS activity. The authors linked this profile to protected endothelial function. In their hypercholesterolemic animals this profile, as well as postC protection was lost. This absence of difference in NOS activity between control and postC hearts, under conditions of hypercholesterolemia, might be due to advanced endothelial injury in these animals – to such an extent that the postC intervention can not induce favourable changes in metabolism.

The dependence of postC-related cardioprotection on the activity of guanylyl cyclase (GC) has been demonstrated by both Yang *et al.* (2005) and Penna *et al.* (2006). Both studies used 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) to inhibit GC activity, which in turn abrogated postC protection. Penna and colleagues (2006) went further by showing an increase in cyclic guanosine-monophosphate (cGMP)-levels in reperfusion, following postC. This observed GC-cGMP activity however seems to be partially dissociated from nitric oxide (NO), since they also found that NOS inhibition resulted in only a partial

reduction in postC cardioprotection, as well as a decrease in cGMP levels (later in reperfusion).

It could be that the NO-cGMP pathway contributes to postC cardioprotection through: 1) the anti-oxidant activity of NO (Wallace, 2005); 2) the abilibilty of cGMP to open the mitochondrial ATP-dependent potassium channel (mK^+_{ATP}-channel) (Qin *et al.*, 2004); and 3) reduced intracellular Ca^{2+} overload and hypercontracture (for a brief review on hypercontracture in reperfusion see Piper *et al.*, 2004).

1.4.2.6. Postconditioning and the mitochondria

The mitochondrial K^+ $_{ATP}$- dependent channel

One of the first studies on postconditioning demontrated the importance of the mK^+_{ATP}-channel in this form of cardioprotection (Yang *et al.*, 2004). It seems that opening of the mK^+_{ATP}-channel is associated with the activation of the survival kinases (to be discussed later), since blocking it also diminished the levels of phosphorylated protien kinase B (PKB/Akt) and extracellular signal-regulated kinase (ERK) in the mitochondria (Zhao, *et al.*, 2006). This same study ruled out the possible role of the sarcolemmal K^+_{ATP}-channels, since blocking them had no effect on postC in cardiac muscle cells. The significance of the mK^+_{ATP}-channels was further underscored by the finding that administration of a mK^+_{ATP}-channel blocker in reperfusion (including and excluding the postC protocol) leads to blockade of the infarct sparing effect of postC (Penna *et al.*, 2006).

The mitochondrial permeability transition pore

Currently the mitochondrial permeability transition pore (mPTP) is receiving much attention as a / the possible end-effector in both postC, as well as IPC. The concept of a permeable pore in the mitochondrial membrane, the opening of which precedes cell death, is not a new concept. In 1988 Crompton & Costi published evidence for a mitochondrial pore which can be opened by Ca^{2+}, inorganic phosphates (P_i) and oxidative stress. A recent study (Kim *et al.*, 2006) has however shown that it is ROS and the sudden normalisation of pH (in the ischaemia / reperfusion setting) that open the channel. In this scenario, Ca^{2+} overload is a consequence of mPTP opening, and not a stimulus. Three main molecules have been implicated in the structure of this pore: the voltage-dependent anion channel (VDAC) located in the outer mitochondrial membrane, the adenine nucleotide translocase (ANT) in the inner membrane and cyclophilin-D (CypD) in the mitochondrial matrix. It is thought that these molecules form a complex which is “deformed” by stressors, such as

oxidants, to form an open pore through which relatively large molecules can pass freely. The end-result is a loss in mitochondrial membrane potential, uncoupling of oxidative phosphorylation and a loss of ATP, which, in turn leads to dysregulation of ion homeostasis and necrotic cell death. Rupture of the outer membrane could also result in leakage of pro-apoptotic molecules (such as cytochrome *c*) into the cytosol, mediating an apoptotic mode of cell death (for a review on this topic see Crompton, 2000).

The importance of the mPTP in ischaemia / reperfusion injury has repeatedly been demonstrated. Hausenloy *et al.* (2003) showed that this pore is especially critical in the reperfusion phase, since inhibiting it right at the onset of reperfusion conferred cardioprotection. It is therefore not surprising that Argaud and colleagues (2005) found that postC was associated with a delay in Ca^{2+}-induced mPTP opening. They speculate that this could be due to the decrease in ROS associated with postC. Bopassa *et al.* (2006) also demonstrated a role for the preservation of the mPTP in its closed conformation, in the cardioprotection elicited by both postC and low pressure reperfusion, in the isolated rat heart. The inhibition of the mPTP elicited by these interventions was however lost with inhibition of phospatidylinositol 3-kinase (PI3-kinase), implicating a connection between mPTP and PI3-kinase. Zhao *et al.* (2006) also report a link between postC-cardioprotection, mK^{+}_{ATP}-channel activity, the increase in phosphorylated survival kinases (PKB and ERK) in the mitochondria and mPTP activity.

Cyclophilin-D (and per implication the CypD-dependent mPTP) has been shown to be involved in Ca^{2+} and ROS mediated cell death, especially necrosis (Nakagawa *et al.* 2005 & Baines *et al.* 2005). The importance of this molecule in postC has also been demonstrated by Lim *et al.* (2007). They found that CypD-deficient mice could not be cardioprotected by IPC, postC or pharmacological mimetics of these mechanical interventions, since these hearts were already “protected”. Control IFS-values were already in the same range as the reduced IFS-values associated with cardioprotection in the wild-type mice.

Juhaszova *et al.* (2004) suggested a model in which numerous cellular pathways associated with survival (such as PKC and PI3-kinase) converge on one “master-switch”, which then regulates the common end-effector of these pathways – namely the mPTP. Their study suggested that this “master-switch” could be glycogen synthase kinase-3β (GSK-3β). Phosphorylation of this kinase inactivates it, leading to cell survival. These

interactions could also be of importance in postC. Tillack *et al.* (2006) reported that a postC protocol increased the phosphorylation of GSK-3β. Using GSK-S9A transgenic mice (in which GSK-3β can not be inactivated), Gomez *et al.* (2007) found that postC could not reduce infarct size. Only administration of the mPTP inhibitor cyclosporin-A could decrease infarct size, also suggesting that GSK-3β is upstream of mPTP. For a review on the mPTP in postconditioning see Gateau-Roesch *et al.* (2006).

1.4.2.7. The protein kinases in postconditioning

Both mitogen activated protein kinase p38 (p38 MAPK), as well as c-Jun NHP_2 terminal kinase (JNK) have been implicated in cell death and apoptosis. Cicconi *et al.* (2003) found that although both these kinases are involved in ROS-mediated apoptosis, they seem to be exerting opposite effects, with p38 MAPK being pro-apoptotic and JNK possibly involved as part of a scavenger pathway. This is contrary to the observation that inhibition of JNK in reperfusion was associated with cardioprotection (Ferrandi *et al.*, 2004). The precise role and functions of these kinases, as well as their roles in IPC, are therefore still quite uncertain (for a review on the kinases in pre- and postconditioning see Hausenloy & Yellon (2006)). Not much is known about their significance in postC either. In neonatal cardiomyocytes Sun and colleagues (2006) found that postC cardioprotection was associated with a reduction in the activity of both p38 MAPK and JNK, indicating that a part of the cardioprotection conferred by postC, might be due to the down regulation of these kinases. However, Feng *et al.* (2006) applying an isoflurane postC protocol (which refers to the administration of isoflurane for the first 15 minutes of reperfusion), found no alterations in the phosphorylation of p38 MAPK in infarct-remodeled myocardium.

The Reperfusion Injury Salvage Kinases

The Reperfusion Injury Salvage Kinases (RISK) refer to the pro-survival kinase pathway duo of PI3-kinase – PKB/Akt and MAPK/ERK kinases (MEK) 1/2 – ERK p42/p44. For a review on the RISK pathway within the setting of reperfusion and ischaemia see Hausenloy & Yellon (2004). These kinases appear to play a role in the salvage of tissue from ischaemia / reperfusion injury, exerting their effects in reperfusion. This is illustrated by Yang *et al.* (2004) who found that the cardioprotection associated with both NECA and bradykinin, given in reperfusion, is dependent on the activity / phosphorylation of both PI3-kinase and ERK p42/p44. Bopassa *et al.* (2006) also found that PI3-kinase activity is of importance in the cardioprotection conferred by low-pressure reperfusion.

Despite their action in reperfusion, they have also been implicated in the mechanism of IPC (Hausenloy *et al.*, 2005). It is therefore no surprise that a large amount of research has already been done on their role in the phenomenon of postconditioning.

The PI3-kinase – PKB/Akt pathway

Most studies that have investigated the importance of PI3-kinase in postC, by using pharmacological inhibitors (Wortmannin and LY-294002), have found it to be an essential feature of the postC cardioprotective mechanism (Yang *et al.*, 2004; Tillack *et al.*, 2006; Fujita *et al.*, 2007). It is therefore not surprising that PKB/Akt, a downstream effector of PI3-kinase, has also been implicated in postconditioning (by analysis of the activity of PKB/Akt (Zhu *et al.*, 2006), or Western blot quantification of phosphorylation (Morrison *et al.*, 2007)). Darling *et al.* (2005) however, found that although ERK p42/p44 is involved in their isolated rabbit heart model, they could not show a role for PI3-kinase (using the inhibitor LY-294002). Together with this inhibitor data, they did not find significant differences in the degree of phosphorylation of PKB/Akt in postC vs control hearts.

Philipp *et al.* (2006) found PI3-kinase to be downstream of both adenosine receptor stimulation and PKC activity. Some of the possible ways in which the PI3-kinase – PKB/Akt pathway exerts its effects, is by stimulating NO production and inhibiting the opening of the mPTP. Manintveld *et al.* (2007) illustrated that together with PI3-kinase, NOS activity is also critical for postC protection. Tsang *et al.* (2004) and Zhu *et al.* (2006) found that the phosphorylation of endothelial NOS (eNOS) is dependent on the activity of PI3-kinase, indicating that NO production is downstream of PI3-kinase. Zhu *et al.* (2006) found that GSK-3ß (which links with the mPTP) seems to be downstream of PI3-kinase – PKB/Akt. Similarly, Tillack *et al.* (2006) demonstrated that the increased phosphorylation of GSK-3ß, associated with postC, is dependent on PI3-kinase activity. This link between PI3-kinase and the mPTP is nicely illustrated by Bopassa *et al.* (2006), who found that PI3-kinase activity is not only necessary for postC protection, but it is also important in the postC-linked inhibition of the mPTP (as measured by analyzing the mPTP's response to Ca^{2+} loading).

The MEK1/2 – ERK p42/p44 pathway

A number of studies have demonstrated a role for the ERK p42/p44 pathway in postC cardioprotection (Yang *et al.*, 2004; Darling *et al.,* 2005; Fujita *et al.*, 2007; Morrison *et al.*, 2007), although results are not as straight forward as with the PI3-kinase – PKB/Akt

pathway. Just as is the case with PI3-kinase – PKB/Akt, all studies done on ERK p42/p44 utilised either the MEK1/2 inhibitors PD-98059 or UO126, or measurement of phosphorylation or activity. Zhu *et al.* (2006) reported an elevation in ERK p42/p44 phosphorylation, associated with postC, but interestingly they did not find increased *in vitro* ERK p42/p44 activity. Taken together with the increased activity of PKB/Akt and the dependence of postC cardioprotection on PI3-kinase, the authors concluded that only PI3-kinase – PKB/Akt is the dominant protective pathway in postC.

Interestingly, Feng *et al.* (2006) and Chiari *et al.* (2005) found that isoflurane postconditioning is dependent on PI3-kinase – PKB/Akt activity, although Feng and colleagues (2006) did not observe any relationship between isoflurane postC and ERK p42/p44 phosphorylation.

More interesting facts on the role of the RISK pathway in postC

In H9c2 cardiac muscle cells Zhao *et al.* (2006) report that blockade of the mK^{+}_{ATP}-channel lead to a loss of postC protection, as well as a decrease in the levels of mitochondrial phosphorylated PKB/Akt and ERK p42/p44. This means that the mK^{+}_{ATP}-channel is upstream of the survival kinases, and might stimulate their translocation from the cytosol to the mitochondria. These observations, combined with an observed decrease in Bax and upregulation of Bcl-2 in the mitochondria, associated with postC, suggest that the RISK kinases act in the mitochondria to inhibit apoptosis.

There is only one study showing the significance of the RISK pathway in human tissue: Sivaraman *et al.* (2007) found that postC protection of isolated human atrial tissue was lost with the inhibition of PI3-kinase and MEK 1/2 - ERK p42/p44.

Schwartz & Lagranha (2006) investigated the role of the RISK kinases, in postC, in the porcine heart. They observed an increased phosphorylation of both ERK p42/p44 and PKB/Akt, associated with postC, which however, was not associated with a reduction in IFS. This anomaly implies that the activity of the RISK pathways, although important, might not be the single most critical component of postC protection in the *in vivo* setting.

It therefore seems reasonable to conclude that the RISK pathway is indeed recruited in postconditioning, as a central pathway mediating protection. There are however studies which did not show the involvement of both, or even any, of the PI3-kinase – PKB/Akt or

MEK1/2 – ERK p42/p44 pathways. An explanation for these contradictory observations is not readily available, but it seems as if the RISK pathway might not be the only and most critical component of postconditioning.

1.4.2.8. The role of pH

The knowledge that transient acidosis confers a degree of protection is not new. Kitakaze *et al.* published a paper in 1988 in which they report that reperfusion with an acidic perfusate (pH 6.6 or 7.0) after 15 minutes of ischaemia protected the isolated ferret heart against stunning. They attributed this protection to the blunting of the effect of Ca^{2+} overload, under acidic conditions. Hori *et al.* (1991) linked acidosis with staged reperfusion. In their model they found that staged increase in perfusion pressure over the first 10 minutes of reperfusion, conferred protection against stunning. They found this protection to be closely related to pH, with a decrease in pH eliciting protection and an increase in pH inhibiting staged reperfusion protection. Recently two research groups reported links between postC and transient acidosis. Fujita and colleagues (2007) found that postC was associated with prolonged transient acidosis (as measured in the coronary venous blood), as well as the phosphorylation / activation of the RISK kinases PKB/Akt and ERK p42/p44. Interestingly, they found that increasing the pH (by infusing sodium bicarbonate ($NaHCO_3$)) abrogated the cardioprotective effect of postC, as well as blocked the phosporylation of RISK. Cohen *et al.* (2007) also investigated the link between postC and acidosis by comparing postC with two different protocols of acidotic reperfusion. They found that both decreased infarct size, provided that the total time of intervention is 2 minutes and the intervention is applied immediately at reperfusion. The authors hypothesize that postC protects through two equally important mechanisms: at initial reperfusion the prolonged acidosis keeps the mPTP closed (a link between acidosis and mPTP inhibition has been shown (Petronilli *et al.* (1993)), while gradual reperfusion is still occurring which can then activate a redox sensitive survival pathway – which then permanently inhibits opening of the mPTP.

1.4.2.9. Other possible role-players in postC

There are also other molecules that have been identified as possibly involved in postC, although they have not received as much attention.

Boengler *et al.* (2007) report that postC protection is lost in STAT3 (signal transducer and activator of transcription 3)-deficient mice, implicating a role for STAT3. They link this to

the loss of postC protection which they observed in old wild-type mice, since they found that older mice also present with decreased levels of STAT3.

Jiang *et al.* (2005) found that postC was associated with a decrease in the expression of tissue factor (TF) and the inhibition of thrombin activity in the area at risk. This means that postC could also exert its protective effect by inhibiting the TF-thrombin pathway.

It is also interesting to note that postC does not confer cardioprotection in old (Boengler *et al.*, 2007), hypercholesterolemic (Zhao *et al.*, 2007) or metabolic syndrome hearts (Reubner *et al.*, 2006). This is similar to reports concerning the effects of aging and hyperlipidemia in IPC protection (Juhaszova *et al.*, 2005; and Ferdinandy 2003). It seems these conditions attenuate the cardioprotective potential of endogenous protection mechanisms. Interestingly, Reubner *et al.* (2006) found that postC in a rat strain with metabolic syndrome (Wistar-Ottawa-Karlsburg-W-rats (WOKW)) could not elicit an increase in the phosphorylation of GSK-3β, suggesting that metabolic syndrome blocks postC cardioprotection by inhibiting its signal transduction pathway. These findings could limit the clinical application of postC, or postC mimetics.

1.5. Phosphatases in ischaemia / reperfusion

In all cells there is a balance between the levels of phosphorylated and unphosphorylated proteins. This balance is controlled by two broad classes of enzymes: kinases (which phosphorylate proteins) and phosphatases (which dephosphorylate proteins). This balance is of importance, since in many cases the phosphorylation state of a protein determines the activity of that protein. As illustrated in the previous section, research has largely focussed on the role and importance of kinases in the setting of ischaemia / reperfusion. However, the role of the phosphatases in this setting has received much less attention. This section will primarily focus on the serine / threonine phosphatases type 1 and type 2A (PP1 and PP2A). For a review on the serine / threonine phosphatases, including types 2B and 2C see Wera & Hemmings (1995). A more recent review by Gallego & Virshup (2005) focusses on the regulation of specifically PP1, PP2A and PP5.

An example of the importance of the phosphatases in cardiac physiology is illustrated by Carr *et al.* (2002). They found that a shift in the kinase / phosphatase balance (by overexpressing PP1 in murine hearts) was associated with a reduction in cardiac function,

dilated cardiomyopathy and premature mortality. They speculate that changes in PP1 activity could also be involved in the pathology of heart failure in humans, and that these changes might be due to dysfunctional regulation of PP1 – which is itself also dependent on phosphorylation / dephosphorylation reactions.

1.5.1. Phosphatases and protection

It seems that phosphatase inhibition is generally associated with an improvement in the outcome of ischaemia / reperfusion (Xiuhua *et al.*, 1997; Weinbrenner *et al.*, 1998 and 1998; Armstrong *et al.*, 1998; Barancik *et al.*, 1999). This is illustrated by the study of Isotani *et al.* (2002), who reported that the phosphatase inhibitor okadaic acid (OA) protected rat kidneys against ischaemia / reperfusion injury, when administered early (within the first few minutes) in reperfusion. However, contradictory observations have been made. For example, Hausenloy *et al.* (2002) utilised cyclosporin A (CsA) to investigate the role of the mPTP in ischaemic preconditioning. CsA is an inhibitor of mPTP activity and associated with cardioprotection, but also inhibits the phosphatase calcineurin (also known as PP2B). To determine whether calcineurin inhibition was responsible for CsA-induced cardioprotection, FK506 was administered, which also inhibits calcineurin, without acting on the mPTP. Their results indicated that FK506 did not induce a decrease in infarct size, when given in reperfusion (5 minutes before the onset of reperfusion, for the duration of 20 minutes), clearly excluding calcineurin inhibition as a protective intervention during reperfusion. However, Weinbrenner *et al.* (1998) found that FK506 exerted a potent infarct sparing effect, when administered from 15 minutes before 30 minutes ischaemia until the end of ischaemia. It seems that the timing and / or duration of phosphatase inhibition might be determining factors in the eventual effect of an inhibitor.

Ladilov *et al.* (2002) found that PP1 and PP2A has contrasting effects on tissue damage and cell survival. They found that inhibition of PP2A only (by administration of a low dose, 5 nmol/L, of okadaic acid before sustained ischaemia) increased the protective effets of a hypoxic preconditioning (HP) protocol. When both PP1 and PP2A were inhibited (with cantharidin administered before ischaemia, in the presence or absence of HP), all protection against functional deterioration or intracellular Ca^{2+} loading (in isolated cardiomyocytes) was lost. The authors concluded that PP1 could be a mediator of HP-protection, in contrast to PP2A activity, which exerts a detrimental effect on HP-protection.

1.5.2. Phosphatase activity and ischaemia / reperfusion

It is possible that protection against ischaemia / reperfusion damage by inhibition of phosphatases, is mediated by an increase in the phosphorylation states of various pro-survival proteins in the cell. Conversely, phosphatase activity *per se* could be protective if it mediates inactivation (through dephosphorylation) of pro-death effectors. The big question is which proteins are involved.

Calcium (Ca^{2+}) homeostasis

In the study by Carr and colleagues (2002) mentioned earlier, they demonstrated that it is possible that overexpressed PP1 exerts its effects on the level of the sarcoplasmic reticulum (SR), by decreasing the level of phosphorylation of phospholamban (PLB), and in doing so contributing to a dysregulation of Ca^{2+} transport. Phosphorylation of phospholamban regulates the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) by alleviating its inhibition of SERCA (Kim *et al.*, 1990; Brittsan, *et al.*, 2000), with a resulting increase in Ca^{2+} movement out of the cytosol into the SR.

This interaction between phosphatase activity and Ca^{2+} regulation was also noted by Neumann and coworkers. In 1993 they found that okadaic acid (OA; a PP2A inhibitor) exerted a postive inotropic effect on isolated guinea-pig papillary muscles, associated with an increase in the phosphorylation of, amongst other proteins, PLB, troponin inhibitor (TnI) and myosin light chains (MLC). Similar effects were also reported to be elicited by cantharidin (Neumann *et al.*, 1995; Boknik *et al.* 2002).

Schaffer & Punna (1993) made the observation that the rat heart sarcolemma has a high activity of both PP1 and PP2A, which contributes to the regulation of Ca^{2+} pump activity. In corroboration of this, Neumann and colleagues found that (besides PLB modulation) both OA and cantharidin increased the sarcolemmal Ca^{2+} channel current (Neumann *et al*, 1993; and 1995), although canthadirin exerted only a weak modulatory effect on the L-type Ca^{2+} channel (Neumann *et al.*, 1995). It should be kept in mind that the effects associated with an inhibitor is also potentially determined by the cell system being studied. In this regard, Knapp *et al.* (1998) found that cantharidin did not increase intracellular Ca^{2+} levels in arterial smooth muscle cells, contrary to the observations reported in cardiomyocytes (Neumann *et al.*, 1995).

It could also be speculated that phosphatases influence Ca^{2+} homeostasis through other ion-channels and transporters. In this regard Chen *et al.* (1995) found that phosphatases similiar to PP2A and calcineurin dephosphosphorylates sodium (Na^{+}) channels in the rat brain, which then leads to an increase in current through these channels. It is noteworthy that Ladilov (2002) speculated that the protection conferred by HP might be via regulation of Na^{+} overload.

Structural maintenance

Another possible explanation for the protection induced by phosphatase inhibition is via its effects on structural integrity. During anoxia there is a progressive reduction in protein phosphorylation, probably due to the decrease in substrate (ATP) levels, as well as a reduction in kinase activity, while the phosphatases remain active (Kobryn & Mandel; 1994). Preservation of cytoskeletal phosphorylation, through inhibition of phosphatases, may therefore increase a cell's resistance against ischaemic injury.

Fernandez *et al.* (1990) illustrated that PP1 is involved in regulation of actomyosin filaments. In their model PP1 inhibition (by OA) protected nonmuscle cells against A-kinase induced actin reorganization. Armstrong *et al.* (1997 & 1998) found that the administration of phosphatase inhibitors (such as OA, calyculin A and fostriecin) immediately before, or during, an anoxic insult, could protect isolated cardiomyocytes, suggesting that this could be due to a decrease in the dephosphorylation of structural, or cytoskeletal, proteins during anoxia. In support of Armstrong *et al.*'s speculation, Loktionova & Kabakov (1993) found that both OA and cantharidin increased the stability of ATP-depleted endothelial cells' cytoskeleton, prevented F-actin degradation and preserved cell morphology. The authors attributed these observations to the arrest of HSP27 (heat shock protein 27; a protein associated with actin resistance against various stressors) dephosphorylation.

Preservation of the activity of pro-survival proteins and kinases

Weinbrenner *et al.* (1998) found fostriecin (used at a concentration to inhibit PP2A) to be protective (as demonstrated by a decrease in IFS in the isolated rabbit heart), even when administered during ischaemia. They suggest that this protection could be due to an increase in the phosphorylation state of a substrate in a protective signal transduction pathway, possibly PKC-specific substrate. Intriguingly, Armstrong and colleagues (1998) however found that fostriecin protection was independent of PKC activation. Xiuhua *et al.*

(1997) also speculated that OA protection could be mediated through an increased phosphorylation of PKC.

Protection through an increase in the phosphorylation of proteins, recruited in survival kinase pathways, could therefore also contribute to protection. In this regard, Armstrong *et al.* (1997) found that blockade of the mitochondrial K^{+}_{ATP}-channel (by 5-hydroxydecanoate) attenuated fostriecin-mediated protection in pig cardiomyocytes (this dependence on the mK^{+}_{ATP}-channel could however not be demonstrated in rabbit myocytes).

Calyculin A (an inhibitor of PP1 and PP2A) administration before or during anoxia, has been implicated in both myocardial cell protection, as well as an increase in phosphorylation of p38 MAPK and HSP27 (Armstrong *et al*, 1998; and 1999). This protection was lost with the administration of a p38 MAPK inhibitor (SB203580). The role of p38 MAPK phosphorylation in protection is however controversial. Mackay & Mochly-Rosen (2000) found that inducing increased phosphorylation of p38 MAPK during anoxia, using the tyrosine phosphatase inhibitor vanadate, increased cardiomyocyte susceptibility to ischaemic injury. In the setting of IPC, it has been found that, a decrease in p38 MAPK phosphorylation during ischaemia and reperfusion is in fact associated with cardioprotection (Marais *et al.*, 2005; and Moolman *et al.*, 2006). Barancik *et al.* (1999) found that administration of OA, before a regional ischaemic insult in an *in vivo* swine model, conferred an infarct sparing effect, associated with an increase in the phosphorylation and activity of JNK. The inhibitor, however had no effect on the phosphorylation and activity of either p38 MAPK or ERK p42/p44. It is clear that a great deal of confusion and uncertainty still prevails regarding the role of p38 MAPK in the setting of ischaemia / reperfusion.

1.5.3. Phosphatases in ischaemic preconditioning

In light of the above discussed, it is logical that many studies (including some already mentioned) have focussed on the possibility that phosphatases are somehow involved in preconditioning. Weinbrenner *et al.* (1998) however found that although PP2A inhibition *per se* had an infarct sparing effect comparable to IPC, neither PP1 nor PP2A was inhibited in preconditioned hearts. Despite these findings, it is conceivable that phosphatase activity could modulate IPC. This was demonstrated by Fenton and colleagues (2005). By applying OA (at a concentration to inhibit both PP1 and PP2A)

during the preconditioning stimulus, the effectiveness of the IPC protocol was restored in old rat hearts. It is noteworthy that OA (on its own) could only confer cardioprotection in the old hearts when administered both before and after ischaemia. In young hearts however, OA in reperfusion alone was sufficient. This indicates the existence of a protection threshold, which must be overcome before an intervention (such as phosphatase modulation) can successfully elicit cardioprotection.

Except for the possible modulation of the phosphorylation status of proteins already mentioned, Cai & Semenza (2005) also investigated PKB/Akt activity in preconditioning. It should be noted that PKB/Akt activity is largely regulated by PI3-kinase / PTEN (phosphatase and tensin homologue deleted from chromosome 10). Cai and Semenza (2005) found that IPC induces down regulation of PTEN during sustained ischaemia. This then (together with the oxidative inactivation of residual PTEN in reperfusion) favoures the activation of PKB/Akt – which mediates cardioprotection. Besides the role of PTEN, PP2A has also been implicated as a regulator of PKB/Akt activity (Andjelkovíc *et al.*, 1996).

1.5.4. Conclusion

Most studies on the subject have found that the inhibition of phosphatase activity during, or just before, ischaemia / reperfusion confers cardioprotection – suggesting a deleterious role for PP1 and PP2A in this setting. Mechanisms for this observed protection probably involves the preservation of, or increase in, the phosphorylation levels of certain proteins involved in cell survival. Modulation of the activity of these proteins could have an effect on structural stability, ion homeostasis, and the activation of pro-survival pathways. Unfortunately the lack of suitably specific phosphatase inhibitors has hampered efforts to identify the roles of specific phosphatases and proteins. Therefore, the precise role of the phosphatases in cell survival in ischaemia / reperfusion, as well as their importance in the mechanisms of known cardioprotective interventions such as IPC and postC, is still a topic of ongoing investigation.

1.6. Motivation and aims of this study

Since it was described in 2003, a large amount of research has been done on the phenomenon of postconditioning. As discussed in the preceding literature review, PostC cardioprotection has been demonstrated in all species tested and in various experimental setups. There are however, still discrepancies concerning the precise algorithms associated with cardioprotection. Although the retrogradely perfused (Langendorff) isolated rat heart has been widely used in postC research, investigations in the setting of the isolated working heart model is lacking, with only one reported study (Sasaki *et al.*, 2007).

Concerning the mechanisms by which postC protects, most reasearch has been aimed at the pro-survival mechanisms known to be active in IPC, such as adenosine, PKC, the mK^{+}_{ATP}-channel, etc. Despite the focus on the roles of pro-survival kinases in postC, much less has been published on the involvement of other kinases and no work has been done on the possible role of the phosphatases. In fact, relatively little research has been done on the phosphatases in ischaemia / reperfusion *per se.*

The aim of this study was therefore to contribute to some of these outstanding issues by:

1. Development of an effective cardioprotective postC protocol in the isolated rat heart, using both the Langendorff and working heart models. End-points investigated were infarct size and functional recovery. Comparing postC in these two models could shed light on the factors that determine the efficacy of postC under different conditions.
2. Characterization of the signalling kinase profiles associated with postC, at different time points in reperfusion, in the two perfusion models. We focused our investigation on the widely publicized RISK pathway (i.e. PKB/Akt and ERK p42/p44), as well as the less investigated (in the setting of postC) p38 MAPK.
3. Investigation of the possible role of the phosphatases, PP1 and PP2A, in postconditioning and ischaemia / reperfusion *per se.* This was done by investigating the effect of co-administration of the PP1 and PP2A inhibitor, cantharidin, on postC-induced cardioprotection, using infarct size and activation of PKB/Akt, ERK p42/p44 and MAPK p38 as end-points.

Chapter 2: Material and Methods

“The heart is deceitful above all things
and beyond cure.
Who can understand it?”

Jeremiah 17:9
The Bible

Chapter 2: Material and methods

This study can be divided into three sections. The methods used in each section will be descibed separately. They are:

1. The development of a cardioprotective postconditioning protocol.
2. Analysis of the involvement of the reperfusion injury salvage kinase (RISK)-pathway in the setting of postconditioning.
3. The effect of a phosphatase inhibitor in the setting of postconditioning and reperfusion.

2.1. Animals

Male Wistar rats, weighing between 210 and 350 g, were used. Animals were given free access to food and water until the time of experimentation. All rats were anaesthetized by administration of an intra-peritoneal injection of pentobarbital, at a dose of approximately 80 mg / rat.

All experiments were done in accordance to the "Guide for the care and use of laboratory animals" published by the US National Institutes of Health (NIH publication no 85–23, revised 1985). The project was also approved by the Ethics committee of the University of Stellenbosch (Faculty of Health Sciences).

2.2. Perfusion technique of the isolated rat heart

Following proper sedation, hearts were rapidly excised and arrested in ice-cold Krebs solution. Immediately thereafter, the aortas were cannulated and in all instances the hearts were perfused with Krebs-Henseleit bicarbonate buffer, containing (in mM): NaCl 119; $NaHCO_3$ 24.9; KCl 4.74; KH_2PO_4 1.19; $MgSO_4$ 0.6; Na_2SO_4 0.59; $CaCl_2$ 1.25 and glucose 10. The buffer was continuously gassed with a 95 % O_2 / 5% CO_2 combination in order to ensure adequate oxygenation of the buffer and also to maintain a pH of 7.4. The temperature of the heart was monitored by inserting a temperature probe into the coronary sinus. Two different modes of perfusion were employed, namely retrograde Langendorff perfusion (the balloon model) and the working heart model. In both models a basic protocol of 30 minutes stabilisation, variable time periods of ischaemia and a total of 30 minutes reperfusion, was followed.

2.2.1. Retrograde Langendorff perfusion (Balloon model)

In this model the aorta was cannulated and hearts retrogradely perfused with Krebs-Henseleit bicarbonate buffer at a pressure of 100 cmH_20. Temperature was maintained at approximately 37 °C during stabilisation, ischaemia and reperfusion.

A water filled cling-film balloon, connected to a pressure transducer (Viggo Spectromed), was inserted into the left ventricle, via the left atrium. By setting the pressure in this balloon, the end-diastolic pressure was maintained between 0 and 10 mmHg. Myocardial function was quantified in terms of heart rate (HR) and left ventricular developed pressure (LVDP). The LVDP can be defined as the difference between the measured systolic pressure and the set diastolic pressure. These functional parameters were recorded on a computerized system throughout the duration of the experiment. Another important functional parameter that was used, is the rate pressure product (RPP, i.e. LVDP x HR).

2.2.2. The working heart model

After mounting the aorta onto the aortic cannula, the pulmonary vein of the heart was also cannulated. The heart could then be perfused either retrogradely or in the working heart mode. The term “work” refers to the heart functioning against a set afterload of 100 cmH_20 and a preload of 15 cmH_2O (applied through the pulmonary vein cannula). Hearts were not electrically paced.

Functional parameters monitored in this model were the intra-aortic pressure and heart rate, as well as the coronary and aortic flow rates. The intra-aortic pressure and heart rate were measured with a pressure transducer (Viggo Spectromed) inserted into the aortic cannula. Data were recorded on a computerized system and left ventricular work performance calculated using the formula described by Kannengieser *et al.* (1979):

$$0.002222\,(P_{AO} - 11.25)(CO)$$

where, P_{AO} = aortic pressure

CO = cardiac output (aortic output + coronary flow rate)

Coronary and aortic flow rates were manually measured by timed collection of the perfusate passing through the coronary system and the aorta (during work mode).

The temperature of the hearts was monitored to ensure that it did not rise above 37 °C during work. Temperature was maintained at approximately 36.5 °C during the ischemic period and elevated to 37 °C during reperfusion.

2.3. Application of ischaemia

As will be expanded on later in this chapter, different protocols required different modes of ischaemia. These different modes of ischaemia are as follows:

- **Global ischaemia (GI)** This refers to total cessation of coronary perfusion to the heart. This was achieved by perfusion of the heart in the Langendorff mode and closure of the aortic cannula.
- **Regional ischaemia (RI)** In this form of ischaemia, only the left coronary artery was ligated. This was done by ensnaring the proximal part of the left coronary artery using a silk suture, in such a way that it could be loosened again to reperfuse the affected tissue. This was done by tightening the snare using two pieces of interlocking tubing. Occlusion of the artery was confirmed by measuring the resultant reduction in the coronary flow. This form of ischaemia is used for the determination of infarct size.

2.4. Determination of infarct size

At the end of the RI experiments the left coronary artery, which at this stage was open to allow reperfusion, was securely ligated again. This was followed by the slow infusion of Evans Blue suspension (0.5 %) into the heart, via the cannulated aorta. By doing this the Evans Blue solution is forced through the coronary arteries that are still open and unaffected by the occlusion. Care was taken to ensure that a sufficient volume of Evans Blue (between 0.5 and 1.0 ml) was infused to clearly delineate the area affected by the closed suture from the rest of the ventricular tissue. The heart was then removed from the system and frozen overnight, before being sliced into thin slices of equal thickness (about 2 mm). These slices were then stained by incubation in 1% w/v triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 15 minutes, at room temperature. Pitts *et al.* (2007) describes this as a staining reaction in which the TTC reacts with active dehydrogenases in the viable tissue to form a brick-red precipitate. The slices were then removed from the TTC-buffer solution and fixed in a 10% v/v formaldehyde solution. This was done in order to enhance the contrast between the stained and unstained areas. At this point the damage to each slice of tissue becomes visible: the blue area showed viable and undamaged tissue, the white, unstained area was dead, infarcted tissue and, together

with the surrounding brick-red area, indicated the area at risk. Quantification of the different surface areas was done by using planimetry. The areas of the tissue slices were then summated to determine the infarct size (IFS) and area at risk (AR) for the whole left ventricle. Infarct size was expressed as a percentage of the area at risk. Planimetry was done using the UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas), which is available from the internet at http://ddsdx.uthscsa.edu/dig/itdesc.html.

2.5. Western blot analysis

As will be described in more detail later in the text, hearts were freeze–clamped at different time points in reperfusion using precooled Wollenberger tongs, whereafter the tissue samples were plunged into and stored in liquid nitrogen. These collected tissue samples were then used for protein identification and quantification analysis as follows:

Protein extraction: Frozen tissue samples were initially pulverized in a stainless steel mortar under liquid nitrogen cooled conditions, whereafter further homogenization was done (using a Polytron homogenizer) in lysis buffer. The lysis buffer contained (in mM): Tris-HCL (pH 7.5) 200; EGTA 10; EDTA 100; sodium chloride (NaCl) 1000; β-glycerophosphate 1; tetrasodiumpyrophospate 2.5; sodium vanadate (Na_3VO_4) 10; phenylmethylsulphonylfluoride (PMSF) 100; leupeptin 10 µg/ml; aprotonin 10 µg/ml; Triton X-100 1%. Following homogenization, samples were centrifuged at 1000 g for 10 minutes. The supernatant was then separated from the pellet and used for further experimentation.

The amount of proteins extracted was quantified using the Bradford technique (Bradford; 1976). The lysis samples were then diluted in Laemmli sample buffer to a final protein concentration of 2.2 µg/µl. The final samples were then boiled for 5 minutes and stored at –20 ºC.

Protein separation: Proteins were separated according to their molecular weight using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) setup. After brief boiling (see above) and centrifugation (to ensure all the samples were at the bottom of the eppendorff tubes) of the samples, 20 µg of protein from each sample was loaded in a 4 % stacking polyacrylamide gel and separated on either a 12 % (for p38 MAPK and ERK

p42/p44) or a 10 % (for PKB/Akt) polyacrylamide gel, using the standard Bio-RAD Mini-PROTEAN II System.

Western blotting: After separation, proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Immobilon^{TM} P, Millipore) in a tank electrotransfer setup. In this setup the membrane-gel stack is immersed in transfer buffer, through which electrical current is applied. The transfer buffer contained (in mM): Tris 25; glycine 192; methanol 20%. After transfer, membranes were regularly stained with Ponceau red (which is a reversible protein stain) to confirm that adequate transfer had taken place.

Blocking of the membrane: Following transfer, the non-specific protein binding sites on the membrane were blocked by incubating the membrane in a 5% fat-free milk in Tris-buffered saline (TBS)-0.1 % Tween 20 solution for at least one hour.

Incubation with antibodies: In all experiments p38 MAPK, ERK p44/p42 and PKB/Akt were investigated. Membranes were therefore probed with primary antibodies directed against: total p38 MAPK and dual phospho-p38 MAPK (Thr180/Tyr182); total ERK p44/p42 and phospho-ERK p44/p42 (Thr202/Tyr204); total PKB/Akt and phospho-PKB/Akt (Ser473). All antibodies used were from Cell Signalling Technology. Primary antibodies were diluted in TBS-Tween solution (1:1000) and incubated with the membranes for at least 5 hours.

Following primary antibody-binding, membranes were incubated for 1 hour in an ECL anti-rabbit immunoglobulin G, Horseradish peroxidase-linked (from donkey) secondary antibody (Amersham), diluted in TBS-Tween solution (1:4000). This conjugated antibody then bound to the already bound primary antibody.

Membranes were briefly rinsed with 3 changes of TBS-Tween, followed by at least 4 x 5 minutes washing with large volumes of TBS-Tween solution after blocking, between antibody incubations, and before final visualisation.

Visualisation: After incubation with the secondary antibody, membranes were covered with ECL^{TM} detection reagents for 1 minute, and then exposed to an autoradiography film (Hyperfilm ECL, RPN 2103). The detection reagents react with the Horseradish peroxidase

(linked to the secondary antibody) in a luminescence reaction and the resulting light emmision is “captured” on the radiography film.

Films were then densitometrically analysed (UN-SCAN-IT$_{TM}$ version 5.1, Silkscience). For comparison purposes, samples from negative control hearts (exposed to only 30 minutes perfusion) were included in each blot and used for normalization of the unknown samples (i.e. calculation of the ratio between the sample and negative control). Normalized data was expressed in arbitrary units (AU).

2.6. Statistical analysis

All results are given as mean ± standard error of the mean (SEM). Where only two parameters were compared, comparisons were done with an unpaired T-test. For comparison of multiple parameters, one-way analysis of variance (ANOVA) was applied, followed by the Bonferroni correction as post hoc test. P values of < 0.05 was considered significant.

Chapter 3: Development of a cardio-protective protocol

"The entire question of the optimal algorithm and durations of reperfusion and ischemic segments of the PoC (postconditioning) stimulus is open."

Vinten-Johansen J, Zhao Z-Q, Jiang R, Zatta AJ, Dobson GP.

***J Appl Physiol* 2007; 103:1441–1448.**

Chapter 3: Development of a cardioprotective protocol

3.1. Background and motivation

A relatively large number of studies have already been done on postconditioning of the rat heart. Most of these studies have also shown that postconditioning can confer cardioprotection in the rat heart, both *ex vivo*, as well as *in vivo.* It is however, also clear from the studies reported, that some groups experienced difficulties in eliciting protection, suggesting that the rat heart may be sensitive to the postC protocol employed. A general overview of postconditioning work that has been done in specifically the rat heart is summarised in table 1.

The first phase of this project therefore logically entailed the development of a postconditioning protocol in the *ex vivo* rat heart, capable of eliciting a significant degree of cardioprotection. Up to date all studies published on postconditioning in the isolated rat heart, except one, utilised a retrogradely perfused Langendorff setup. Sasaki and coworkers (2007) utilised a working heart setup to investigate a unique postconditioning protocol of 1 minute reperfusion and 5 minutes ischaemia. Although this protocol protected against reperfusion arrythmias, they could not demonstrate increased functional recovery or a reduction in cell damage (as measured by CK release). Therefore, since research on postconditioning in the working *ex vivo* heart is lacking, we foucussed our investigation on postconditioning in our working heart setup. A number of different postC protocols were applied and their efficacy evaluated in terms of functional recovery during reperfusion and infarct size. The protocol that showed the most potential in the working heart, was also evaluated in the retrograde perfusion (Langendorff) model.

During the course of our study, we realised that there were three different variables that had to be considered in the establishment of a postconditioning protocol. These variables and our experimental approaches are summarized in table 2.

Table 1: *Summary of studies done on postconditioning in the rat heart. Although most of these could demonstrate a cardioprotective role for postconditioning, there are still discrepancies in the literature.For a more detailed discussion, see chapter 1.*

Authors	**Model used**	**End-point**	**PostC protocol**	**Success**
Kin *et al*., 2004	*In vivo*	Infarct size	3 or 6 x 10 sec	Yes
Wang *et al*., 2007	*Ex vivo*	Functional recovery	6 x 10 sec	Yes
Penna *et al*., 2006	*Ex vivo*	Infarct size	5 x 10 sec	Yes
			Reperfusion:15, 20, 25, 30 sec Ischaemia: 20, 15, 10, 5 sec	Yes
Tang *et al*., 2006	*In vivo*	Infarct size	6 x 10 sec	Yes
			20 x 10 sec	Yes
			60 x 10 sec	No
Manintveld *et al*., 2007	*In vivo*	Infarct size	3 x 30 sec	Yes
Bopasse *et al*., 2006	*Ex vivo*	Markers of necrosis	3 x 30 sec	Yes
Galagudza *et al*., 2004	*Ex vivo*	Fibrillation	2 minutes ischaemia after 15 minutes reperfusion	Yes
Dow & Kloner, 2007	*In vivo*	Infarct size	4 x 10 sec	No
			4 x 20 sec	No
			8 x 30 sec	No
			20 x 10 sec	No
Crisostoma *et al*., 2006	*Ex vivo*	Functional recovery	6 x 10 sec	Yes
Kaljusta *et al*., 2007	*In vivo*	Infarct size	3 x 10 sec	Yes in one of two laboratories
	Ex vivo	Infarct size	3 x 10 sec	No
			3 x 30 sec	No
			2 x 60 sec	No
			6 x 10 sec	No

***Table 2**: The different variables that were taken into consideration in the development of a cardioprotective postC protocol in the* ex vivo *rat heart, in our laboratory.*

Variable	Options
Time and mode of ischaemia	35 minutes of regional ischaemia induced by coronary ligation.
	20 or 25 minutes of global no-flow ischaemia.
Temperature during ischaemia and initial reperfusion (first 10 minutes)	36.0 – 36.5 °C.
	36.5 – 37.0 °C.
Postconditioning cycles	6 × 10 seconds applied regionally, i.e via the suture ligating the coronary artery
	6 × 10 seconds applied globally, i.e via manipulation of global flow to the heart.
	3 × 30 seconds applied regionally or globally.
	3 × 20 seconds applied regionally.
	4 × 15 seconds applied globally.

3.2. Materials and methods

As previously described: male Wistar rats between 210 and 350 g were sacrificed, the hearts rapidly excised and aortas cannulated. Hearts were perfused with Krebs-Henseleit buffer for the duration of the experiment, except for periods of global no-flow ischaemia.

Both infarct size, as well as functional recovery, were investigated as end-points. The basic perfusion protocol that was applied consisted of a 30 minute stabilisation period before an ischaemic insult, which was followed by 30 minutes of reperfusion. All postconditioning protocols were applied at the very onset of reperfusion, without any delay. To facilitate the interpretation of the data, the relevant protocols are described in the "Results" section.

3.3. Results

3.3.1. The working heart model

3.3.1.2. Global ischaemia

Initially we focused our investigation on developing a postC protocol that could elicit an increase in functional recovery of the working rat heart. To do this we applied either a 25 or a 20 minute period of no-flow global ischaemia (temperature 36.5 °C), followed by either uninterrupted reperfusion (i.e. non-postconditioned; NonPostC), or 6 x 10 seconds reperfusion / ischaemia applied by manipulating the global coronary flow with the aortic cannula (postC). We chose this postC protocol since, it has been reported in various studies to confer cardioprotection.

In this model two different sets of experiments were conducted: a 20 minute GI postC group, with its NonPostC control group, as well as a 25 minute GI postC group, with its corresponding NonPostC group (Fig. 1).

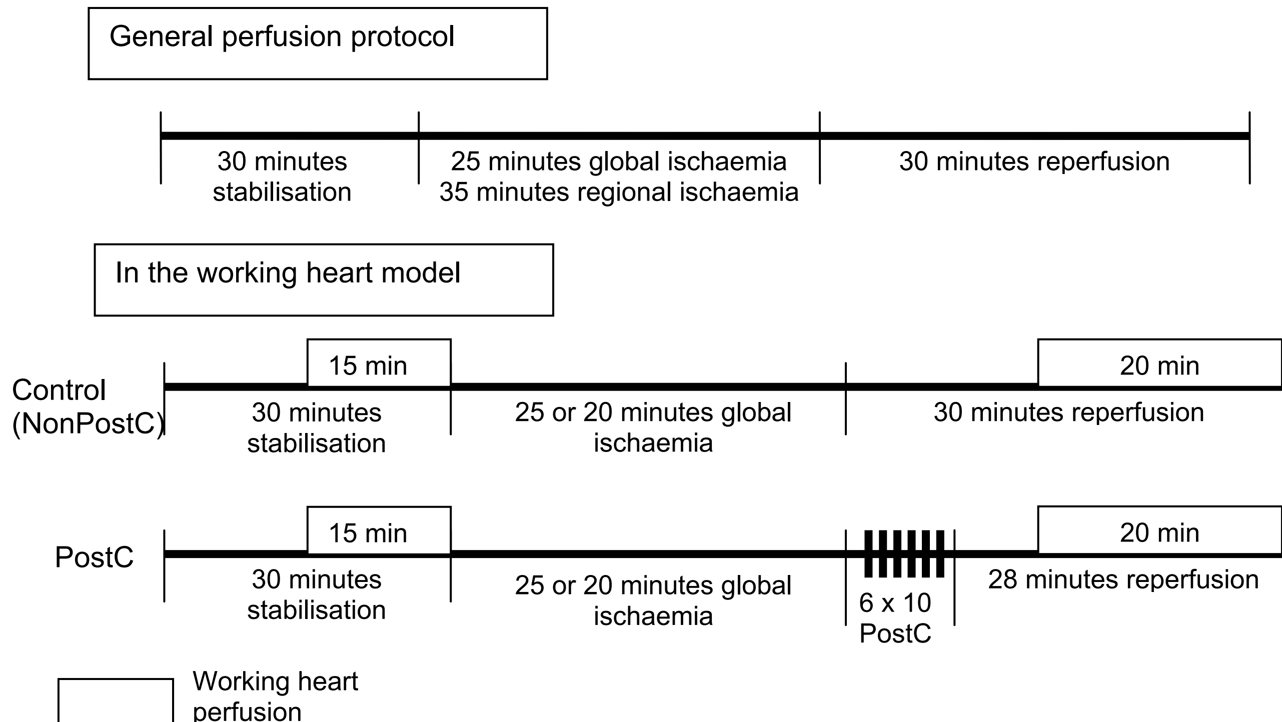


Figure 1*: Experimental protocol to investigate the ability of postC to increase post-ischaemic functional recovery in the working heart model, following a global ischaemic insult.*

It was decided to simplify data presentation by omitting coronary flow (CF) data, except in instances in which a significant difference in functional parameters was present which might be influenced by the coronary flow rate. Both the postC (n=10), as well as NonPostC (n=9) groups, in the 25 minute GI study, generated acceptable aortic – and cardiac outputs before the onset of ischaemia (NonPostC group: AO = 38.56 ± 1.91ml/min, CO = 53.50 ± 2.19 ml/min; postC group: AO = 41.70 ± 1.90 ml/min, CO = 56.95 ± 2.19 ml/min). The percentage recoveries of both of these parameters are shown in figure 2. NonPostC hearts recovered to an AO of 1.78 ± 1.35 ml/min and a CO of 9.56 ± 1.92 ml/min. This level of recovery was comparable to that observed in the PostC group (AO = 0.0 ml/min and CO = 8.85 ± 0.8 ml/min).

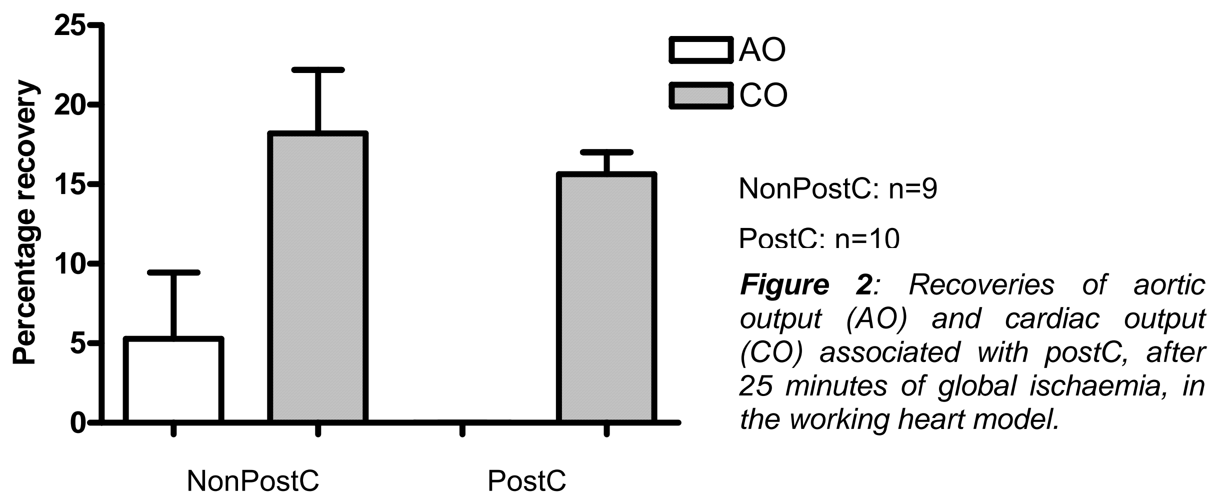


***Figure 2**: Recoveries of aortic output (AO) and cardiac output (CO) associated with postC, after 25 minutes of global ischaemia, in the working heart model.*

The recoveries in aortic output and cardiac output associated with postC after 20 minutes GI followed the same pattern (figure 3). The 20 minute GI postC group (n = 4), had a mean aortic output of 37.25 ± 2.056 ml/min, together with a cardiac output of 52.25 ± 3.62 ml/min at the end of stabilisation. The aortic output measured in the NonPostC group (n=4) was 47.25 ± 3.198 ml/min, with a cardiac output of 64.13 ± 3.25 ml/min. There were no significant differences in recovery between the NonPostC and PostC groups. In fact, in the NonPostC hearts AO recovered to 8.0 ± 6.06 ml/min, compared to a strikingly low post-ischaemic AO in the PostC group of 1.5 ± 1.5 ml/min. Post-ischaemic CO in the NonPostC and PostC groups were 18.38 ± 6.63 ml/min and 10.50 ± 2.53 ml/min respectively.

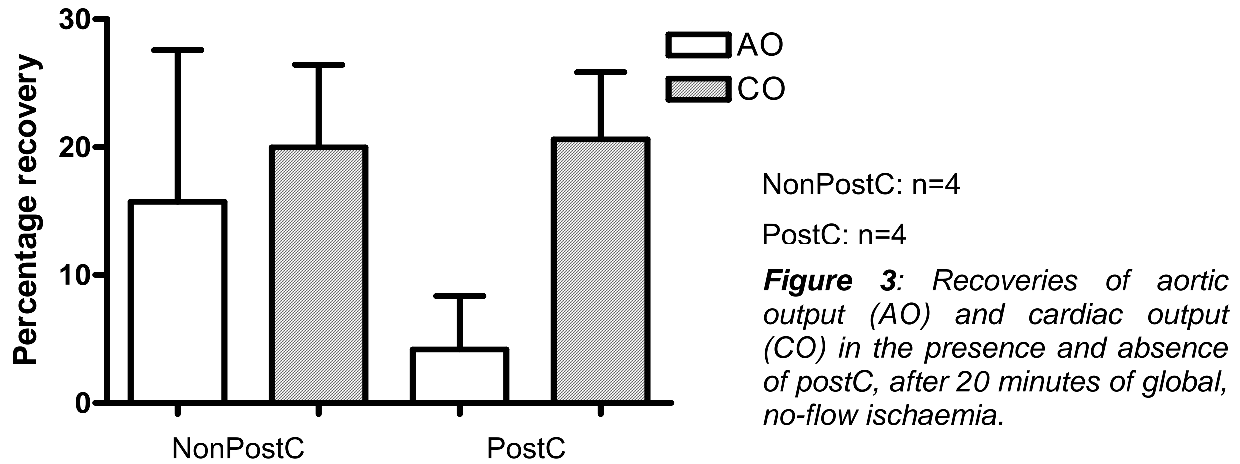


***Figure 3**: Recoveries of aortic output (AO) and cardiac output (CO) in the presence and absence of postC, after 20 minutes of global, no-flow ischaemia.*

The postC intervention we applied was disappointingly ineffectual at eliciting functional recovery after both 20 and 25 minutes of global ischaemia. This could be due to either a suboptimal postC algorythm, or the inherent inability of postC to protect against functional decline. To study postC in the working heart further we therefore decided to investigate a slightly different ischaemic model.

3.3.1.3. Regional ischaemia

In view of our failure to elicit postconditioning in the globally ischaemic working rat heart, it was decided to switch from global ischaemia to regional ischaemia, using both functional recovery and infarct size as end-points. In all experiments where infarct size was used as end-point, 35 minutes of regional ischaemia was applied (by using a suture to occlude the coronary artery). Hearts were broadly divided into one of two groups: Either control hearts, which received uninterrupted full reperfusion after ischaemia (NonPostC), or postconditioned hearts (postC). In this latter group a number of different postC protocols were applied at reperfusion (fig. 4).

In addition to testing different postC algorythms, the best method of applying the postC reperfusion / ischaemia cycles also had to be determined. In this regard two possiblities were investigated: either manipulating the flow of perfusate to the heart as a whole, or manipulating the flow to only the tissue exposed to ischaemia. Manipulating the flow to the whole heart would technically be the easiest option, and it would mean that both the ischaemic and non-ischaemic myocardium would be exposed to the postC intervention. On the other hand, opening and closing the occluder would be more challenging, especially since it would impede effective thermal regulation during the postC intervention.

The advantage however, is that it would target only the tissue exposed to the ischaemic insult.

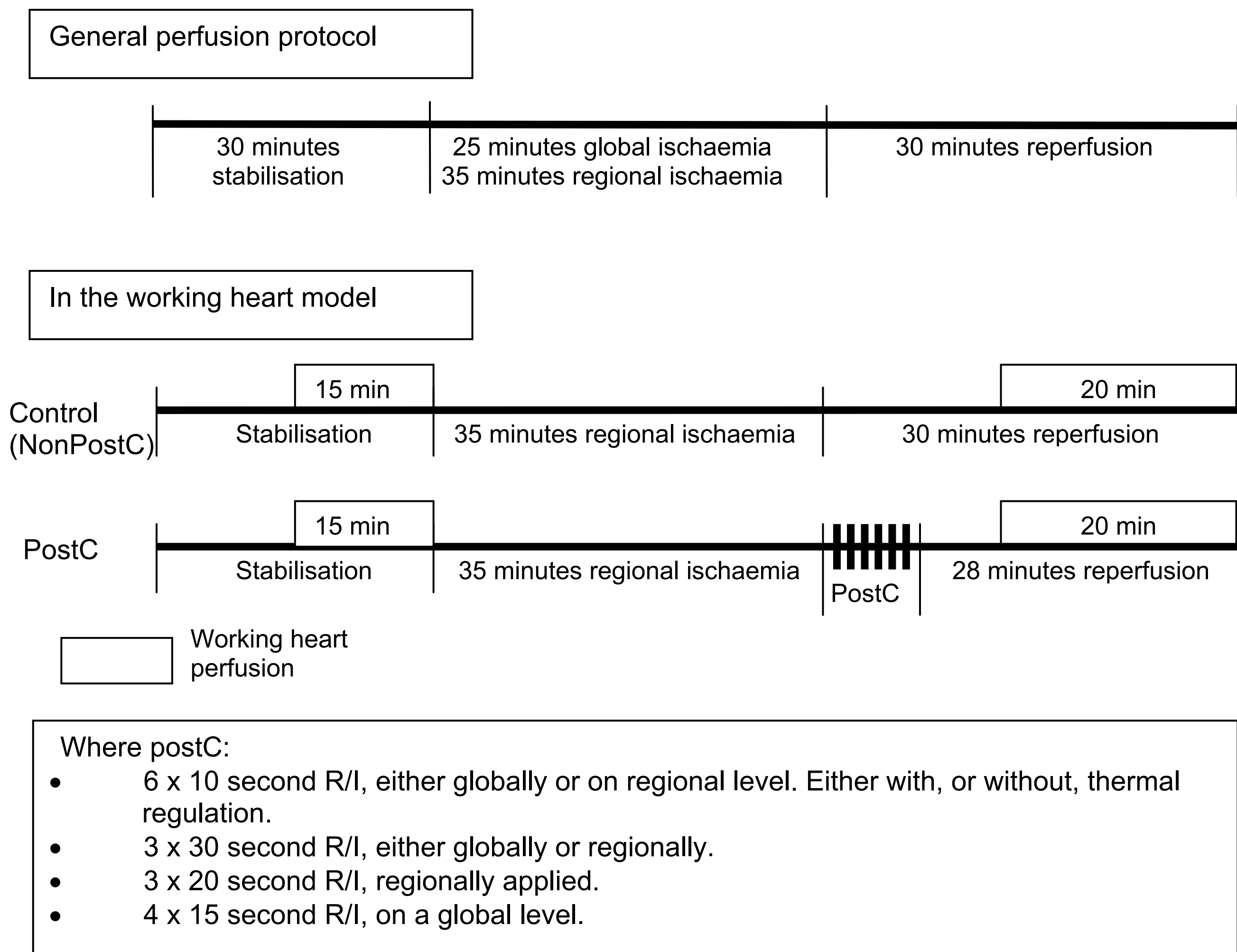


***Figure 4**: Experimental protocol for the investigation of postC in the setting of regional ischaemia. All hearts underwent a 30 minute period of stabilisation, 35 minutes of regional ischaemia and a total of 30 minutes reperfusion. Different postC protocols were tested for their cardioprotective ability, as evaluated by infarct size and functional recovery. Protocols differed in terms of number of cycles, duration of cycles and thermal regulation.*

The following protocols were applied:

- 6 x 10 second cycles of reperfusion and ischaemia, applied by opening and closing the snare around the coronary artery (i.e. on a regional level). Technically this required that, for the 2 minute duration of the postC protocol, the hearts were exposed to the cooler ambient room temperature. To correct for a possible thermal effect, two sets of controls were therefore perfused: one set with a temperature maintained at

approximately 36.5 °C, while the other set was also exposed to the ambient room temperature for the first 2 minutes of reperfusion.

- 6 x 10 second cycles of reperfusion and ischaemia, applied by manipulating the total flow of buffer to the heart as a whole (i.e. on a global level). This protocol had the advantage that the hearts' temperature could be maintained throughout the postC intervention (as the hearts remained in the waterbath-warmed environment after opening the suture). However, no rigorous temperature control was done at this stage.
- 3 x 30 second cycles of reperfusion and ischaemia, applied either on a regional or a global level.
- 3 x 20 second cycles of reperfusion and ischaemia, applied by directly manipulating the snare around the coronary artery (i.e. on a regional level).
- 4 x 15 second cycles of reperfusion and ischaemia, applied by manipulating global flow to the heart.

In all the above mentioned protocols temperature was maintained at an ischaemic temperature of approximately 36.5 °C, there was however no rigorous thermal control during reperfusion.

- 6 x 10 second cycles of reperfusion and ischaemia, applied on a global level. This protocol differed from the above mentioned protocol in terms of its thermal control. In these hearts, the temperature during the postC protocol was maintained between approximately 36.5 °C and 37.3 °C. This was higher than the temperature during ischaemia (36.5 – 36.7 °C). It should be mentioned that the temperature fluctuates significantly during the postC protocol, necessitating active regulation to maintain the temperature within certain limits. Control hearts (without postC intervention) were also maintained at elevated temperatures during especially the first two minutes of reperfusion.

Initial results for the 6 x 10 second postC intervention, by opening and closing the coronary suture, are shown in figure 5. Two NonPostC groups were included: The first (NonPostC1) did not take possible temperature fluctuations during the postC intervention into consideration (n=4; AO before ischaemia: 32.50 ± 0.5 ml/min; CO before ischaemia: 48.00 ± 0.00 ml/min). Although the second NonPostC (NonPostC2) group (n = 7; AO = 36.57 ± 1.50 ml/min; CO = 51.29 ± 1.94 ml/min) underwent the exact same procedure during reperfusion as the postC hearts (i.e. exposed to ambient room temperature for the first 2

minutes of reperfusion), this did not lead to significant changes in infarct size (NonPostC1: 26.46 ± 6.02% compared to NonPostC2: 33.61 ± 3.80%). Both NonPostC groups had similar area at risk values (NonPostC1: 42.70 ± 4.0% and NonPostC2: 53.19 ± 3.25%). The functional parameters in the postC group also fell within acceptable limits (n = 11; AO = 38.45 ± 1.63 ml/min; CO = 53.68 ± 1.80 ml/min).

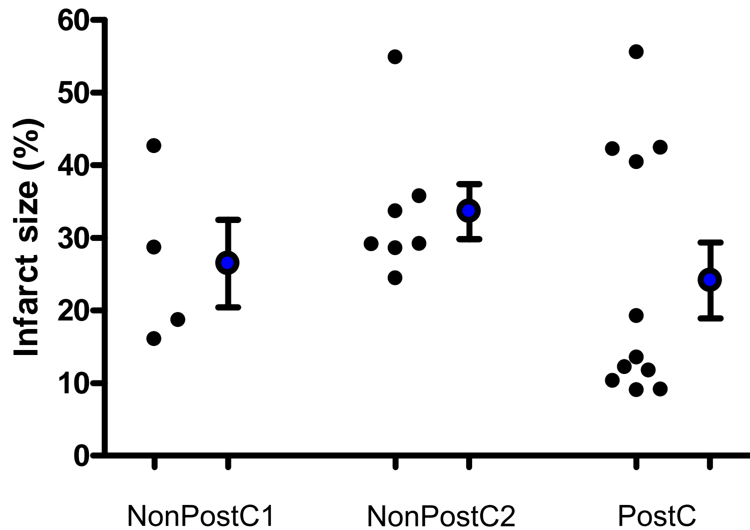


Figure 5*: Infarct sizes when 6 x 10 second postC cycles are applied on a regional level. NonPostC1 does not take temperature fluctuations during the initial reperfusion into account, while NonPostC2 does.*

Since no significant differences in infarct size were present, both of the NonPostC groups were combined as a single control group (n = 11; AO = 35.09 ± 1.12 ml/min; CO = 50.09 ± 1.30 ml/min) for the other experiments where postC was applied on a regional level.

Two other regional postC protocols were tested: a 3 x 20 second protocol (n = 4; AO = 37.00 ± 2.08 ml/min; CO = 54.35 ± 2.65 ml/min) and a 3 x 30 second intervention (n = 6; AO = 36.00 ± 1.37 ml/min; CO = 53.42 ± 1.28 ml/min). Infarct sizes for these protocols are shown in figure 6.

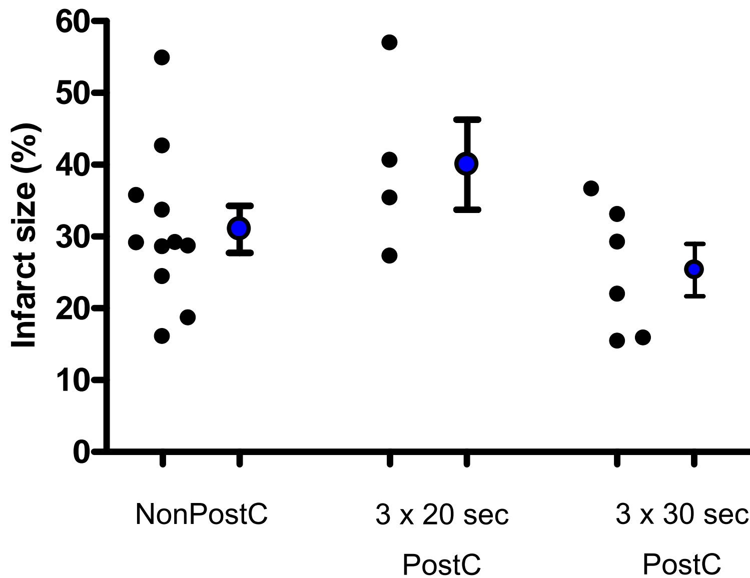


***Figure 6**: Infarct sizes associated with 3 x 20 second and 3 x 30 second postC protocols, applied by manipulating the coronary artery occluder.*

None of the three regional postC protocols were associated with a significant decrease in infarct size (figures 5 and 6: NonPostC: 31.01 ± 3.26%; 6 x 10 sec: 24.12 ± 5.22%; 3 x 20 sec: 40.01 ± 6.27%; 3 x 30 sec: 25.31 ± 3.66%). Area at risk size was comparable between the four different groups (NonPostC: 49.37 ± 2.88%; 6 x 10 sec: 51.47 ± 4.35%; 3 x 20 sec: 46.79 ± 4.88%; 3 x 30 sec: 47.69 ± 3.25%). These protocols conformed to the general protocol described in the literature (see chapter 1). We however found that none of these postC interventions could exert an infarct-sparing effect. From the three postC protocols tested it seems that the 6 x 10 sec protocol showed the most promise, since more than 50% of the hearts subjected to this protocol presented with infarct sizes lower than 20%.

In all postC protocols applied regionally, no significant differences in functional recovery were observed between postC and NonPostC hearts (i.e. recovery in aortic output and cardiac output). Functional data is shown in table 3.

As with infarct size reduction, although no significant differences were observed, the postC 6 x 10 sec protocol showed the most potential to increase functional recovery (6 x 10 sec: AO recovery = 46.58 ± 9.27%, CO recovery = 60.45 ± 7.80%; compared to NonPostC: AO = 34.47 ± 7.02%, CO = 52.94 ± 5.44%; 3 x 20 sec: AO = 20.88 ± 5.68%, CO = 44.60 ± 3.66%; and 3 x 30 sec: AO = 21.17 ± 6.31%, CO = 43.78 ± 5.69%).

Table 3*: Functional data before and after sustained ischaemia in the presence of different postC interventions, applied regionally. No significant differences were present between the groups. AO: aortic output; CO: cardiac output.*

Protocol	Baseline AO (ml/min)	Post-ischaemic AO (ml/min)	Baseline CO (ml/min)	Post-ischaemic CO (ml/min)
NonPostC	35.09 ± 1.12	12.18 ±2.64	50.09 ± 1.30	26.59 ± 2.95
6 x 10 sec	38.45 ± 1.63	17.75 ± 3.73	53.68 ± 1.80	32.38 ± 4.27
3 x 20 sec	37.00 ± 2.08	7.8 ± 2.40	54.35 ±2.65	24.30 ± 2.63
3 x 30 sec	36.00 ± 1.37	7.92 ± 2.51	53.42 ± 1.28	23 ± 3.27

It is therefore however clear that none of the postC protocols applied by manipulating regional flow (i.e. 6 x 10 sec, 3 x 20 sec and 3 x 30 sec) could confer cardioprotection, as measured by infarct size and functional recovery. It might be that this lack of benefit is due to the method of postC application (i.e. regional).

In order to investigate the efficacy of globally applied postC (by manipulating the flow of perfusate to the whole heart), three different postC protocols were tested: 6 x 10 sec, 3 x 30 sec and 4 x 15 sec. Although these protocols were not a precise repeat of the regionally applied postC protocols (the very unsuccessful 3 x 20 second regional postC was replaced by a 4 x 15 second protocol), this gave us the opportunity to investigate more postC algorithms.

The baseline values for these series' are shown in table 4. All of the hearts' basic functional measurements fell within acceptable limits.

***Table 4**: Baseline functional parameters of hearts that underwent postC applied globally. As was the case in the regionally applied intervention,there is a single series of control hearts (NonPostC) that were exposed to the same procedure as the experimental groups.*

PostC protocol	N – value	Aortic output (ml/min)	Cardiac output (ml/min)
NonPostC	22	37.23 ± 0.89	52.50 ± 1.17
6 x 10 seconds	26	38.15 ± 1.16	53.85 ± 1.57
4 x 15 seconds	10	34.70 ± 1.90	49.75 ± 1.59
3 x 30 seconds	3	39.33 ± 3.71	55.33 ± 4.41

There was no significant recovery in aortic output or cardiac output in the 6 x 10 second and 4 x 15 second protocols (table 5).

***Table 5**: Post-ischaemic function, as well as functional recovery in hearts treated with 6 x 10 sec, 4 x 15 sec and 3 x 30 sec postC algorythms, applied by manipulating global flow to the hearts. AO: aortic output; CO: cardiac output. * P < 0.05 vs NonPostC.*

Protocol	Post-ischaemic AO (ml/min)	AO recovery (%)	Post-ischaemic CO (ml/min)	CO recovery (%)
NonPostC	6.34 ± 1.03	16.62 ± 2.71	19.05 ± 1.49	36.13 ± 2.59
6 x 10 sec	6.52 ± 0.91	16.95 ± 2.19	20.42 ± 1.37	37.80 ± 2.17
4 x 15 sec	7.80 ± 1.91	22.52 ± 5.44	20.34 ± 2.05	40.99 ± 4.14
3 x 30 sec	13.07 ± 3.73	32.47 ± 7.70	36.40 ± 4.57	65.30 ± 3.18*

There was however a significant increase in cardiac output in the 3 x 30 second postC hearts (NonPostC = 36.13 ± 2.59% vs 3 x 30 sec = 65.30 ± 3.18%), together with an increase in the recovery of coronary flow (NonPostC = 81.52 ± 3.84% vs 3 x 30 sec = 143.70 ± 18.71%) (figure 7). In the absence of aortic output recovery, it therefore seems plausible, that the observed CO recovery can be explained by the substantial increase in coronary flow.

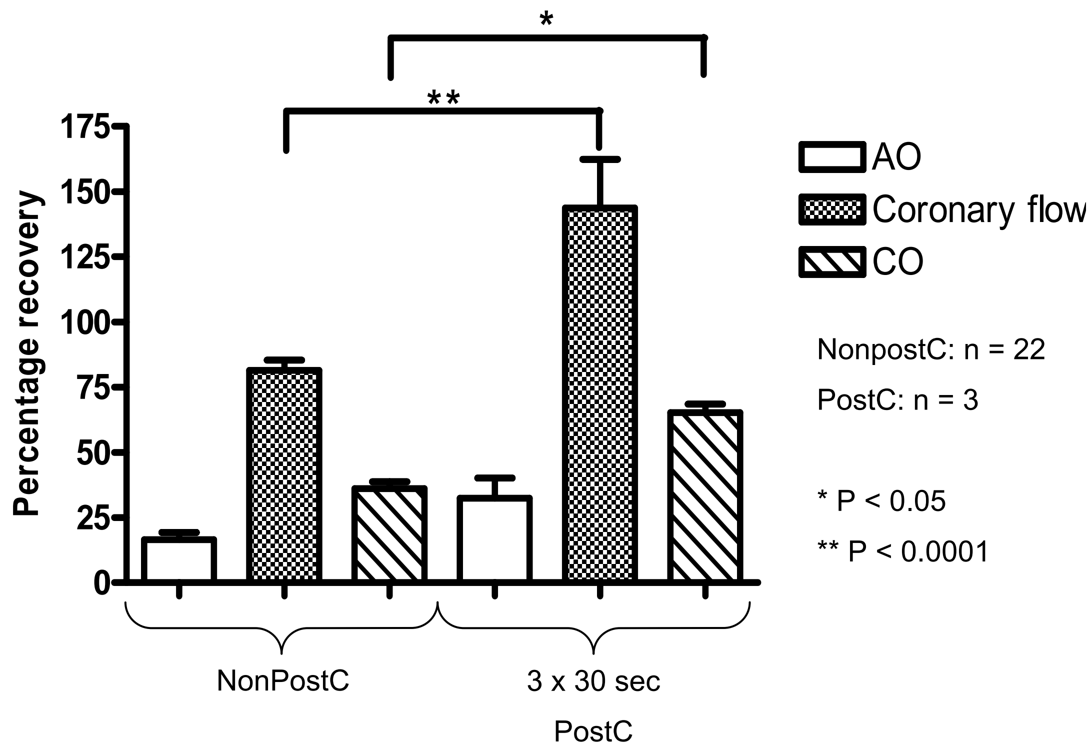


***Figure 7**: Histogram of percentage recovery of functional parameters of 3 x 30 second globally applied postC vs NonPostC hearts. There is a significant increase in the coronary flow and cardiac output after 35 minutes regional ischaemia in the postC hearts.*

No significant decrease in infarct size was associated with any of the different postC protocols (shown in figure 8; NonPostC = 27.84 ± 2.36% vs 6 x 10 sec = 27.03 ± 2.27%; 4 x 15 sec = 33.31 ± 4.26%; 3 x 30 sec = 25.28 ± 10.39%). In all four experimental groups area at risk size was similar (NonPostC = 48.65 ± 1.66%; 6 x 10 sec = 48.55 ± 3.00%; 4 x 15 sec = 48.17 ± 2.72%; and 3 x 30 sec = 44.56 ± 1.13%).

Despite the global application of the postC intervention (with its technical and thermal advantages), the postC protocols tested could not elicit any form of cardioprotection. This meant that another parameter, besides algorythm and method of application, had to be considered in the postC protocol.

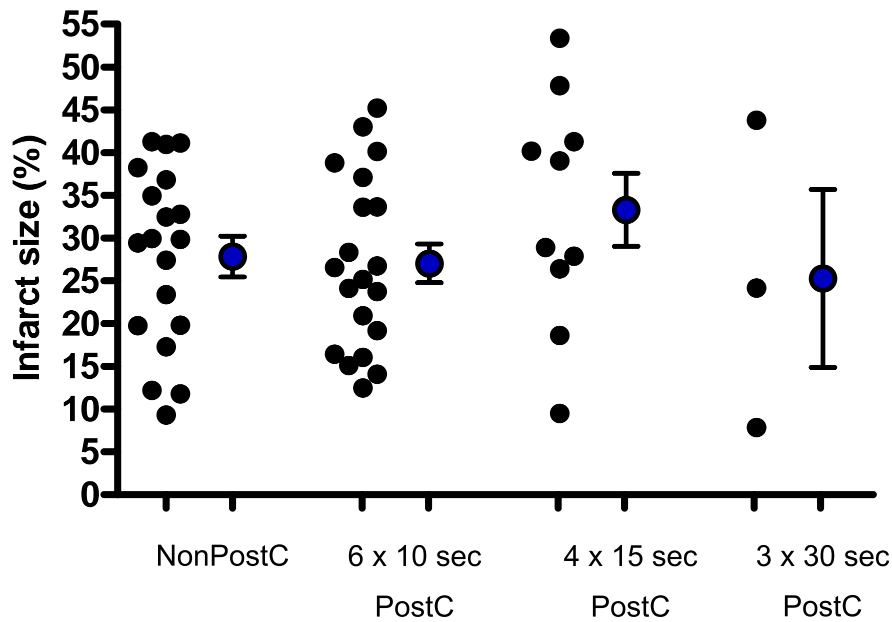


Figure 8*: Infarct sizes associated with various postC protocols, applied via global flow regulation. There were no differences in infarct size between NonPostC, 6 x 10 second, 4 x 15 second and 3 x 30 second postC groups.*

Because of its predominance in the literature we decided to pursue the 6 x 10 second postC protocol. Because of practical considerations, we decided to try and implement this protocol by manipulating global flow to the hearts. As illustrated in figure 8, this protocol had not been showing any success up until now. We therefore slightly changed the original experimental protocol by increasing the temperature of the hearts during ischaemia and the first minutes of reperfusion (to approximately 36.5 °C – 37.3 °C). Special attention was given to maintaining this elevated temperature during the postC protocol itself. This change meant that a new control series, under the same thermal regulation, had to be included. There were no significant increases in functional recovery in the NonPostC (n = 11; AO = 39.18 ± 2.91 ml/min; CO = 54.91 ± 3.07 ml/min) vs PostC (n = 13; AO = 40.38 ± 1.97 ml/min; CO = 56.92 ± 2.50 ml/min) group. Post-ischaemic function and percentage recovery from baseline values are shown in table 6.

***Table 6**: Post-ischaemic functional data and percentage recovery in NonPostC and 6 x 10 sec postC, globally applied and under strenuous thermal regulation. This postC protocol could not elicit functional recovery. AO: aortic output; CO: cardiac output.*

Protocol	Post-ischaemic AO (ml/min)	AO recovery (%)	Post-ischaemic CO (ml/min)	CO recovery (%)
NonPostC	4.91 ± 1.84	11.89 ± 4.39	19.55 ± 3.48	35.95 ± 5.97
6 x 10 sec	2.65 ± 1.24	5.82 ± 2.58	20.03 ± 2.19	35.26 ± 3.72

Despite the inability of the protocol to conserve post-ischaemic function, it did elicit an infarct sparing effect (figure 9; PostC = 17.74 ± 2.72% compared to NonPostC = 35.81 ± 3.67%). Both groups had similar area at risk sizes (PostC = 52.24 ± 2.38 and NonPostC = 48.08 ±2.96%).

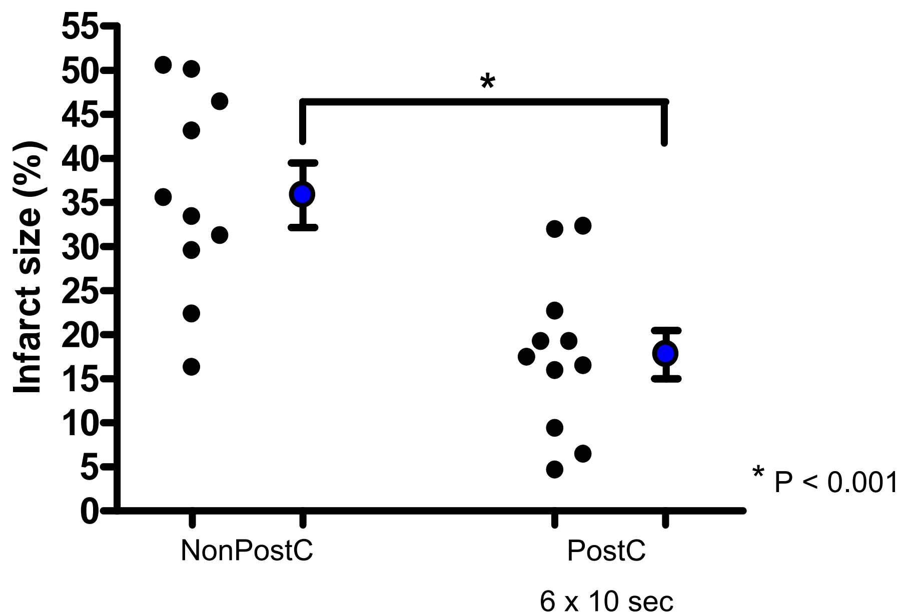


***Figure 9**: Infarct size reduction with the application of a 6 x 10 sec global postC protocol, at a temperature of 36.5 °C – 37.3 °C.*

In order to investigate the possible effect of temperature on postC efficacy the temperature of the hearts were measured regularly throughout ischaemia (every 5 minutes) and during the first 10 minutes of reperfusion (every 2 minutes). The measured temperature profile is

depicted in figure 10. The “Unregulated” – group refers to hearts that were allowed to remain at cooler temperatures, while it was attempted to maintain the “Regulated” – group at an elevated temperature during ischaemia and especially reperfusion.

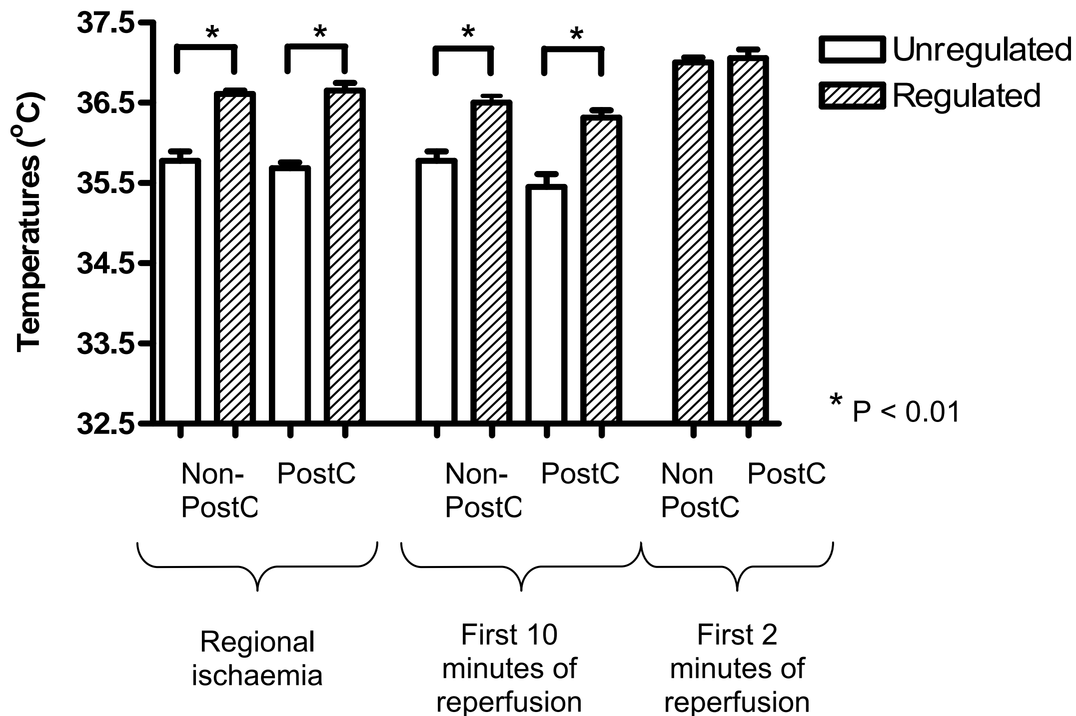


Figure 10: *Temperature profiling of NonPostC hearts, or hearts exposed to 6 x 10 sec global reperfusion / ischaemia at the onset of reperfusion. PostC hearts that showed a decrease in infarct size (the “Regulated” group) were in fact exposed to significantly higher temperatures than thermally “Unregulated” postC hearts. Especially the first 2 minutes of reperfusion (postC time) was very warm in the thermally regulated groups. Unfortunatley, no thermal data for the first 2 minutes of reperfusion was collected in the “Unregulated” hearts.*

3.3.2. The retrogradely perfused Langendorff model

3.3.2.1. Global ischaemia

In the retrogardely perfused (Langendorff) model two different ischaemic insults were utilised (fig. 11) : a 30 minute period of global ischaemia (with only functional recovery as end-point) and 35 minutes of global or regional ischaemia (with infarct size as an additional end-point). In this model, only the 6 x 10 second reperfusion / global ischaemia protocol was applied as a postC intervention. Temperature was maintained at approximately 37 °C throughout the whole duration of the experiment. As was the case in the working heart model, special attention was given to maintain temperature during the postC protocol. The same reperfusion temperatures were also maintained in the control (NonPostC) hearts.

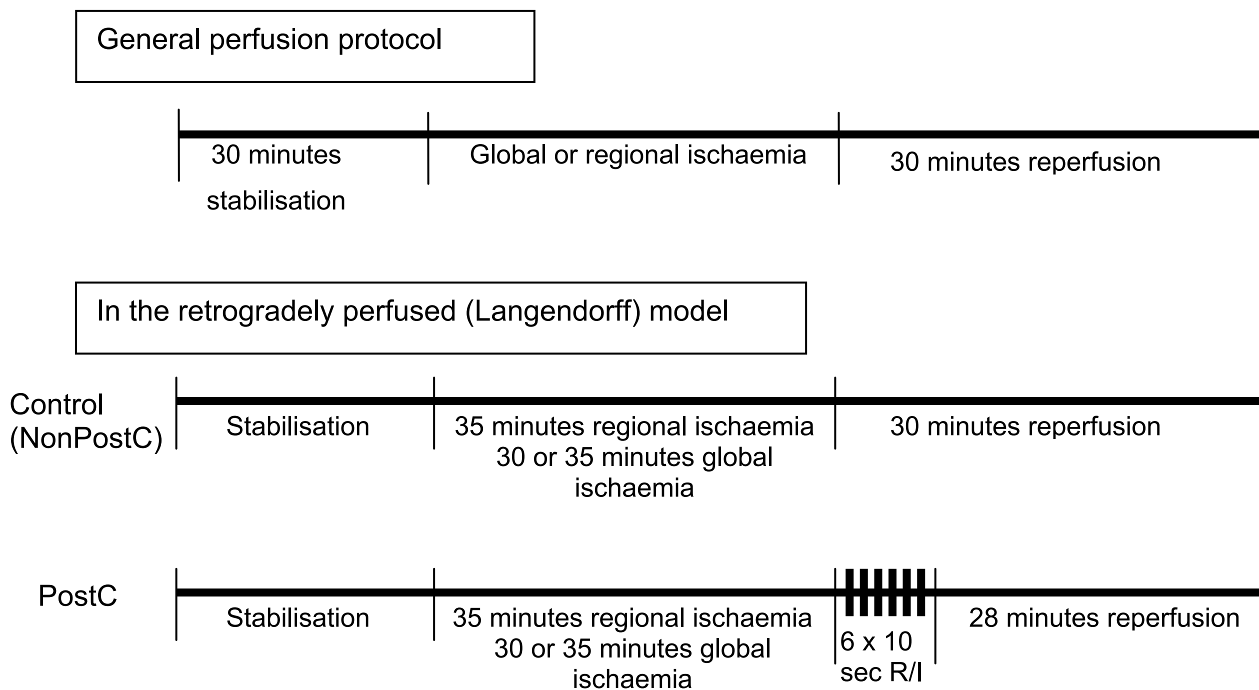


Figure 11: *Experimental protocols applied in the retrogradely perfused Langendorff model. Functional recovery was evaluated after 30 and 35 minutes of global ischaemia. Infarct size and functional recovery was analyzed after 35 minutes regional ischaemia. Only one postC protocol was evaluated: 6 x 10 sec globally applied reperfusion and ischaemia. Temperature was maintained throughout at 37 °C.*

Two different parameters, left ventricular developed pressure (LVDP) and rate pressure product (RPP; i.e. the product of the heart rate and the LVDP), were used to assess functional recovery in the presence or absence of postconditioning. Functional data in NonPostC and PostC hearts, before and after global ischaemia (30 minutes of 35 minutes), are shown in table 7.

***Table 7**: Functional data recorded in hearts exposed to a 6 x 10 sec postC intervention, as well as control (NonPostC) hearts, before and after 30 minutes and 35 minutes global ischaemia. LVDP: Left ventricular developed pressure; RPP: Rate Pressure Product; GI: global ischaemia. For 30 minutes GI: NonPostC: n=18; PostC: n=13. For 35 minutes GI: NonPostC: n=7; PostC: n=7.*

** P < 0.01, NonPostC vs postC after 30 minutes global ischaemia.*

Intervention		Baseline		Post-ischaemic	
		LVDP (mmHg)	RPP	LVDP (mmHg)	RPP
30 min GI	NonPostC	98.50 ± 3.30	33850 ± 1084	15.33 ± 3.32	4119 ± 911.8
	6 x 10 sec	106.0 ± 3.67	35590 ± 1199	28.69 ± 2.42*	8646 ± 842.1*
35 min GI	NonPostC	111.4 ± 8.09	38917 ± 3751	31.00 ± 7.42	12418 ± 3354
	6 x 10 sec	98.71 ± 6.69	33924 ± 2875	48.14 ± 4.73	15870 ± 1469

Hearts exposed to a 30 minute period of global ischaemia, followed by a 6 x 10 seconds postC protocol showed a significant recovery in RPP (fig. 12), compared to NonPostC hearts (PostC = 24.61 ± 2.53% vs NonPostC = 12.27 ± 2.63%). It is noteworthy that there is also a striking (although not significant) increased LVDP recovery in postC vs NonPostC hearts (27.80 ± 2.96% vs 15.64 ± 3.31%). In absolute values, hearts exposed to a postC protocol after 30 minutes global ischaemia was associated with significantly elevated LVDP (NonPostC: 15.33 ± 3.32 mmHg vs postC: 28.69 ± 2.42 mmHg) and RPP (NonPostC: 4119 ± 911.8 vs postC: 8646 ± 842.1) values compared to NonPostC hearts (table 7).

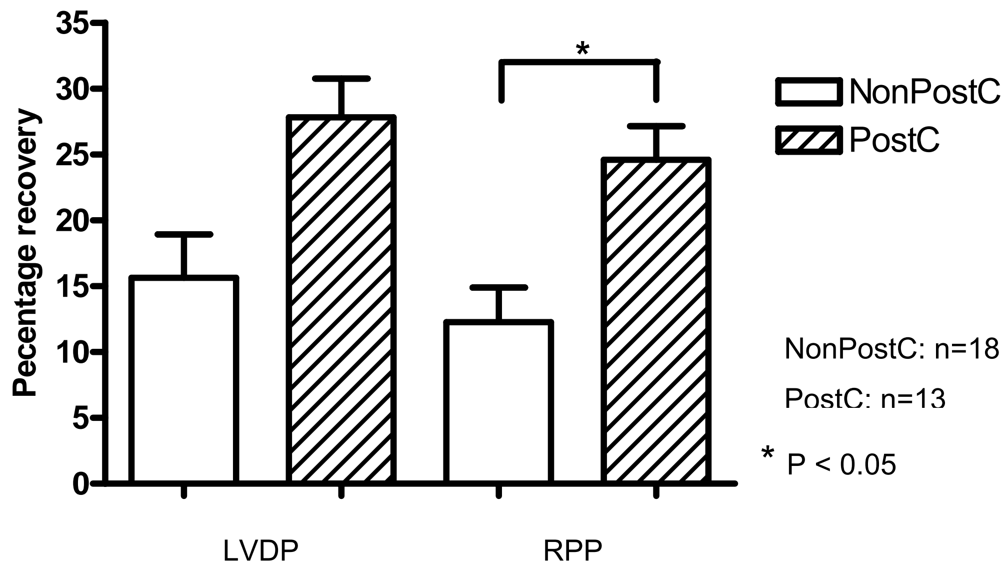


***Figure 12**: Functional recovery after 30 minutes global ischaemia, as expressed in terms of left ventricular developed pressure (LVDP) and rate pressure product (RPP) in Langendorff model hearts treated with a 6 x 10 second globally applied postC protocol.*

Interestingly, in hearts exposed to 35 minutes global ischaemia (fig. 13), LVDP recovery was significantly higher in postC (48.49 ± 3.14%) than NonPostC (28.40 ± 7.02%) hearts, while the difference in RPP recovery, although noticeable, was not significant (PostC = 47.64 ± 4.28% vs NonPostC = 31.35 ± 7.12%). Care was taken to maintain the temperatures of the hearts during ischaemia (NonPostC: 36.94 ± 0.05 °C; PostC: 37.00 ± 0.04°C) and the first 10 minutes of reperfusion (NonPostC: 36.98 ± 0.05 °C; PostC: 36.74 ± 0.12 °C).

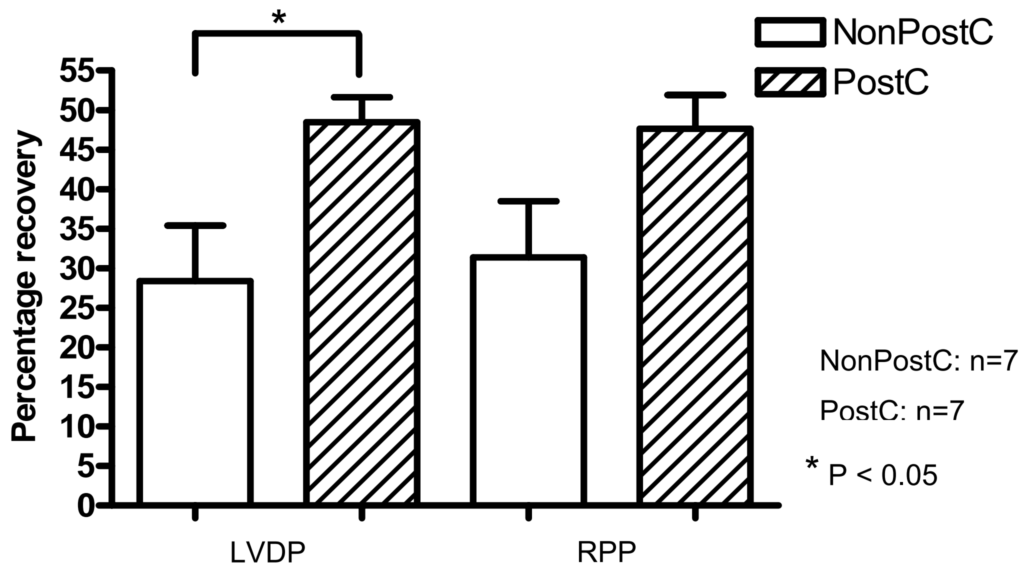


***Figure 13**: Functional recovery after 35 minutes of global ischaemia in hearts treated with postC (6 x 10 seconds) and nonPostC hearts, in the Langendorff model. Although there is a notable RPP recovery in postC hearts, it is only LVDP that shows significant recovery.*

3.3.2.2. Regional ischaemia

As was the case in the working heart model, we also decided to broaden our investigation in the Langendorff model to include infarct size analysis. We used our findings from the working heart model to guide our experimental approach in the Langendorff model. We therefore used the protocol which showed postC-mediated cardioprotection in the working heart model, in the Langendorff model. Hearts were subjected to 35 minutes regional ischaemia, followed by 30 minutes reperfusion. PostC entailed 6 x 10 seconds reperfusion / ischaemia at the onset of reperfusion. Temperature was monitored and maintained at approximately 37 °C throughout ischaemia and reperfusion.

This experimental protocol elicited a significant reduction infarct size in postC (27.81 ± 2.49%) compared to NonPostC (47.99 ± 3.31%) hearts (fig.14). Both the postC and NonPostC groups presented with similar area at risk sizes (postC = 47.10 ± 1.92% and NonPostC = 47.85 ± 2.80%).

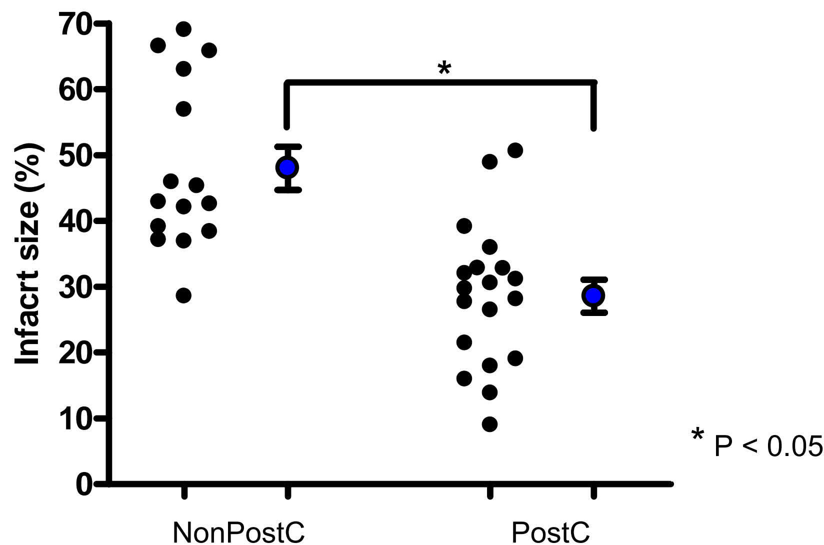


Figure 14*: In the Langendorff model, a postC protocol of 6 x 10 seconds global reperfusion / ischaemia was associated with a significant decrease in infarct size after 35 minutes of regional ischaemia.*

Functional parameters measured in both groups are shown in table 8. As was obserevd in the working heart model, postC applied after a regional ischaemic insult was not associated with increased recovery of any of the functional parameters (NonPostC: LVDP = 78.76 ± 2.46%, RPP = 76.78 ± 1.86% compared to PostC: LVDP = 83.31 ± 2.66%, RPP = 83.41 ± 2.75%).

Table 8*: Functional parameters measured in NonPostC and PostC (6 x 10 sec global reperfusion / ischaemia cycles) hearts exposed to 35 minutes of regional ischaemia. PostC was not associated with a significant increase in post-ischaemic function. LVDP: Left ventricular developed pressure; RPP: Rate Pressure Product; RI: regional ischaemia. NonPostC: n=17; PostC: n=24.*

Intervention		Baseline		Post-ischaemic	
		LVDP (mmHg)	RPP	LVDP (mmHg)	RPP
30 min RI	NonPostC	101.8 ± 3.54	33722 ± 1305	80.88 ± 2.70	26271 ± 1090
	6 x 10 sec	99.21 ± 2.86	31174 ± 876.8	81.88 ± 2.59	25876 ± 998.3

3.4. Discussion

3.4.1. Working heart model

3.4.1.1. Global ischaemia: Functional recovery

In our initial attempts to develop a postC protocol that would elicit significant functional recovery in the working heart model, the same postC protocol was applied, but after different periods of global ischaemia (20 or 25 minutes). We chose a widely used postC protocol of 6 x 10 seconds reperfusion / ischaemia (Kin *et al.*, 2004; Penna *et al.*; 2006; and Wang *et al.*, 2007), which was applied by regulating the flow of perfusate to the heart as a whole. In both these experimental groups the postC intervention failed to elicit a significant recovery in function, as assessed by aortic output and cardiac output (figures 2 and 3).

At the time when this study was initiated, no other group had published data on postconditioning using the working heart model. Since then one group has used this particular model, but in a very unorthodox manner, compared to the postC experiments described in the retrogradely perfused Langendorff model. The model described by Sasaki *et al.* (2007) differ from ours in several respects: They perfused hearts at an afterload of 80 cmH_2O, while ours worked at a higher pressure of 100 cmH_2O. Both models shared the same preload of 15 cmH_2O. They applied 15 minutes of global ischaemia, while pacing their hearts at 330 beats per minute, which makes their model more applicable to study stunning, rather than irreversible myocardial damage. We chose 20 and 25 minutes of ischaemia, since previous work in our lab has shown that both these insults were associated with functional recovery in IPC hearts (Lochner *et al.*, 2003). Perhaps the most important difference between our experimental setup and that of Sasaki *et al.* (2007) is the postC protocol itself: they applied a unique postconditioning protocol of 1 minute reperfusion, followed by 5 minutes of global ischaemia. Although this protocol could reduce reperfusion arrythmias, it was not associated with functional recovery or a decrease in creatine kinase (CK) release – all of which indicate the limited cardioprotective ability of this protocol. Thus, regardless of the differences in postC protocol, it would seem that functional recovery is not a reliable indicator of cardioprotection in postC.

3.4.1.2. Regional ischaemia: Infarct size and functional recovery

In light of the failure of postC to elicit functional recovery after a global ischaemic insult in our working heart model, it was decided to focus on the reported infarct sparing effect of postconditioning. Since it has been widely reported that postconditioning does reduce infarct size, we pursued this end-point quite vigorously by testing various experimental conditions and postC protocols in our working heart model. Three parameters were taken into consideration: the method of postC intervention, the number of cycles applied and the temperature during this intervention. A period of 35 minutes regional ischaemia was used in all experiments. This ischaemic insult yields an infarct size of 30 – 60% of the area at risk (Lochner *et al.*, 2003).

- **The method of postC intervention:** This refers to how the reperfusion and ischaemia cycles were applied – either by manipulating the suture itself, and therefore the regional flow to the area at risk; or by manipulating the flow of perfusate to the whole heart. We applied three different postC protocols by manipulating the regional flow: 6 x 10 sec, 3 x 20 sec and 3 x 30 sec. None of these postC protocols elicited either a decrease in infarct size (figures 5 & 6), or an increase in functional recovery. The 6 x 10 sec protocol however did seem to show a trend toward a decrease in infarct size (PostC: 24.12 ± 5.22% vs NonPostC: 31.01 ± 3.26%), although the relatively wide distribution of postC data points precluded significant difference.

  Three protocols were also applied by manipulating global flow: 6 x 10 sec, 4 x 15 sec and 3 x 30 sec (fig. 8). None of these protocols were associated with a significant decrease in infarct size either. Admittedly, more hearts could have been done in the 3 x 30 sec group, but since our previous results in this particular model (using regional manipulation) were not encouraging either, we decided to abandon this protocol. There was however, a significant increase in the cardiac output of postC hearts, in the 3 x 30 sec group (fig. 7). This is probably not due to actual functional recovery, but rather to an increase in coronary flow. Indeed, there was a significant increase in coronary flow in postC hearts, in the absence of aortic output recovery. In our hands coronary flow regularly shows an increase after regional ischaemia (data not shown), due to physical tearing of the heart tissue by the suture.

  Although altering the mode of reperfusion / ischaemia induction failed to offer a protective postC protocol, we did learn that technically it was easier to apply a global reperfusion / ischaemia regime, than a regional one. There are primarily two reasons

for this: Firstly, repeated regional manipulation presents the risk that the heart or coronary artery might be damaged or torn during the postC protocol. Secondly, thermal regulation is much easier in the global model, since the heart is maintained in a buffer-filled waterjacket cylinder. With regional manipulation the hearts are exposed to the ambient temperature for the duration of the postC intervention.

- **The number of cycles applied:** Two protocols have been shown to be particularly successful in the rat heart: either a 6 x 10 second protocol (Kin *et al.*, 2004; Wang *et al.*, 2007; and Penna *et al.*; 2006), or a 3 x 30 second protocol (Bopassa *et al.*, 2006; and Manintveld *et al.*, 2007). We therefore focused our study on protocols within these limits, i.e. 6 x 10 sec, 4 x 15 sec, 3 x 20 sec and 3 x 30 sec. Another possibly important parameter to take into account is the total duration of the postC protocol (as speculated by Iliodromitis *et al.*, 2006). In this regard out postC protocol also fell within the published limits of either 2 minutes or 3 minutes. Despite the fact that our experiments mimicked those described by others, we failed to repeat their results (placing us in the same company as Dow & Kloner, 2007; and Kaljusta *et al.*, 2007). Despite the absence of functional recovery or reduction in infarct size associated with any of the protocols, it did seem as if the 6 x 10 sec protocol was the most promising. As already mentioned it decreased IFS when applied regionally (fig. 5), while it lead to a very wide distribution of infarct sizes in the global approach (fig. 8).
- **Temperature during ischaemia and reperfusion:** In view of the above, we decided to focus our search for an infarct sparing postC protocol in the working heart model on the 6 x 10 sec globally applied reperfusion / ischaemia protocol. Although this protocol had not been associated with a decrease in infarct size in our previous experiments (postC: 27.03 ± 2.27% vs NonPostC: 27.84 ± 2.36%), we decided to test it again – but with a slight elevation in temperature during the postconditioning protocol (from approximately 36.5 °C to between 36.5 °C and 37.3°C). This required a more strenuous regulation and monitoring of the temperature, than had been employed up until this point. Surprisingly, we now found that the postC protocol was associated with a significant reduction in infarct size (fig. 8; postC: 17.74 ± 2.72% vs NonPostC: 35.81 ± 3.67%). This decrease in infarct size was however still not associated with improved functional recovery.

Analysis of the temperature data that was collected showed that in the cardioprotective postC protocol there was a significant increase in temperature, compared to the previous postC protocol, during ischaemia (36.32 ± 0.09 °C vs 35.45 ± 0.16 °C), as well

as the first 10 minutes reperfusion (36.65 ± 0.1 °C vs 35.68 ± 0.07 °C). The question is whether an increase of approximately 1 °C during ischaemia / reperfusion could really be responsible for the observed changes in infarct size? Work that has been done on the effect of temperature in ischaemia / reperfusion has focussed on much larger alterations in temperature, in the setting of the protective effect of hypothermia. For example, Riess *et al.* (2004) found that perfusion and ischaemia of guinea pig hearts at 17 °C was associated with a decrease in infarct size. Hypothermia (in the order of 29 °C), before and during hypoxia, was also associated with the preservation of myocardial function in rabbits (Ning *et al.*, 2003). Another demonstration of the protection afforded by a slight decrease in temperature is given by Hale *et al.* (1997). They found that a reduction of 6 °C during ischaemia can elicit an infarct sparing effect, although such a degree of hypothermia 5 minutes before reperfusion had no cardioprotective effect. Schwartz *et al.* (1997) showed that even slight fluctuations in ischaemic epicardial temperature (35.5 – 41 °C) exerted a major effect on infarct size in canine hearts. These authors observed a positive correlation between infarct size and epicardial temperature. It is therefore clear that the myocardium is sensitive to fluctuations in temperature, but if it is sensitive to fluctuations of less than 1 °C seems to be unknown. All these studies also indicated a protective role for hypothermia, while in our hands upregulation of temperature during the postC protocol seemed to be a prerequisite for protection. We could only speculate that, if postC is an active process as suggested by Tsang *et al.* (2004) and Bopassa *et al.* (2006), the slight increase in temperature might favour increased protective enzymatic processes in the myocytes. It might also be possible that the answer lies in the temperature fluctuations during the ischaemia / reperfusion cycles. Recently Khaliulin *et al.* (2007) reported the existence of temperature preconditioning, in which three cycles of hypothermia (26 °C) prior to sustained ischaemia elicits a even more potent cardioprotective effect than ischaemic preconditioning.

Further research is required to elucidate the role of temperature in postC efficacy. These studies could entail the comparison of the protective effects of postC at defined different temperatures. Further research would probably be dependent on computerized continuous temperature measurements throughout the experiments, as well as very stable temperature regulation.

Despite the absence of an adequate explanation, we had now for the first time, established a postC protocol that could elicit an infarct sparing effect in the working heart model.

3.4.2. Retrogradely perfused Langendorff model

3.4.2.1. Global ischaemia: functional recovery

To further investigate the effect of postC on functional recovery, we decided to also investigate postC in the retrogradely perfused heart model. Previous work in our lab had shown the Langendorff model to be resilient enough to show functional recovery even after 35 minutes global ischaemia, although elevated functional recovery in preconditioned hearts were only observed after 30 minutes global ischaemia (Lochner *et al.*, 2003). In this model we tested the effects of the postC protocol, that we had found to be cardioprotective in the working heart model. As was the case in the working heart model, two different global ischaemic insults (30 and 35 minutes) were tested to determine the ability of our thermally regulated, globally applied 6 x 10 sec postC protocol to elicit functional recovery.

In our study we found that postC was associated with a significant increase in functional recovery after both 30 and 35 minutes global ischaemia. Interestingly, after 30 minutes GI the RPP was significantly increased in postC hearts (fig. 12), while after 35 minutes GI only LVDP showed significant recovery (fig. 13) in the absence of a difference in heart rate recovery values (data not shown). Since RPP (the product of both heart rate and LVDP) could be considered more representative of functional performance, these differences might be due to the severity of the ischaemic insult. In fact, postC after 30 minutes global ischaemia was associated with a significant increase in heart rate recovery (NonPostC: 54.19 ± 9.90% vs postC: 88.64 ± 3.32%), with post-ischaemic heart rate values within acceptable limits (in beats per minute: NonPostC: 187.2 ± 35.05 bpm; postC: 297.5 ± 10.73 bpm).

Although we demonstrated functional recovery after postconditioning in the Langendorff model, it should be noted that no functional recovery was observed in later experiments (data not shown) to determine the kinase profiles associated with postC (described in chapter 4). This emphasises the unreproducibility of the protective effects attributed to postC in the rat, specifically its ability to salvage function. It is noteworthy that in their review on postconditioning, Zhao & Vinten-Johansen (2006) did not even mention functional recovery as a possible end-point to investigate. However, several others assessed functional recovery associated with postconditioning, in the retrogradely

perfused Langendorff model, and showed improvement (Bopassa *et al.*, 2006; Crisostoma *et al.*, 2006; and Wang *et al.*, 2007). In the study by Bopassa *et al.* (2006) they applied the unique protocol of 1 minute reflow followed by 3 x 30 sec ischaemia / reperfusion – which induced a significant recovery in function after 30 minutes of global ischaemia. Both Crisostoma *et al.* (2006) and Wang *et al.* (2007) found that a postC protocol similar to the one we applied, elicited an increase in functional recovery in rats after both 20 and 25 minutes (in male rats, according to Crisostoma *et al.*, 2006) and 30 minutes (Wang *et al.*, 2007) global ischaemia. Many researchers however did not attempt to use functional recovery as an end-point, while others were unable to demonstrate postC-related recovery in function. Couvreur *et al.* (2006) could not show that postC protected against stunning after 10 minutes ischaemia in both the *in vivo* canine and rabbit hearts. In the isolated rabbit heart Darling and coworkers (2005) could also not show postC – induced functional recovery.

A question which does arise is why the same postC protocol could elicit functional recovery in the Langendorff model, but not in the working heart? Possible explanations are discussed in the comparative study between the two models in the setting of IPC (Lochner *et al.*, 2003). Two very plausible explanations concern the oxygen utilisation in the working heart model. The working rat heart has to function against a preset preload and afterload; extra stress which the Langendorff model does not have to contend with. It could therefore be that the working heart's oxygen demand exceeds its supply during reperfusion, undermining function. It has also been shown that the working heart generates significantly more free radicals during reperfusion than the Langendorff model – possibly due to an increase in oxygen consumption (Damerou *et al.*, 1993). This increased oxidative stress could also influence post-ischaemic function. Despite these explanations it is still worrisome that postC could not increase post-ischaemic function in the working heart, in contrast to ischaemic preconditioning (Lochner *et al.*, 2003). It could be that ischaemic preconditioning elicits a more potent cardioprotection effect than postconditioning, an observation also made by Kin *et al.* (2004). This was also reflected by the more outspoken reduction in infarct size induced by preconditioning (discussed in the next section).

3.4.2.2. Regional ischaemia: infarct size and functional recovery

In these experiments we did not test any variations in the postC protocol, rather we attempted to investigate the infarct-sparing effect of the postC protocol, that we had developed in the working heart model, in the Langendorff model. As described in the

working heart model, hearts were exposed to 35 minutes regional ischaemia, followed by 6 x 10 second reperfusion / ischaemia cycles, applied globally. Temperature was monitored and regulated during ischaemia and reperfusion, at the same level as in the working heart setup. Results mimicked those found in the working heart model. The postC protocol exerted an infarct – sparing effect (fig. 14), without any significant functional recovery.

Comparison of the infarct sparing effect of our postC protocol in the working heart model and the Langendorff model reveals two interesting differences. In the working heart model our postC protocol decreased infarct size by 50.46%, which is more than the 40.49% reduction in the Langendorff model. We also see a significantly higher IFS, for a given intervention, in the Langendorff compared to the working heart model (Langendorff postC: 28.56 ± 2.50% vs work heart postC: 17.74 ± 2.72%, $p < 0.05$; and Langendorff NonPostC: 47.99 ± 3.31% vs work NonPostC: 35.81 ± 3.67%, $p < 0.05$; also depicted in figure 15).

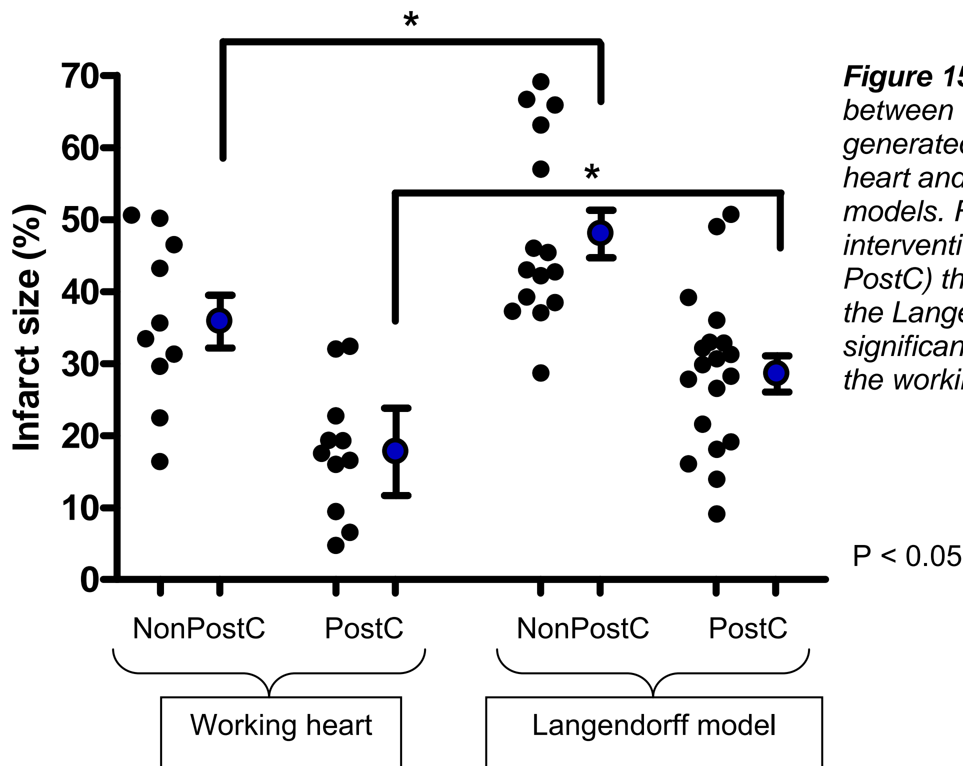


***Figure 15**: Comparison between the infarct sizes generated in the working heart and Langendorff models. For a given intervention (NonPostC or PostC) the infarct sizes in the Langendorff model are significantly higher than in the working heart model.*

These observed differences might be due to the intrinsic differences between the two models. As already discussed: the working heart model is exposed to different stressors

and energy demands than the Langendorff model. Nonetheless, our postC protocol was associated with a significant reduction in infarct size in both models.

The first group to report on the infarct sparing effect of postC in the rat was Kin *et al.* (2004), who tested two postconditioning protocols, 3 and 6 cycles of 10 seconds reperfusion / ischaemia, both of which conferred the same degree of reduction in infarct size. Interestingly, these authors reported that although the decrease was significant compared to control hearts, the infarct sizes were still significantly greater than in the presence of ischaemic preconditioning. More specifically: both postconditioning protocols decreased infarct size from approximately 50% in control hearts to 40%, in contrast to IPC wich reduced infarct size to approximately 20% of the area at risk. Similiarly Tang and colleagues (2006), found that although postC could reduce infarct size significantly (6 x 10 sec: 34% smaller than control; and 20 x 10 sec: 47% reduction), this protection was not as potent as that imparted by preconditioning (both in the early and late phases, which reduced IFS by 72% and 70% respectively). Using a 3 x 30 sec postC protocol, Manintveld and coworkers (2007) also reported that postC reduces IFS after 45 or 60 minutes of ischaemia, but by less than 50%. In contrast to these reports and maybe more similar to our results, Penna *et al.* (2006) found that a postC protocol similar to ours reduced infarct size from 65 ± 4% to 22 ± 4% (in constant-flow perfused hearts) and from 59 ± 5% to 46 ± 2% (in constant-pressure perfused hearts).

In the light of the above discussed results by other groups, it seems as if postC protection, although present, is not very potent (especially compared to the protection associated with IPC). In fact, it seems that in our model, postC exerted quite a strong infarct sparing effect compared to other studies.

This observed relatively potent infarct sparing effect in our postC model , however now raises another question: Why could we not demonstrate a stronger effect on functional recovery? In fact, in the working heart model which showed the greatest extent of infarct reduction, we did not find any functional recovery. This observed lack of the expected direct relationship between functional performance and infarct size has been made by other researchers as well (Cohen *et al.*, 1999; and Lochner *et al.*, 2003). Three possible explanations have been put forward to explain this dissociation. The phenomenon of stunning (discussed in chapter 1) following ischaemia would transiently suppress functional ability, even in the presence of a small infarct. The precise effect of postC on stunning has not yet definitively been determined - Couvreur *et al.* (2006) found that postC

could not protect against stunning, while Kin *et al.* (2005) concluded that it is protective. Interestingly Cohen *et al.* (1999) found that IPC could elicit functional recovery – but only after 3 days of reperfusion. But even this observed functional recovery was not proportional to measured IFS. These authors also speculate that it could be that the salvaged myocardium (in the cardioprotective setting of IPC) is not functionally normal. Functional recovery could also be determined by the physical site of necrosis / tissue damage in the heart. Cohen *et al.* (1999) speculate that damage to the subendocardial fibers might have a deleterious effect on overall fiber shortening, thus undermining cardiac function.

3.4.3. Summary

During the course of developing a cardioprotective postconditioning protocol in the isolated rat heart we tested different possible postconditioning algorithms, which we applied by manipulating either global or regional flow to the heart. Temperature (during ischaemia and especially the first ten minutes of reperfusion) appears to be very important in the efficacy of the protocol.

We found that 6 cycles of 10 seconds reperfusion / ischaemia, applied by manipulating global flow to the heart, at the very onset of reperfusion and maintained at a target temperature of 36.6 °C – 37.3 °C, conferred significant cardioprotection.

This protocol was associated with a signifcant infarct sparing effect in both the working heart and retrogradely perfused models, after 35 minutes regional ischaemia. It could however not elicit functional recovery in the working heart model, although it was associated with functional recovery in the less strenuous Langendorff model.

To our knowledge we are the first group to show that postconditioning can be used as a reperfusion intervention to decrease infarct size in the isolated working rat heart. Its failure to elicit functional recovery in this experimental model, is however disappointing.

Chapter 4: Postconditioning: role of signalling kinases

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny…'"

Isaac Asimov

www.quotationspage.com

Chapter 4: Postconditioning: role of the signalling kinases

4.1. Background and motivation

Several possible mechanisms have been implicated in the mechanism of postC. In the intact animal some of the proposed mechanisms involve blood borne factors, such as neutrophils (Zhao *et al.*, 2003; Kin *et al.,* 2004; Mykytenko *et al.*, 2007). Postconditioning has however, also been demonstrated in the isolated heart system, implying a role for factors endogenous to myocardial tissue. Although various factors have been identified, the RISK (reperfusion injury salvage kinase) – pathway is especially noteworthy, since it seems this pathway is central in cardioprotection during reperfusion (particularly in IPC (Hausenloy *et al.*, 2005), for a review on RISK in ischaemia / reperfusion see Hausenloy & Yellon; 2004).

A number of researchers have shown that increased PI3-kinase activity is associated with postC cardioprotection (Yang *et al.*, 2004; Fujita *et al.*, 2007). A notable exception is Darling *et al.* (2005), who could not show a functional role for PI3-kinase activity, nor increased PKB/Akt phosphorylation associated with postC. Despite this, Darling *et al.* (2005), and other authors (Yang *et al.*, 2004; Fujita *et al.*, 2007), have found that ERK p42/p44 also seems to be involved in postC. However, Zhu *et al.*(2006) reported that ERK p42/p44 activity was not increased in postC. Schwartz & Lagranha (2006) reported the intruiging results that although they found increased phosphorylation of PKB/Akt and ERK p42/p44 in postC hearts, this phosphorylation was not associated with cardioprotection. Thus, some uncertainty still exists as to the precise role of the RISK-pathway in the cardioprotective mechanism of postC.

Another kinase of possible importance is p38 MAPK, since it has been shown to be involved in IPC (although its precise role is yet to be elucidated (Hausenloy & Yellon (2006)). Only one study by Sun and colleagues (2006) has shown a possible role for p38 MAPK in postconditioning. They found it to be decreased in neonatal cardiomyocytes exposed to a postC protocol.

In view of the existing literature, it seems possible that either PKB/Akt or ERK p42/p44 (or probably both) are involved in postC protection, in our isolated rat heart models. We therefore decided to investigate the precise roles of PKB/Akt and ERK p42/p44 in our model of postconditioning. We also aimed to investigate the possible role of p38 MAPK in our postC model, since there seems to be virtually no information regarding this kinase in postC cardioprotection.

4.2. Materials and methods

Hearts from male Wistar rats were cannulated and perfused with oxygenated Krebs-Henseleit buffer (see chapter 2). For this investigation we used both the working heart, as well as the retrogradely perfused Langendorff models. In view of our postC – development experiments (see chapter 3) we used the protocol which we had found was best associated with postC-mediated cardioprotection (i.e. reduced infarct size and increased functional recovery). In brief: hearts were exposed to 30 minutes stabilisation, 35 minutes ischaemia (regional or global, depending on the perfusion model) and maximum 30 minutes reperfusion. PostC was elicited by the application of 6 x 10 second global reperfusion / ischaemia cycles, at a temperature of between 36.5 and 37.3 °C.

In order to assess the role of the kinases in postC, two different lines of investigation were pursued:

1. In both perfusion models hearts were either exposed to a postC protocol, or conventional full and immediate reperfusion (NonPostC). Hearts were then freeze-clamped at different time points during reperfusion, and later analyzed, using Western blotting.
2. Following the above mentioned approach, the importance of the kinases that had been found to be phosphorylated in postC was further investigated by administering appropriate inhibitors during reperfusion.

The protocols used will be described in detail in the “Results” section. All protein analyses also included control hearts, i.e. hearts that only received 30 minutes of retrograde perfusion. Densitometry data from these hearts were used as reference for the normalization and comparison of data from the experimental groups (see chapter 2).

4.3. Results

4.3.1. The kinase profile associated with postC

This study was done in both working heart and Langendorff models. Hearts were exposed to 35 or 20 minutes of ischaemia (global or regional) and then freeze-clamped and plunged into liquid nitrogen at either 10 or 30 minutes reperfusion. The frozen tissue samples were then later analyzed using Western blotting techniques (as described previously), probing for p38 MAPK, ERK p42/p44 and PKB/Akt (in all cases antibodies were used for both total and phosphorylated protein). Data was analyzed as described in chapter 2.

4.3.1.1. The working heart model

The experimental protocols applied in the working heart model are shown in figure 16.

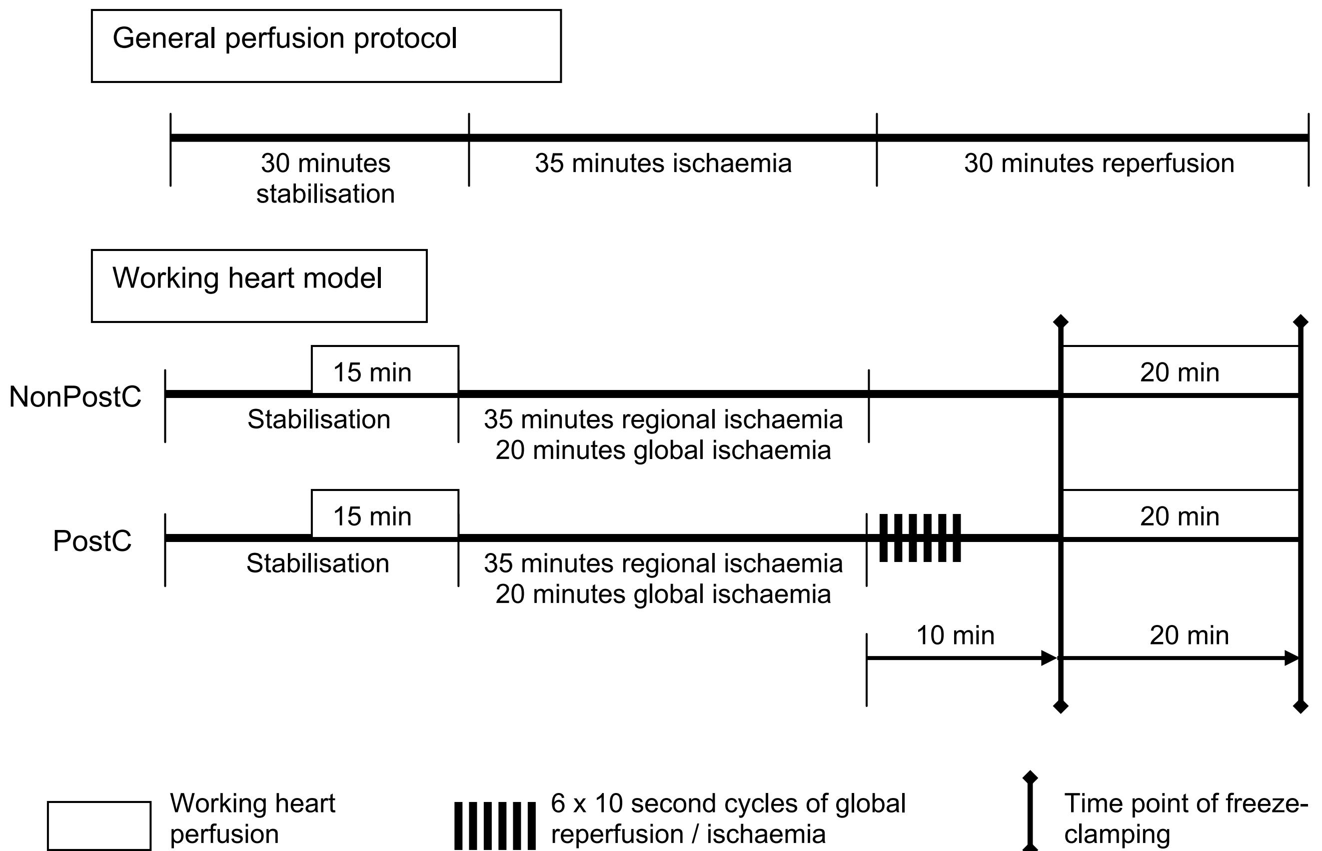


***Figure 16**: Perfusion protocols used to describe the expression and activation of p38 MAPK, ERK p42/p44 and PKB/Akt associated with postC at 10 minutes and 30 minutes reperfusion, in the working heart.*

In order to investigate the kinase profile in the working heart model at 30 minutes reperfusion, hearts were initially exposed to 20 minutes global ischaemia only. Whole hearts were then freeze-clamped after 30 minutes reperfusion and stored in liquid nitrogen.

In the PostC group 3 hearts were perfused (AO before ischaemia = 36.00 ± 2.31 ml/min; CO before ischaemia = 52.25 ± 3.62 ml/min). Three hearts were also analyzed in the NonPostC group (AO = 40.67 ± 3.71 ml/min; CO = 56.67 ± 4.89 ml/min). Total and phosphorylated protein profiles of the kinases under investigation are shown in figure 17. It is noticeable that reperfusion *per se* increased the phosphorylation of all the kinases investigated: p38 MAPK (approximately 2 fold in NonPostC and PostC hearts), PKB/Akt (just more than 3 fold in both groups) and ERK p42/p44 (especially ERK p44 with a ~2.5 fold increase, but also ERK p42 with a ~1.5 fold increase). At this stage in reperfusion there however seems to be no differences between the postC and NonPostC hearts, with regard to phosphorylated p38 MAPK, ERK p42/p44 and PKB/Akt. Similarly, the total protein content of each kinase evaluated did not differ between the two groups (fig. 17).

In view of these negative results, it was decided to investigate the kinases at 10 minutes reperfusion, after exposure to 35 minutes regional ischaemia. After 10 minutes reperfusion (in the presence and absence of postC), just before freeze-clamping, the snare (used for the induction of ischaemia) was firmly tied around the coronary artery. The ischaemic zone of the ventricle distal to the suture was then quickly dissected from the rest of the heart and freeze clamped. The rest of the heart was also rapidly freeze-clamped after dissection. Thus, ischaemic, as well as non-ischaemic tissue samples, were harvested from each heart. This was done to investigate possible differences between the ischaemic and non-ischaemic samples, as well as to avoid a possible confounding influence of the non-ischaemic tissue on the ischaemic tissue. Functional baseline values for the hearts used in this investigation are shown in table 9. Since we found that slightly elevated temperatures during ischaemia and early in reperfusion are important in the infarct sparing effect of postC, care was taken to regulate the temperature of these hearts (NonPostC: RI temperature = 36.60 ± 0.03 °C; reperfusion temperature = 36.88 ± 0.04 °C, and PostC: RI temperature = 36.66 ± 0.02 °C; reperfusion temperature = 36.88 ± 0.05 °C).

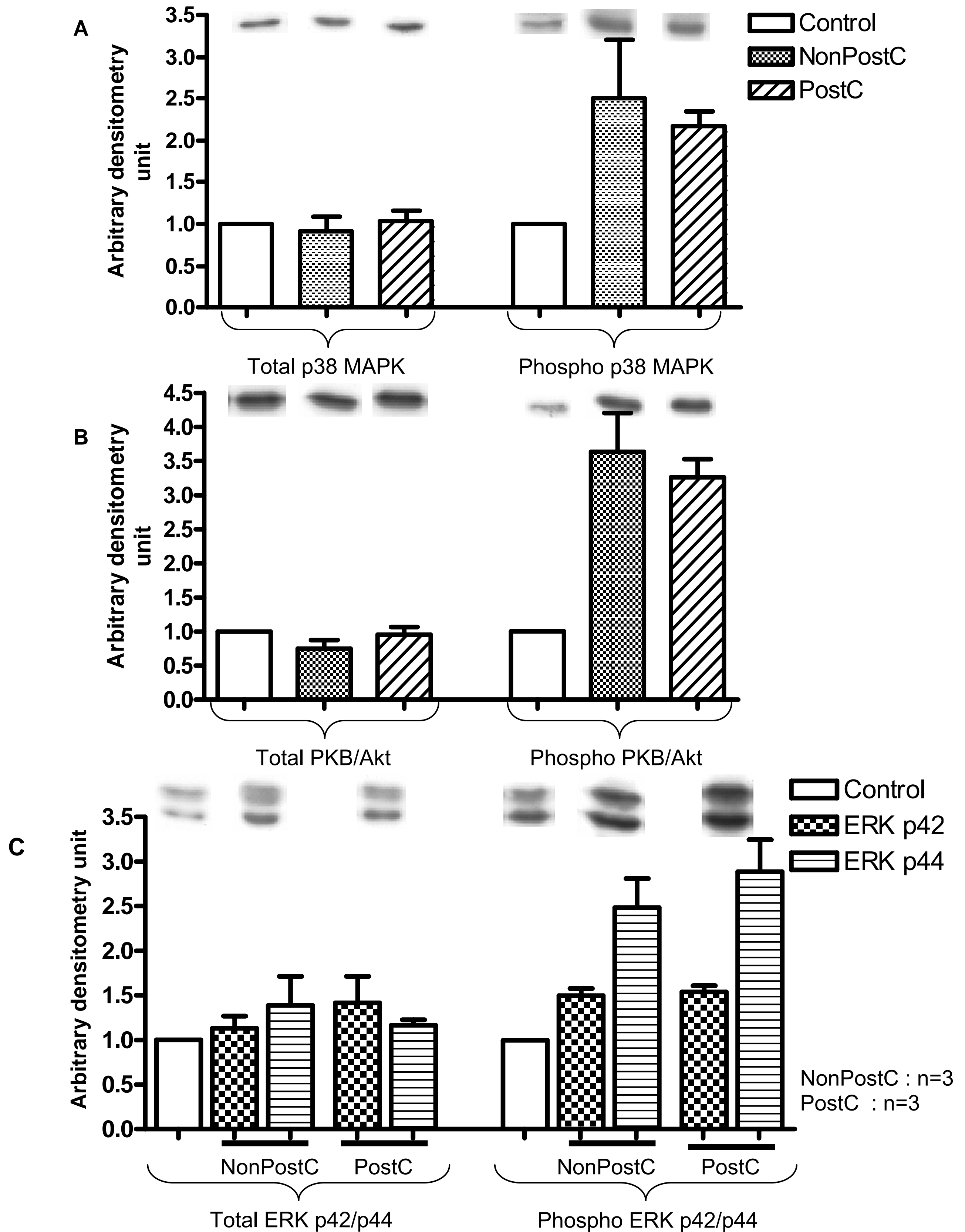


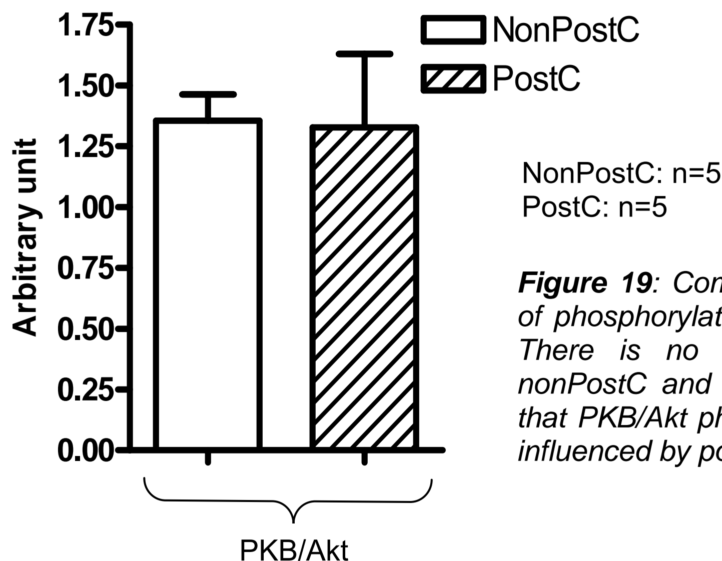
Figure 17*: Total and phosphorylated profiles of p38 MAPK (A), PKB/Akt (B) and ERK p42/p44(C) at 30 minutes reperfusion, after 20 minutes global ischaemia, in the working heart model. There were no significant differences in total protein levels or activity (as assessed by phosphorylation) between postC and NonPostC groups. Control: n=2.*

***Table 9**: Baseline functional performance of hearts used to analyze the kinases at 10 minutes reperfusion, in the presence or absence of postC, in the working heart model.*

PostC protocol	N – value	Aortic output (ml/min)	Cardiac output (ml/min)
NonPostC	6	48.33 ± 3.99	66.58 ± 5.55
PostC (6x 10 sec)	6	48.08 ± 2.11	63.75 ± 3.16

The total protein expression and the phosphorylation states of the kinases under investigation, in the non-ischaemic zone (i.e. the zone of tissue that was not exposed to ischaemia) are shown in figure 18. Very interesting to note is that p38 MAPK shows a significant elevation in phosphorylation in postC compared to NonPostC hearts (postC: 1.19 ± 0.08 AU vs NonPostC: 0.84 ± 0.11 AU, where AU refers to 'arbitrary unit'). This is unexpected, since it is unlikely that postC exerts effects on tissue that was not exposed to ischaemia (and therefore not in need of protection). That was not the only unexpected finding in the non-ischaemic tissue: an increase in the total amount of PKB/Akt was also observed in the postC vs NonPostC hearts.

To ensure that the differences found in the expression of total PKB/Akt (NonPostC: 0.75 ± 0.04 AU vs postC: 1.03 ± 0.07 AU) were not masking differences in the phosphorylation of the kinase, the ratio of phosphorylated to total protein was calculated. There were however still no differences in the phosphorylation levels between the postC and NonPostC groups, when expressed in terms of total PKB/Akt protein (fig. 19).



NonPostC: n=5
PostC: n=5

***Figure 19**: Comparison of the ratio of phosphorylated to total PKB/Akt. There is no difference between nonPostC and PostC – confirming that PKB/Akt phosphorylation is not influenced by postC.*

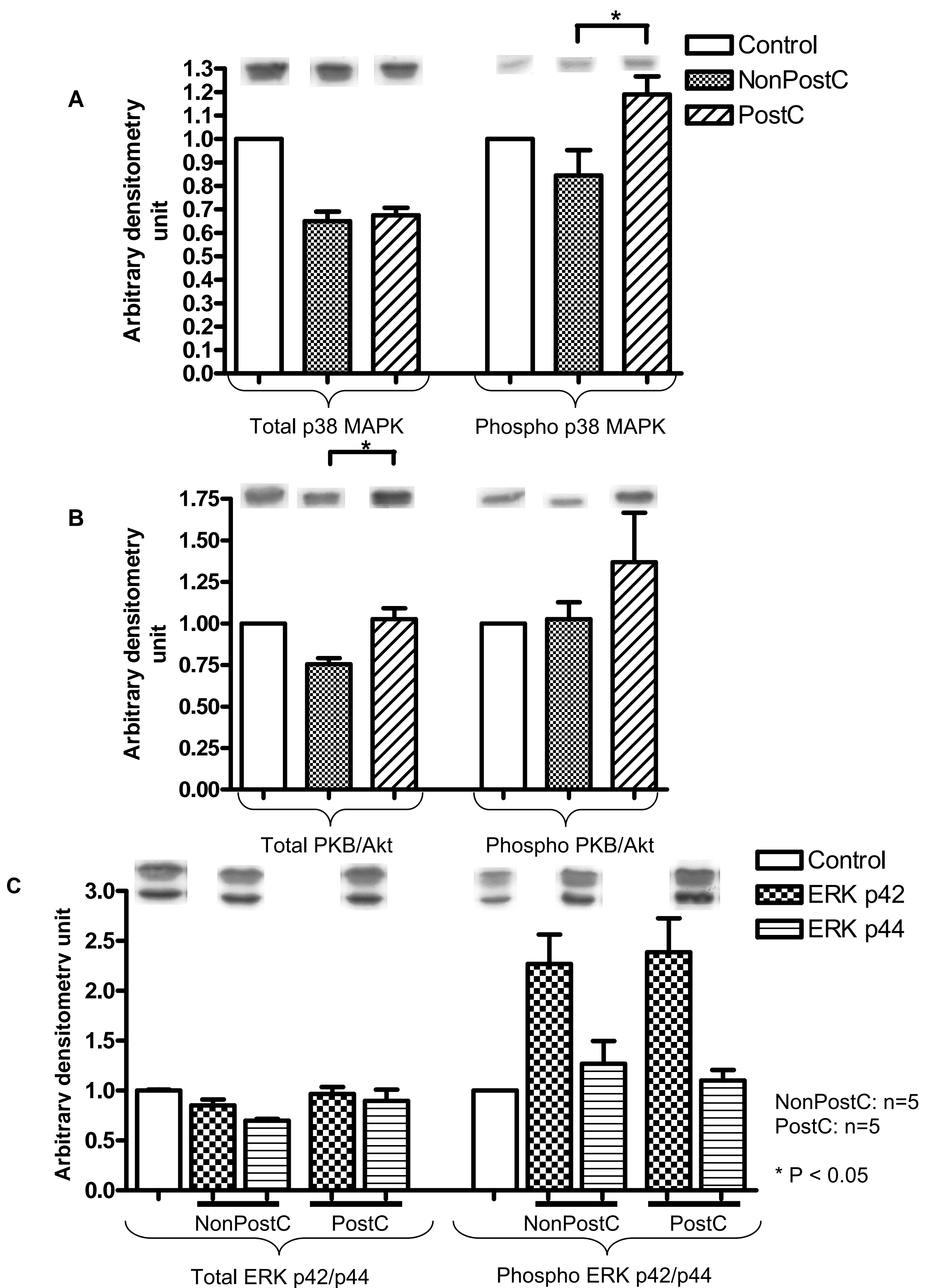


***Figure 18**: Levels of total and phosphorylated p38 MAPK (A), PKB/Akt (B) and ERK p42/p44 (C) at 10 minutes reperfusion in non-ischaemic tissue, after 35 minutes regional ischaemia, in the working heart model. PostC tissue was associated with a significant increase in the phosphorylation of p38 MAPK, as well as a sigificant increase in total PKB/Akt. Control hearts; n=2.*

The profile of total protein expression and phosphorylation in the myocardial tissue exposed to ischaemia showed even less difference between the postC and NonPostC groups, than in the non-ischaemic zone (fig. 20). These results are surprising as it is especially in the tissue that was exposed to ischaemia that one expects postC to exert its effects, if any.

It is interesting to note that in the ischaemic tissue the same trend persists as noted in the tissue freeze-clamped after 30 minutes reperfusion, i.e. a noticeable increase in the phosphorylation of the kinases, in relation to control values, due to reperfusion itself. Phosphorylation of p38 MAPK increased approximately 4 fold in both postC and NonPostC groups, while phospho-PKB presented with an increase of about 6 fold. ERK p42/p44 also increased, but to a lesser degree: ERK p42 ~ 2.5 fold and ERK p44 ~ 1.5 fold.

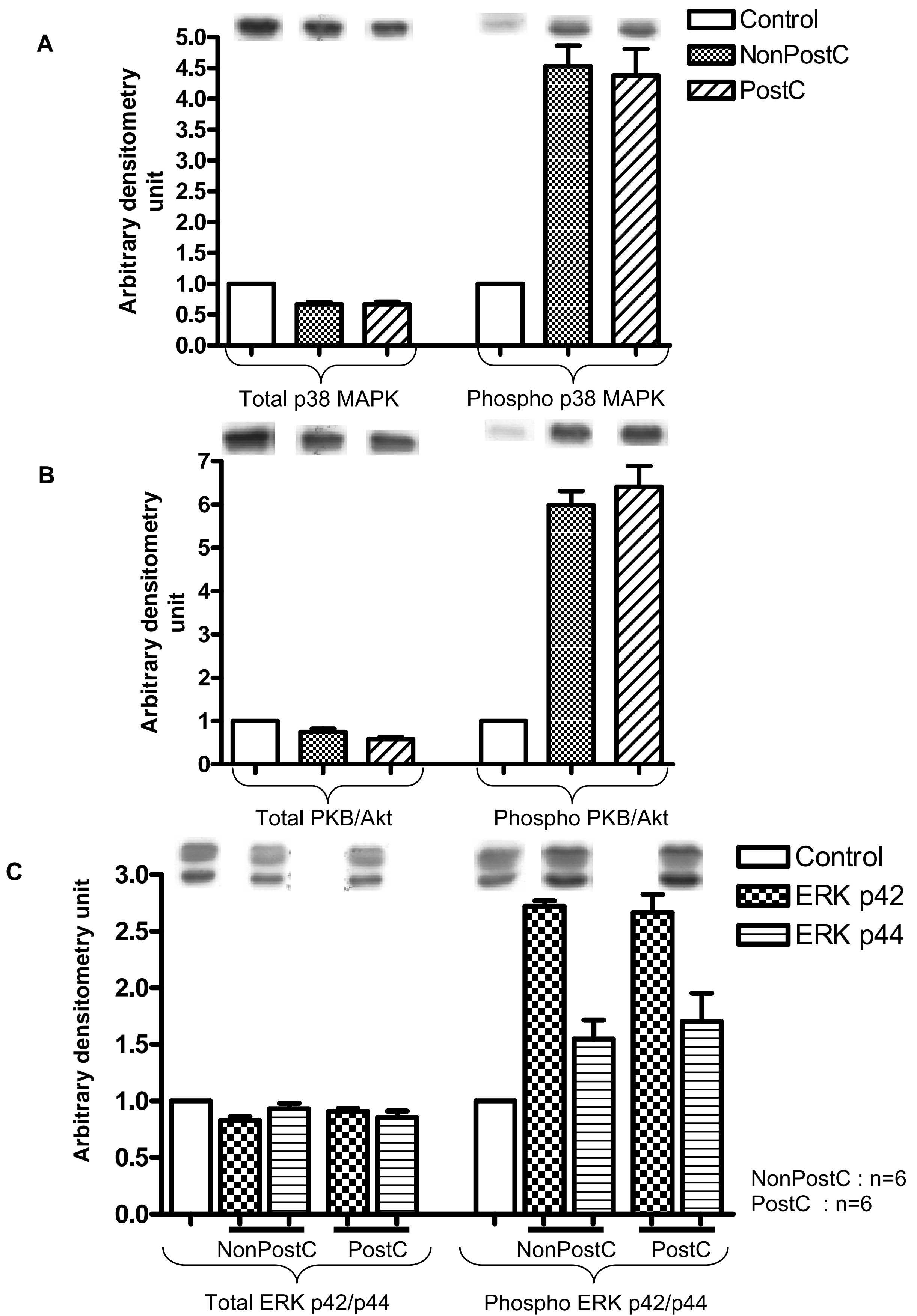


***Figure 20**: Total and phosphorylated levels of MAPK p38 (A), PKB/Akt (B) and ERK p42/p44(C) in tissue exposed to ischaemia, at 10 minutes reperfusion, after 35 minutes regional ischaemia, in the working heart model. There were no significant differences in protein levels or activity between postC and NonPostC groups. Control: n=2.*

4.3.1.2. The retrogradely perfused Langendorff model

We previously observed that the retrogradely perfused heart (Langendorff model) survived a 35 minute global ischaemic insult better than the working heart (Lochner *et al.*, 2003). It was therefore decided to apply a 35 minute global ischaemic insult to the retrogradely perfused hearts. Experimental protocols are depicted in figure 21. In the case of global ischaemia, the whole heart could be freeze-clamped – simplifying the protocol, as well as increasing the amount of tissue available for Western blotting.

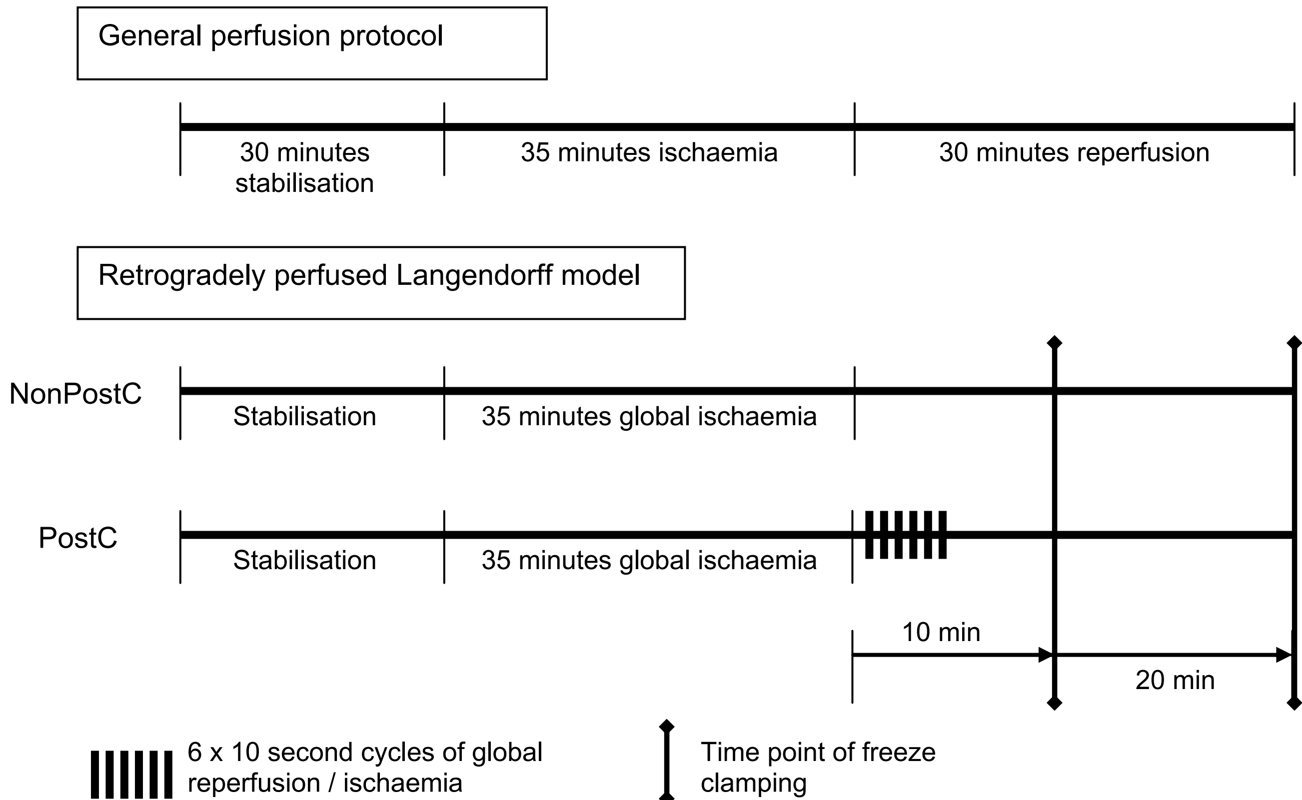


***Figure 21**: Perfusion protocols used to describe the expression and activation of MAPK p38, ERK 1/2 and PKB/Akt associated with postC, at 10 minutes and 30 minutes reperfusion.*

As in the case in the working heart model, no differences in the level of phosphorylation or total protein expression were found between the postC (n = 6; LVDP before ischaemia = 97.17 ± 8.33 mmHg; RPP before ischaemia = 30966 ± 3667) and NonPostC (n = 6; LVDP = 96.83 ± 7.01 mmHg; RPP = 32774 ± 2461) hearts at 30 minutes reperfusion (figure 22). In NonPostC hearts temperatures measured during global ischaemia and the first 10 minutes of reperfusion were 37.14 ± 0.14 °C and 36.96 ± 0.08 °C respectively. The same

magnitude of temperatures were also maintained in postC hearts (GI = 37.00 ± 0.04 °C; reperfusion = 36.73 ± 0.14 °C).

The 10 minute reperfusion analysis in the Langendorff model differed from the working heart model, in that the hearts were exposed to a global ischaemic episode – which meant that whole hearts were freeze-clamped and analyzed. The kinase profiles of hearts which received the postC intervention (n = 4; LVDP = 97.25 ± 5.11 mmHg; RPP = 31320 ± 2436) or no reperfusion treatment (NonPostC: n = 6; LVDP = 99.67 ± 4.88 mmHg; RPP = 31520 ± 2706) are shown in figure 23. Temperatures during ischaemia and initial reperfusion were regulated and monitored as previously discussed (postC: GI = 36.88 ± 0.03 °C; reperfusion = 36.93 ± 0.05 °C, and NonPostC: GI = 37.02 ± 0.05 °C; reperfusion = 36.77 ± 0.14 °C).

At 10 minutes reperfusion in the Langendorff model there was a significant increase in the phosphorylation levels of both ERK p42 and PKB/Akt in the postC compared to NonPostC hearts (ERK p42: postC = 3.13 ± 0.19 AU vs NonPostC = 2.03 ± 0.20 AU, $p < 0.01$; and PKB/Akt: postC = 1.65 ± 0.06 AU vs NonPostC = 0.88 ± 0.23 AU, $p < 0.05$). It therefore seems likely that increased phosphorylation, and activation, of both ERK p42 and PKB/Akt might be involved in the cardioprotective pathways recruited by postconditioning.

As observed in the working heart model, reperfusion on its own was associated with an increase in the phosphorylation of the kinases investigated, although this increase does seem to be less prominent in the in Langendorff model. After 30 minutes reperfusion PKB/Akt shows the most prominent increase: approximately 3 fold. At 10 minutes reperfusion the postC group presented with the most noticeable increases: ~ 2.5 fold in p38 MAPK, ~ 3 fold in ERK p42, ~ 2 fold in ERK p44 and in PKB ~ 1.5 fold.

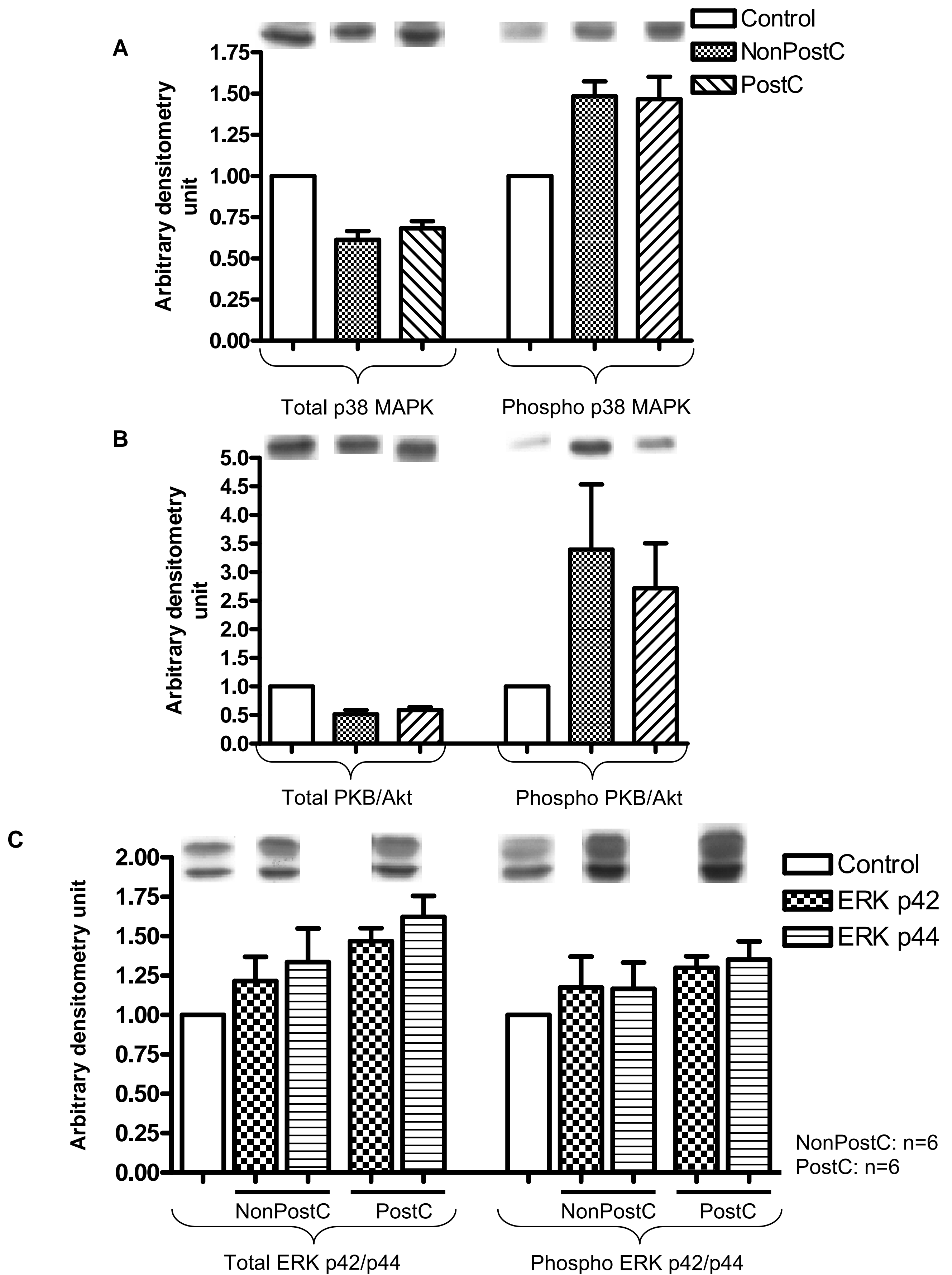


***Figure 22**: Total expression and phosphorylation MAPK p38 (A), PKB/Akt (B) and ERK p42/p44 (C) at 30 minutes reperfusion, after 35 minutes global ischaemia, in the Langendorff model. There are no significant differences between NonPostC and PostC treated hearts. Control hearts; n=1.*

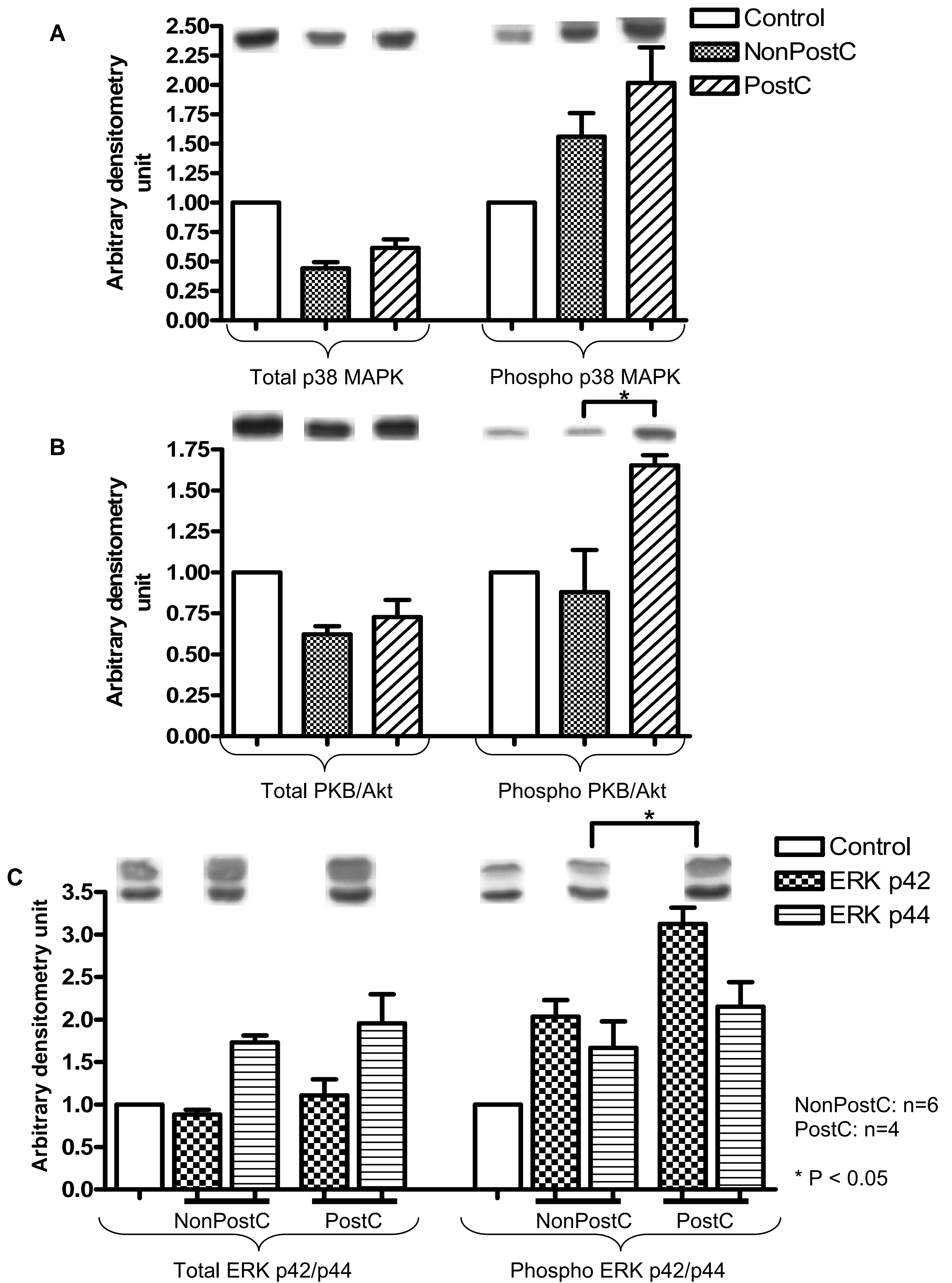


***Figure 23**: Levels of total and phosphorylated p38 MAPK (A), PKB/Akt (B) and ERK p42/p44 (C) at 10 minutes reperfusion, after 35 minutes global ischaemia, in the Langendorff model. PostC tissue was associated with a significant increase in the phosphorylation of PKB/Akt and ERK p42/p44. Control hearts; n=2.*

In summary a number of interesting observations have been made thus far:

I. Irrespective of the perfusion model used (Langendorff model or working heart), postC is not associated with a change in survival kinase and p38 MAPK expression or activation (as assessed by phosphorylation quantification) at 30 minutes reperfusion.

II. Evaluation of kinase activation in regionally ischaemic tissue after 35 minutes regional ischaemia, also yielded disappointing results – even after 10 minutes reperfusion.

III. We could however demonstrate an association between postC and marked activation of ERK p42 and PKB/Akt at 10 minutes reperfusion, after 35 minutes global ischaemia, in the retrogradely perfused Langendorff model.

4.3.2. Investigating the functional importance of PKB/Akt and ERK p42

In the above described phase of the study, using the retrogradely perfused rat heart as model, we found that at 10 minutes reperfusion, PKB/Akt and ERK p42 were significantly phosphorylated in postC. This follow-up study therefore focussed on the role of these two kinases, in the cardioprotection elicited by postC.

The same basic perfusion protocol, as described previously, was followed (fig. 24). Briefly, hearts were mounted on the retrogradely perfused Langendorff system and allowed to stabilise. The hearts were exposed to a 35 minute period of regional ischaemia, followed by either 10 or 30 minutes of reperfusion. To investigate the functional importance of PKB/Akt and ERK p42/p44 in postC, hearts were perfused with either a specific PKB/Akt inhibitor, or an ERK p42/p44 inhibitor (Fig. 24). Both these inhibitors were administered for the final 5 minutes of regional ischaemia and the first 10 minutes of reperfusion, in the presence or absence of postC (6 x 10 second global reperfusion / ischaemia, at between 36.5 and 37.3 °C). The effects of the inhibitors on the efficacy of the postC protocol was then determined in terms of infarct size reduction. Functional recovery during reperfusion was also monitored.

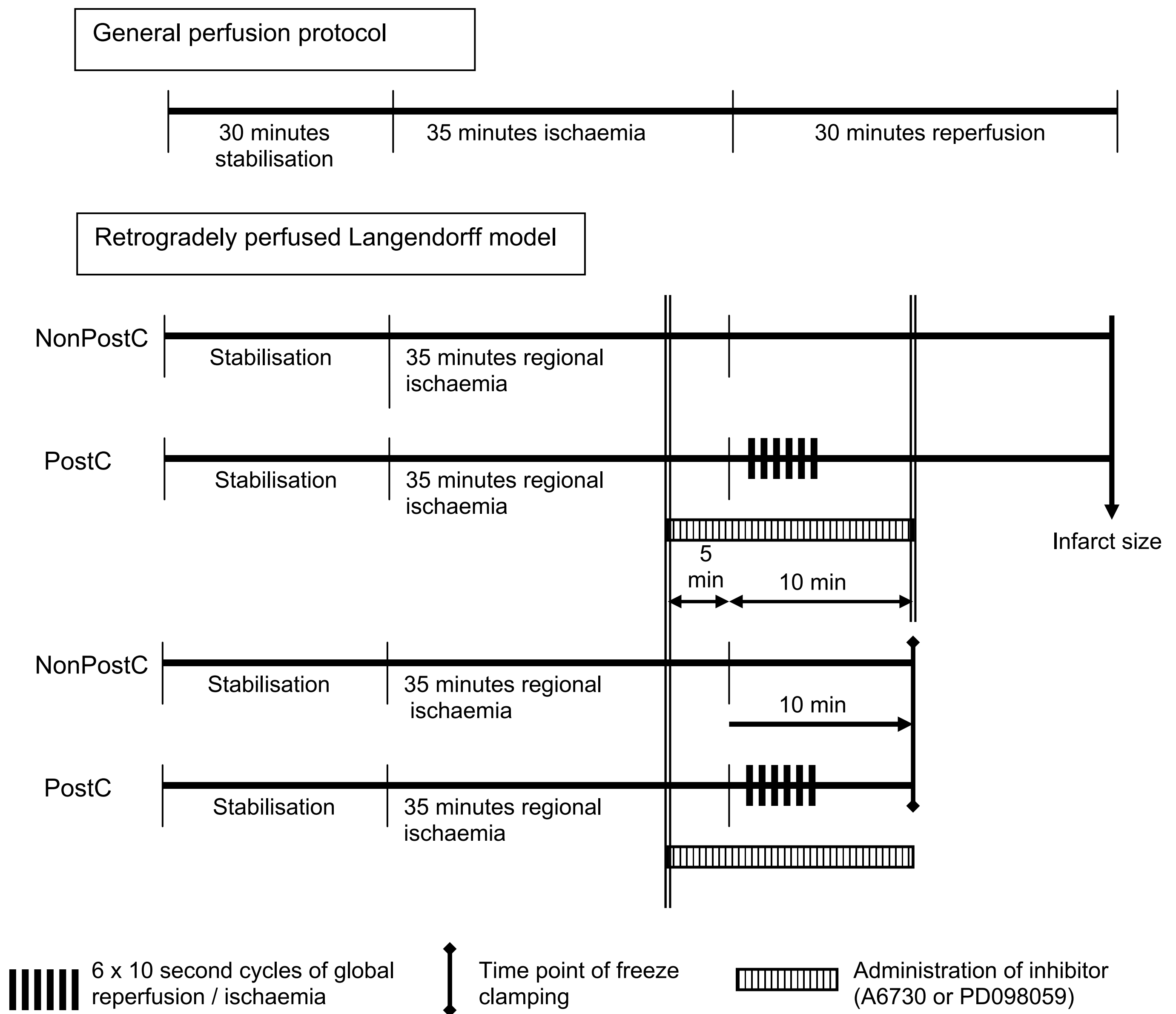


***Figure 24**: Investigating the functional importance of PKB/Akt and ERK p42/p44 activation in the cardioprotective mechanism of postC. Inhibitors for both kinases were administered for the final 5 minutes of ischaemia and the first 10 minutes of reperfusion. Hearts were then either freeze-clamped or reperfused for another 20 minutes, before determination of infarct size.*

4.3.2.1. Effects of ERK p42/p44 inhibition

To inhibit ERK p42/p44 the inhibitor PD098059 (Sigma-Aldrich) was administered at a concentration of 10 µM, dissolved in 0.053% DMSO (Merck).

To verify that the inhibitor was effectively inhibiting ERK p42/p44 phosphorylation, as well as to investigate possible effects of PD098059 on the other kinases of interest, hearts were perfused with the inhibitor, either in the presence (postC: n = 4; LVDP = 100 ± 0.19 mmHg; RPP = 36041 ± 2216) or absence (NonPostC: n = 4; LVDP = 94.00 ± 5.96 mmHg; RPP = 30834 ± 3020) of postC, and then freeze-clamped at 10 minutes reperfusion to be

later analyzed using Western blotting (as described in fig. 24). Although regional ischaemia was applied, the whole hearts were freeze-clamped for analysis (contrary to the method of separating the ischaemic and non-ischaemic zones described previously), since the effects of the inhibitor on the hearts *per* se were investigated. As explained earlier, freeze-clamping the whole heart, gives us the luxury of more tissue for Western blotting analysis. Western blotting analysis of the tissue samples included a postC group, not treated with the inhibitor, which served as a positive control (PostC (+)). As in all other Western blotting experiments, a control group was included that only received 30 minutes perfusion, with no intervention (Control). Data is shown in figures 25 and 26.

The inhibitor was not associated with a significant decrease in the phosphorylation of ERK p44, although there is a strong trend (PostC (+): 4.04 ± 0.17 AU vs PostC+PD: 1.06 ± 0.16 AU). Concerning ERK p42, the inhibitor was associated with a significant reduction in phosphorylation in postC hearts (PostC (+): 4.49 ± 1.28 AU vs PostC+PD: 1.52 ± 0.24 AU, $p < 0.05$). Since it inhibited pERK 42 in postC hearts, it was the right tool to investigate the role of ERK p42 in the mechanism of postconditioning.

Interestingly, PD098059 seemed to inhibit p38 MAPK as well – both in the presence and absence of postC (PostC (+): 2.19 ± 0.64 AU; vs NonPostC+PD: 1.08 ± 0.05 AU, $p < 0.05$; and PostC+PD: 0.74 ± 0.02 AU, $p < 0.01$). As expected, the inhibitor did not show any effect on the total protein levels (figures 25 and 26), when compared with control hearts and postC hearts which did not receive the inhibitor.

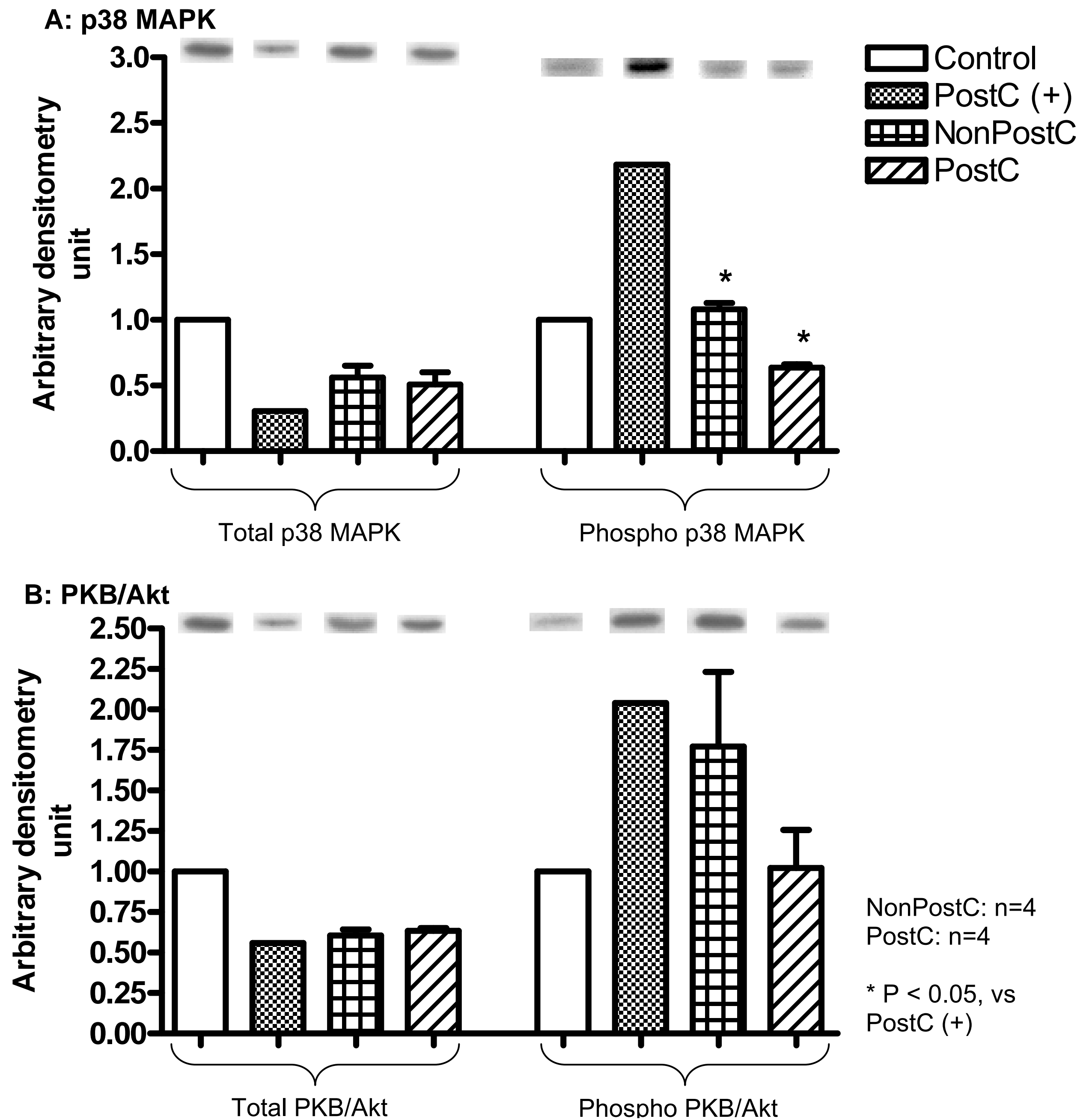
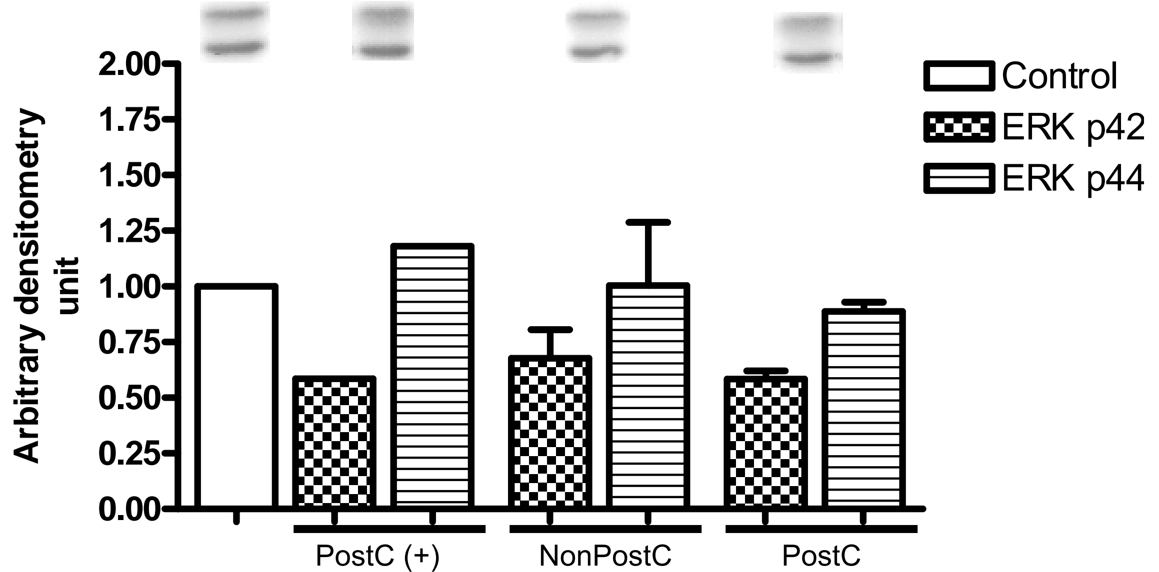


Figure 25*: Effect of PD098059 treatment, in the final 5 minutes of ischaemia and the first 10 minutes reperfusion, on levels of total and phosphorylated p38 MAPK (A) and PKB/Akt (B). Tissue was collected at 10 minutes reperfusion, after 35 minutes regional ischaemia, in the Langendorff model. Control hearts: n=2; Positive control (PostC (+)): n=2.*

A: Total ERK



B: Phosphorylated ERK p42/p44

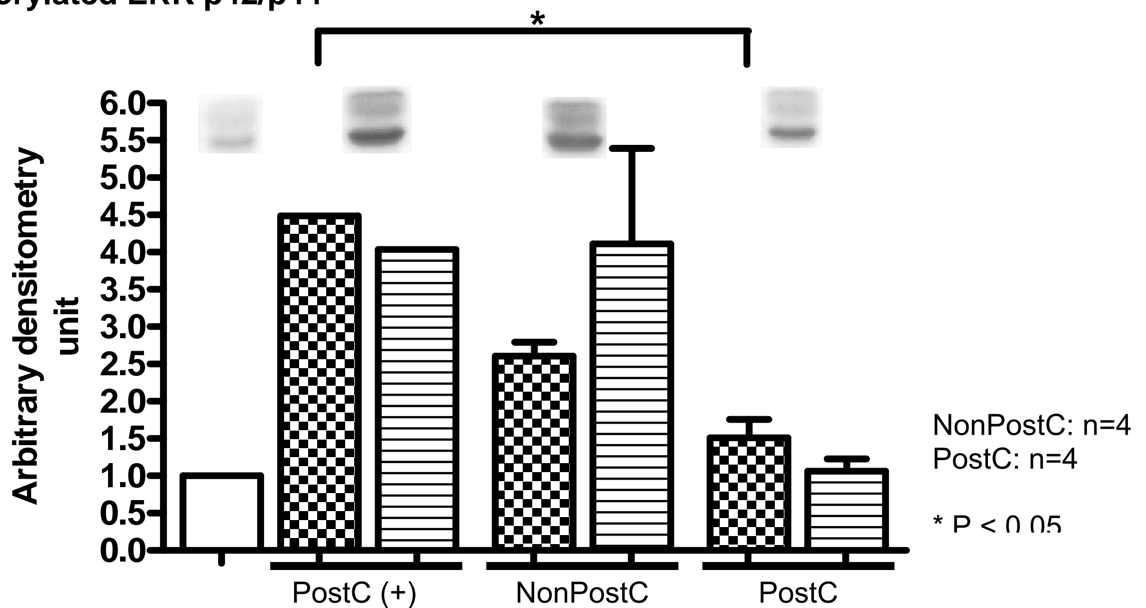


Figure 26*: Effect of PD098059 treatment, in the final 5 minutes of ischaemia and the first 10 minutes reperfusion, on levels of total and phosphorylated ERK p42/p44 (A) and phosphorylated ERK p42/p44 (B). Tissue was collected at 10 minutes reperfusion, after 35 minutes regional ischaemia, in the Langendorff model. Control hearts: n=2; Positive control (PostC (+)): n=2.*

Concerning the effect of MEK1/2 – ERKp42/p44 inhibition on infarct size, data is shown in figure 27. In all four groups depicted in figure 27, similar area at risk values were measured (NonPostC: 45.96 ± 3.62%; PostC: 43.14 ± 3.24%; NonPostC+PD: 43.81 ± 4.48%; and PostC+PD: 48.33 ± 3.29%). Another series of postC and NonPostC hearts were perfused, without PD098059 administration (PostC: n = 13, and NonPostC: n = 9). Just as previously shown, infarct size was significantly lower in the postC than NonPostC hearts (postC: 27.32 ± 2.14% vs NonPostC: 43.26 ± 3.9%; p = 0.001). There was however no significant recovery of any functional parameters in the postC hearts (table 10).

Table 10*: Functional parameters in hearts exposed to 35 minutes regional ischaemia, in the presence and absence of postC, as well as the presence and absence of the ERK p42/p44 inhibitor PD098059. PostC was not associated with significant changes in functional recovery, irrespective of ERK p42/p44 inhibition.*

Intervention		Baseline		Post-ischaemic		Recovery (%)	
		LVDP (mmHg)	RPP	LVDP (mmHg)	RPP	LVDP	RPP
Without PD098059	NonPostC	99.56±5.53	31627±1766	80.89±4.03	25360±1776	82.49±4.64	80.43±3.83
	PostC	100.40±4.39	30828±1645	76.38±4.25	22288±1365	77.67±5.50	73.74±5.08
With PD098059	NonPostC	101.3±5.42	34310±1796	83.57±6.19	27473±2089	84.47±8.01	81.49±6.97
	PostC	103.2±3.95	34101±913	63.33±9.96	20807±3490	63.36±10.98	61.49±10.74

Administration of PD098059 together with NonPostC (n = 7) and postC (n = 9) hearts did not alter the infarct sparing effect of postC. PostC together with PD098059 still significantly reduced IFS, compared to NonPostC hearts, in the presence and absence of the ERK p42/p44 inhibitor (PostC+PD: 23.56 ± 2.82% vs NonPostC+PD: 39.41 ± 3.36%, $p < 0.01$; and NonPostC: 43.26 ± 3.91%, $p < 0.001$). Just as was the case in the absence of PD098059, there were no functional recovery differences between postC and NonPostC hearts (table 10). Postconditioning could therefore not elicit any functional recovery, irrespective of the administration of the MEK1/2 – ERKp42/p44 inhibitor. Temperature during ischaemia (NonPostC: 37.12 ± 0.05 °C; PostC: 37.06 ± 0.05 °C; NonPostC+PD: 36.85 ± 0.17 °C; PostC+PD: 37.04 ± 0.07 °C) and reperfusion (NonPostC: 36.94 ± 0.03 °C; PostC: 36.90 ± 0.04 °C; NonPostC+PD: 37.00 ± 0.17 °C; PostC+PD: 36.94 ± 0.11 °C) was closely monitored and regulated, as previously described.

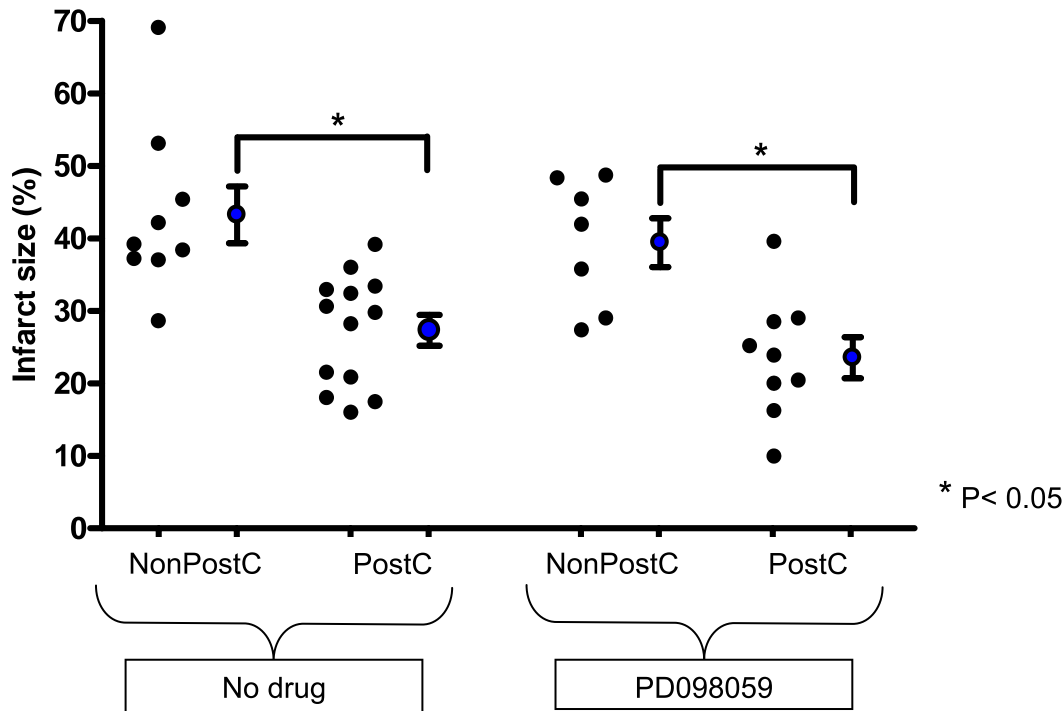


Figure 27*: Infarct sizes in postC and nonPostC hearts, either in the presence or absence of PD098059. The inhibition of MEK1/2 – ERK p42/p44 did not affect the cardioprotective effects of postconditioning.*

4.3.2.2. Effects of PKB/Akt inhibition

The specific PKB/Akt inhibitor, A6730 (Sigma-Aldrich), was used to investigate the importance of PKB/Akt in the cardioprotection associated with postC. To confirm that the inhibitor (at the administered concentration of 2.5 μM, dissolved in 0.025% dimethyl sulfoxide (DMSO)) was inhibiting the phosphorylation of PKB/Akt, hearts were perfused and freeze-clamped at 10 minutes reperfusion, as described in figure 24. As explained earlier, since the effect of the inhibitor on the myocardium *per* se was of interest, whole hearts were harvested for analysis. These hearts received the inhibitor, together with standard uncontrolled reperfusion (NonPostC: n = 4; LVDP = 93.00 ± 5.12 mmHg; RPP = 31351 ± 3339) or the postC intervention (n = 5; LVDP = 85.60 ± 4.50; RPP = 29219 ± 2592). Western blotting analysis of these samples was done together with a positive control (postC (+); n = 2), as well as a control group which received only 30 minutes perfusion (Control). The effect of the inhibitor on the phosphorylation, as well as the total levels, of the kinases under investigation are shown in figures 28 and 29.

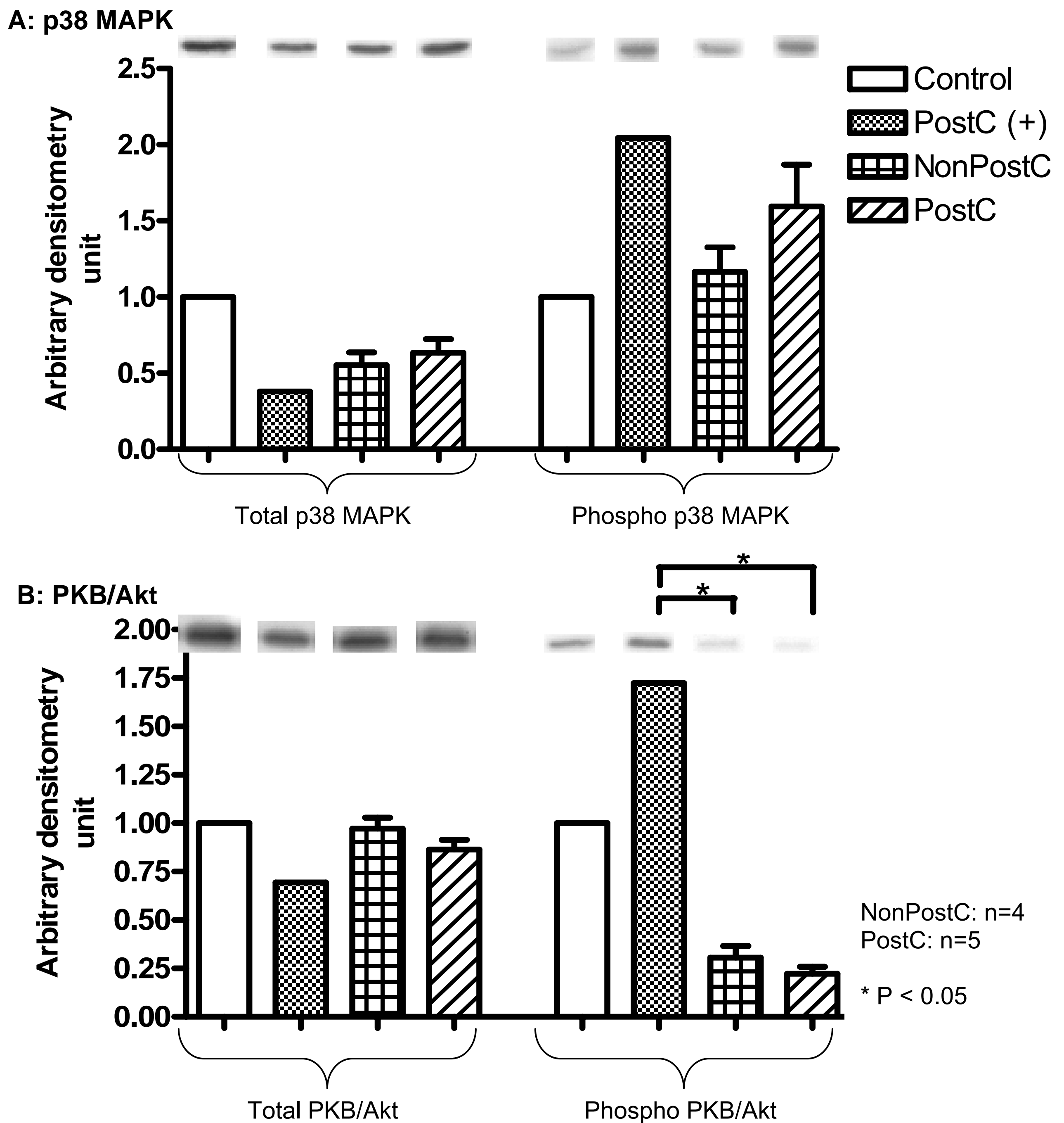


***Figure 28**: Effect of the PKB/Akt inhibitor, A6730, treatment, in the final 5 minutes of ischaemia and the first 10 minutes reperfusion, on levels of total and phosphorylated p38 MAPK (A) and PKB/Akt (B). The inhibitor clearly caused a significant decrease in the phosphorylation of PKB/Akt. Control hearts: n=2; Positive control (PostC (+)): n=2.*

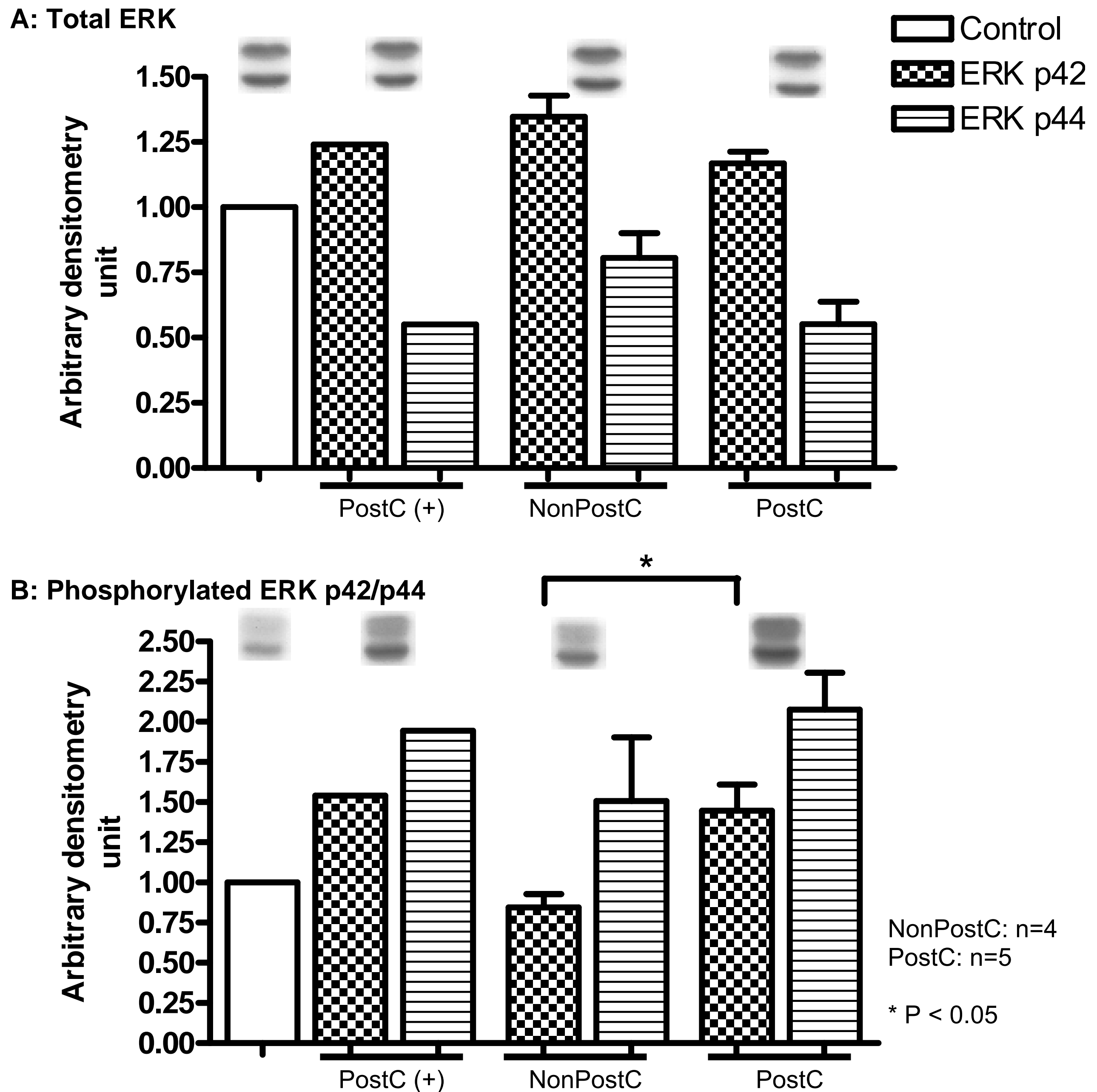


***Figure 29**: Effect of A6730 treatment, on total ERK p42/p44 (A) and phosphorylated ERK p42/p44 (B) profiles. Tissue was collected at 10 minutes reperfusion, after 35 minutes regional ischaemia, in the Langendorff model. The significant increase in phosphorylated ERK p42 in PostC compared to NonPostC hearts is probably due to the postC intervention, and not related to the inhibitor. Control hearts: n=2; Positive control (PostC (+)): n=2.*

The efficacy of the inhibitor is clearly demonstrated in the dramatically reduced levels of phosphorylation of PKB/Akt (fig. 28), in both NonPostC and postC hearts (PostC(+): 1.73 ± 0.07 AU vs NonPostC+A6730: 0.31 ± 0.06 AU and postC+A6730: 0.22 ± 0.04 AU, p <

0.05). This reduction in phosphorylated PKB/Akt occurred while, no differences were observed in the amount of total protein.

The only other significant difference in phosphorylation levels was found in ERK p42 (fig. 29): postC was associated with elevated phosphorylation (PostC: 1.45 ± 0.16 AU vs NonPostC: 0.85 ± 0.08 AU, $p < 0.05$). This is probably not linked to the inhibitor, rather it is likely due to postC itself. This then is a confirmation of our initial findings, that ERK p42 phosphorylation increases with postconditioning. No significant differences were found in the total ERK p42/p44 expression (fig. 29). It is therefore clear that A6730 acted as an effective and specific PKB/Akt inhibitor in our experimental setup.

To evaluate the importance of PKB/Akt in postC, hearts were reperfused in the presence of the PKB/Akt inhibitor, A6730, either in the presence (postC: n=9) or absence of the postC (NonPostC: n=9) intervention. Baseline, as well as post-ischaemic functional values are shown in table 11. As in all postC experiments, temperature was closely regulated (postC: RI = 36.97 ± 0.06 °C; reperfusion = 36.91 ± 0.09 °C, and NonPostC: RI = 37.23 ± 0.11 °C; 36.88 ± 0.10 °C). The same series of inhibitor-free NonPostC and postC hearts, as described in the ERK p42/p44 inhibitor experiments, were used. The effect of the inhibition of PKB/Akt on the infarct sparing effect of postC is shown in figure 30.

There was no significant difference in the area at risk between the drug treated groups (NonPostC: 49.13 ± 3.15% and PostC: 53.00 ± 3.05%). In contrast to ERK p42/p44, inhibition of PKB/Akt was associated with a loss in the infarct sparing effect of postC (NonPostC+A6730: 38.07 ± 4.34% vs PostC+A6730: 35.27 ± 4.42%). It is important to note though, that administration of A6730 was also associated with changes in functional recovery – as depicted in table 11 and figure 31.

Table 11: *Baseline and post-ischaemic functional performance of hearts exposed to 35 minutes regional ischaemia and combinations of NonPostC, PostC, and the presence and absence of the PKB/Akt inhibitor A6730. A6730 dramatically decreased functional recovery, regardless of the presence or absence of postC. * p < 0.001 vs corresponding drug free hearts.*

Intervention		Baseline		Post-ischaemic		Recovery (%)	
		LVDP (mmHg)	RPP	LVDP (mmHg)	RPP	LVDP	RPP
Without A6730	NonPostC	99.56±5.53	31627±1766	80.89±4.03	25360±1776	82.49±4.64	80.43±3.83
	PostC	100.40±4.39	30828±1645	76.38±4.25	22288±1365	77.67±5.50	73.74±5.08
With A6730	NonPostC	95.67±6.24	30267±1713	22.11±2.35	7195±804	24.32±3.05*	24.36±2.91*
	PostC	87.44±6.08	27307±1513	27.22±5.07	6638±1066	30.36±5.08*	24.49±4.36*

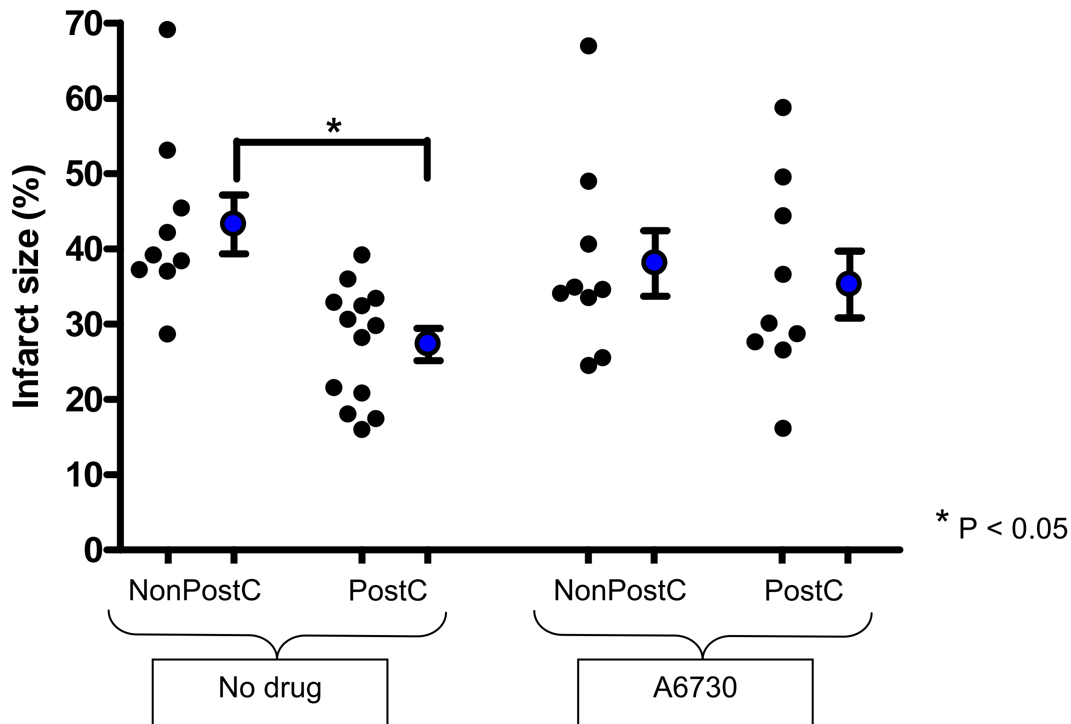


Figure 30*: The effect of PKB/Akt inhibition on the infarct sparing effect of postC. In the absence of the inhibitor postC can reduce infarct size, however with administration of A6730 the postC intervention is no longer cardioprotective.*

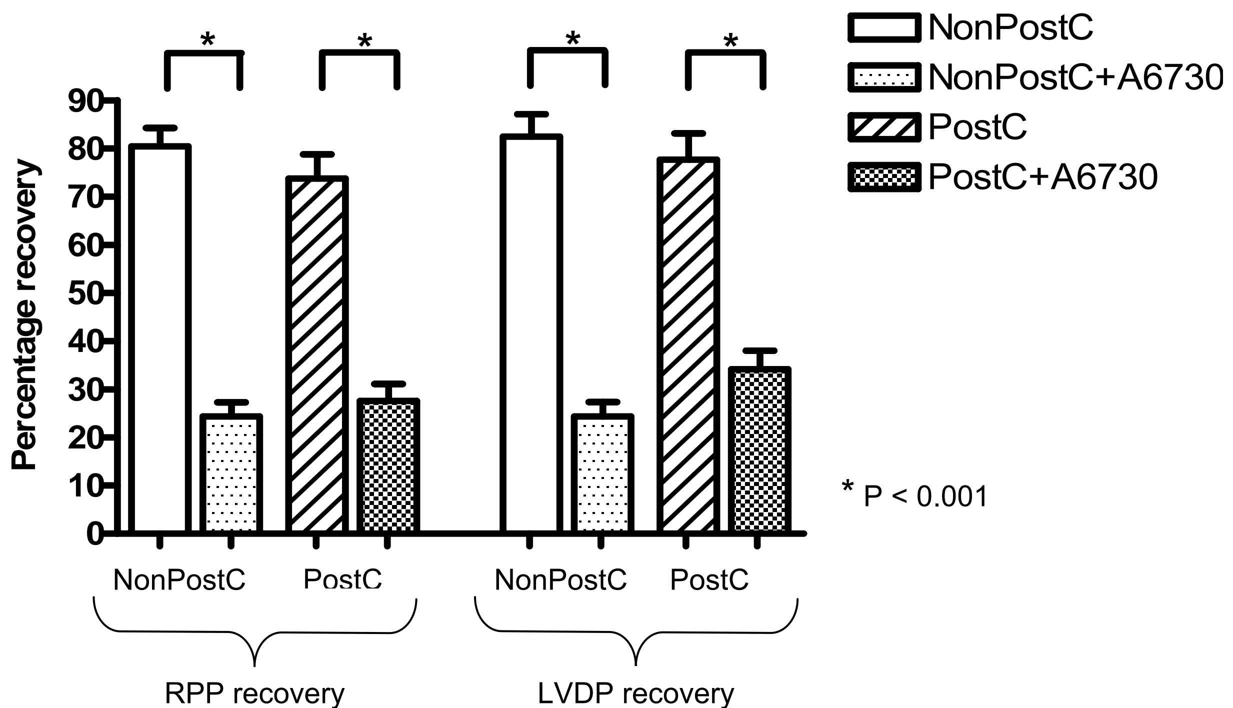


***Figure 31**: Treatment of hearts with the PKB/Akt inhibitor, A6730, in reperfusion, leads to a significant decrease in functional recovery, irrespective of the presence or absence of postC.*

Inhibition of PKB/Akt with A6730, at a concentration of 2.5 μM, significantly reduced functional recovery, compared to hearts that did not receive the inhibitor – irrespective of the administration of a postC intervention.

4.3.2.3. Vehicle controls

For the administration of both PD098059, as well as A6370, DMSO was used as drug solvent. To ascertain whether the concentration DMSO administered to the hearts could exert a confounding effect on the kinase profiles, hearts were perfused with either 54 μl DMSO / 100 ml Krebs-Henseleit buffer (n = 4; corresponding to PD098059 experiments) or 25 μl DMSO / 100 ml buffer (n = 4; as administered with A6730) in the final 5 minutes of regional ischaemia and the first 10 minutes of reperfusion, whereafter hearts were immediately freeze-clamped for Western blot analysis.

The kinase profiles of hearts exposed to only DMSO were compared to hearts that did not receive any DMSO in reperfusion (n = 4). Analysis of all kinases under investigation (as desribed in chapter 2) showed no confounding effect, associated with the DMSO concentrations used, on the phosphorylation of the kinases (data not shown). The

observed effects of the inhibitors on the kinases are therefore solely due to the inhibitors, and not associated with the drug solvent.

4.4. Discussion

Studies on the mechanism of protection associated with ischaemic preconditioning (IPC) have shown a definite role for the survival kinases ERK p42/p44 and PKB/Akt, in the transduction of pro-survival / protective signals, which eventually lead to the recruitment of protective end-effectors (for reviews on the role of the kinases in IPC see Hausenloy & Yellon, 2004; and for a broader review on the mechanisms of IPC see Yellon & Downey, 2003). The advent of postconditioning as a cardioprotective intervention, therefore also prompted intensive research into the role of the kinases, especially the so-called reperfusion injury salvage kinases (RISK). However, consensus concerning the precise role of the kinases in reperfusion and postC-protection has still not been reached (as discussed in chapter 1). In our study we investigated the phosphorylation of PKB/Akt, ERK p42/p44 (i.e. the RISK – pathway) and p38 MAPK associated with postconditioning, in two different experimental models, at two different time-points during reperfusion.

4.4.1. At 30 minutes reperfusion

Initial evaluation of kinase activity, associated with postC, was done after 30 minutes of reperfusion. This was done, since a longer period of reperfusion also offered the opportunity to record functional recovery data. However, at 30 minutes reperfusion we could not demonstrate differences in the phosphorylation of the kinases under investigation, in either the working heart model, or the Langendorff model. This is not too surprising, since all other studies done on the activation of ERK p42/p44 and PKB/Akt focussed their investigation on much earlier time-points in reperfusion, namely from 5 to 15 minutes reperfusion (for example Darling *et al.*, 2005; Tsang *et al.*, 2004 and Zhu *et al.*, 2006). It is therefore clear that the activation of the kinases in postC cardioprotection is transient and limited to the early moments of reperfusion. This early recruitement of the signalling pathways, is in agreement with the the observations made by Kin and co-workers (2004) and Downey & Cohen (2005), that the postC-intervention must be applied either very early in reperfusion, or immediately at the very onset of reperfusion.

4.4.2. At 10 minutes reperfusion

In view of the failure to detect changes in survival kinase activation at 30 minutes reperfusion, the reperfusion time was shortened to 10 minutes. As expected, the effect of the postC – intervention on the signalling kinases is evident at 10 minutes reperfusion. Interestingly, in the regionally ischaemic working heart model we could not demonstrate any differences in the phosphorylation of any of the kinases of interest, in the ischaemic zone. This was contrary to what we found in the Langendorff model, where a significant increase in the phosphorylation of both ERK p42 and PKB/Akt was observed in the postC group. This difference in kinase activity between the two models might be due to the inherent differences between the perfusion models. It must be remembered that, to our knowledge, we are the first to apply postconditioning in the working heart model. Possible differences between the experimental setups, in this context, therefore still need to be explored. This explanation however, does seem unlikely, since the working heart model has been used to show elevated phosphorylation of p38 and PKB/Akt associated with the cardioprotection conferred by red palm oil, in the setting of global ischaemia (Engelbrecht *et al.*, 2006).

Another, more plausible, explanation for the differences in observed kinase profiles, pertains to the samples used for analysis. In the retrogradely perfused Langendorff model whole hearts were exposed to sustained ischaemia, and then snap frozen and later analyzed. This "global" approach has the primary advantages that more tissue is available for analysis, as well as that the damage is more homogeneous than in regional ischaemia. Any differences in protein phosphorylation due to an intervention is therefore "amplified" and easier to detect. This is contrary to the regional ischaemia working heart experiments, in which the heart tissue had to be divided into ischaemic and non- ischaemic samples. Despite the fact that extreme care was exercised in separating the areas, it is possible that the so-called ischaemic area contained undamaged tissue – the result being a lack of detectable differences in kinase phosphorylation.

Intriguingly, there was one unexpected observation in the working heart model. At ten minutes reperfusion there was, in fact, a significant increase in the phosphorylation of p38 MAPK in the non-ischaemic tissue. The precise role and importance of p38 MAPK in cardioprotection is still a matter of debate, while various isoforms complicate matters even further. In IPC both protective and deleterious roles for p38 MAPK have been suggested.

In our laboratory it has been found that p38 MAPK transiently increases during the preconditioning protocol itself, but then it is attenuated during sustained ischaemia and reperfusion, which is associated with protection (Marais *et al.,* 2001 and 2005). Saurin and colleagues (2000) reports that the deleterious effect of increased p38 MAPK during ischaemia might be attributable to activation of the α-isoform. On the other hand, increased phosphorylation of p38 MAPK has also been implicated in IPC (Nagy *et al.*, 2007). Increased phosphorylation of p38 MAPK in reperfusion has also been linked to other cardioprotective treatments, such as dietery supplementation with red palm oil (Engelbrecht *et al.*, 2006; and Van Rooyen *et al.*, 2007), as well as insulin administration in ischaemia / reperfusion (Tiron *et al.*, 2006). For a review on p38 MAPK in the setting of ischaemia / reperfusion and preconditioning, see Steenbergen (2002).

Only two studies evaluated p38 MAPK activation in postconditioning. Sun *et al.* (2006) found postC to be associated with a reduction in p38 MAPK activity, while Feng and colleagues (2006) found p38 MAPK to be unimportant in a isoflurane postC model. In our study, p38 MAPK is clearly not involved in the effect of postC on the ischaemic tissue itself, since it was not significantly phosphorylated at any time point in tissue exposed to ischaemia, in both experimental setups (it should be remembered that global ischaemia was applied in the Langendorff model (fig. 16)). In addition, it seems as if the MEK1/2 – ERK p42/p44 inhibitor, PD098059, also significantly inhibited the phosphorylation of p38 MAPK (fig. 25) in both NonPostC and PostC hearts. Despite this, PD098059 did not abrogate the cardioprotection of postC – indicating that p38 MAPK is indeed not involved.

However, as described above, p38 MAPK has been implicated in cardioprotection. It could be speculated that increased activation of p38 MAPK in neighbouring non-ischaemic tissue might somehow contribute to overall cardioprotection via a paracrine interaction with the ischaemic tissue. It could also be that the postC protocol itself is stimulating an increase in p38 MAPK phosphorylation – as is the case in the IPC protocol (Marais *et al.*, 2001) – without this effect actually being of importance in the eventual cardioprotection. It would be interesting to run a series of postC-intervention “controls”, in which a postC protocol is applied in the absence of sustained ischaemia / reperfusion. This could then shed light on the possible effect of the intervention *per se* on p38 MAPK phosphorylation.

Another interesting finding at 10 minutes reperfusion, is the significant elevation of total PKB/Akt levels in the non-ischaemic tissue of postC working hearts. Two possible

mechanisms could be involved, namely: either an increase in protein expression, or a change in the degradation of proteins. It is however unlikely that either of these mechanisms could come into play after just 10 minutes reperfusion. This significant increase was not noted at 30 minutes reperfusion, or in the ischaemic tissue and not at all in the Langendorff model. Furthermore, the ratio of phospho – PKB/Akt to total PKB/Akt also did not differ between the postC and NonPostC groups, indicating that, as shown in the phosphorylation profile of PKB/Akt, there was indeed no significant postC-related phosphorylation of PKB/Akt at 10 minutes reperfusion in the non-ischaemic tissue, in the working heart model. Whatever the cause, the increase in total PKB/Akt does not appear to be significant.

As previously mentioned, at 10 minutes reperfusion, after 35 minutes global ischaemia in the Langendorff model, we did observe significant increases in the phosphorylation of both ERK p42 and PKB/Akt in the postC group, compared to a NonPostC group. This is in accord with several other studies. Many researchers have shown a link between PI3-kinase – PKB/Akt activity, early in reperfusion (between 7 and 15 minutes reperfusion), and the cardioprotection conferred by postconditioning (in rats: Tsang *et al.*, 2004; Zhu *et al.*, 2006; Bopassa *et al.*, 2006; as well as in canine hearts: Fujita *et al.*, 2006), as well as pharmacological postconditioning with isoflurane administered in reperfusion (Feng *et al.*, 2006). This activation of PKB/Akt was also shown in diseased hearts, i.e. infarct – remodeled (Feng *et al.*, 2006) and hypertrophied (Zhu *et al.*, 2006) hearts. Darling *et al.* (2005), intriguingly found that PI3-kinase is unimportant in postC, and suggested that it is rather ERK p42/p44 that is of importance. Increased phosphorylation and activation of ERK p42/p44 has indeed been implicated in postconditioning (Fujita *et al.*, 2006; Yang *et al.,* 2004). Interestingly, Zhu *et al.* (2006) found this ERK p42/p44 phosphorylation to be dependent on PI3-kinase activity, in agreement with Yang *et al.* (2004) who speculated that PKB/Akt could be upstream from ERK p42/p44 in a form of pharmacological postconditioning (bradykinin or an A_1/A_2 adenosine agonist, NECA, applied at reperfusion). Postconditioning with isoflurane however, does not seem to lead to the increased phosphorylation of ERK p42/p44 (Feng *et al.*, 2006).

To further investigate the functional importance of the observed increase in phosphorylation of PKB/Akt and ERK p42/p44, we utilised inhibitors co-aministered with the postC intervention.

4.4.3. Inhibition of PKB/Akt using A6730 in reperfusion: effect on cardioprotection

Despite discrepancies in the literature, it generally seems as if especially PKB/Akt, but also ERK p42/p44, are involved in the mechanism of postconditioning. The phosphorylation profiles that we observed at 10 minutes reperfusion in the Langendorff model, therefore fits into the work that has already been done. We however, wanted to establish the involvement of these kinases in cardioprotection, by investigating the effect of pharmacological inhibition on the infarct sparing effect of postconditioning.

For inhibition of PKB/Akt, the inhibitor A6730 (Sigma-Aldrich) was used. This compound is also known as Akt-I-1/2 and has been shown to be a specific inhibitor of the PKB/Akt isoforms Akt1 and Akt2 (Barnett *et al.*, 2005). In cells it has been shown to block the phosphorylation of Akt Thr^{308} and Ser^{473}, affecting downstream components. To our knowledge, this is the first study that utilised this inhibitor in the isolated rat heart. All other studies done on the role of PKB/Akt in postC utilised either Wortmannin or LY-294002, both of which are PI3-kinase inhibitors (Yang *et al.*, 2004; Fujita *et al.*, 2007). This then, is the first study to specifically focus on PKB/Akt, not involving upstream PI3-kinase.

As expected, the inhibition of PKB/Akt using the inhibitor A6730 at reperfusion, did abrogate the infarct sparing effect of the postC-intervention (fig. 30), indicating PKB/Akt as a necessary role player in the postC cardioprotection mechanism.

Interestingly, Barnett and co-workers (2005) reported that this inhibitor has an IC_{50} value of 2.7 μM for Akt1 and 21 μM for Akt2. We however demonstrated a significant decrease in phosphorylation of PKB/Akt (ser473), compared to positive postC controls, at a concentration of 2.5 μM inhibitor (fig. 28). In fact, it could even be argued that the concentration we used might have been too much, since it significantly compromised functional recovery after sustained ischaemia, in both NonPostC and postC hearts (table 11 and figure 31). This decrease in functional recovery after the administration of A6730 further confirms inhibition of PKB/Akt, since PKB/Akt has been implicated in cardiac contraction and relaxation (Matsui *et al.*, 2001; Condorelli *et al.*, 2002; and Kim *et al.*, 2003). In this regard, Matsui and coworkers (2001) found in rats tranfected with a constitutively active PKB/Akt mutant, that PKB/Akt exerts a protective effect in the setting of ischaemia / reperfusion by decreasing cell death and increasing the functional

performance of viable cells. The inotropic effect of PKB/Akt is due to amongst others, increased sensitivity of the myofilaments to Ca^{2+} (Cittadini *et al.*, 2006), as well as optimisation of Ca^{2+} handling (Kim *et al.*, 2003; and Cittadini *et al.*, 2006). The latter has been linked to increased Ca^{2+}-current through the L-type Ca^{2+} channel (Kim *et al.*, 2003), as well as post-translational upregulation of SERCA2 (Kim *et al.*, 2003; and Cittadani *et al.*, 2006). The wide variety of cellular activities regulated by PKB/Akt complicates investigation into its precise role in cardioprotective mechanisms, such as postC. Regardless, use of this inhibitor at the concentration we administered, did demonstrate the importance of PKB/Akt in the cardioprotective mechanism of postC.

4.4.4. Inhibition of ERK p42/p44 using PD098059 in reperfusion

Contrary to expectations, inhibition of ERK p42/p44 using PD098059 in reperfusion, did not diminish the infarct sparing effect of postC (fig. 27), which suggests that ERK p42/p44 is not important in the mechanism of postC, in our model. Others have also shown that ERK p42/p44 is not involved in postC. For example, Feng *et al.* (2006) did not find elevated phosphorylation of ERK p42/p44 in response to isoflurane postconditioning in infarct-remodeled rat hearts. Our findings fit in with the phenomenon where increased phosphorylation of a kinase does not necessarily imply an increase in enzyme activity or functional importance of the kinase. This observation has been made in the setting of IPC: In 2000 Behrends *et al.* reported that MAPK phosphorylation does not correlate with the decrease in infarct size elicited by IPC. Bell and colleagues (2007) found that both ERK p42/p44 and PKB/Akt activity is of importance in angiotensin II preconditioning, both in the early and late phases of protection. However, at 6 hours reperfusion (between the two phases), increased phosphorylation of both kinases were observed in the absence of cardioprotection. In the case of postconditioning: Schwartz & Lagranha (2006) could not elicit a significant decrease in infarct size in swine hearts, although they could demonstrate an increase in the phosphorylation of both ERK p42/p44 and PKB/Akt (to the same extent as was measured in a cardioprotective preconditioning protocol). Although Zhu *et al.* (2006) demonstrated a cardioprotective effect for postC in remodeled rat hearts, associated with an increased phosphorylation of PKB/Akt and ERK p42/p44, only PKB/Akt showed increased activity in postC hearts, using *in vitro* kinase activity assays. ERK p42/p44 however, was not activated following the postC intervention, leading them to conclude that ERK p42/p44 is not primarily involved in postC protection. It is therefore possible that in our model, postC is associated with an increased phosphorylation of ERK

p42, without it actually being part of the cardioprotective mechanism – as suggested by the results obtained with PD098059.

Another possible confounder that might have had an effect on our results relates to the inhibitor, PD098059. This inhibitor was first described in 1995 by Dudley *et al.* as a specific inhibitor of the Mitogen Activated Protein Kinases (MAPKs), specifically ERK, by inhibiting the MAPK activating enzyme, MEK. In this original report, it was stated that PD098059 does not inhibit the MAPK homologues JNK and p38. Interestingly, later in 1995 Alessi and colleagues demonstrated that PD098059 does not inhibit the already phosphorylated form of MAPK kinase 1 (MAPKK1, more specifically MEK), but prevents the activation and phosphorylation of MAPKK1. Since these first descriptions of PD098059, it has been used as the main ERK p42/p44 inhibitor in studies to dissect signalling pathways. Another inhibitor which is commonly used is U0126. Just like PD, this inhibitor acts as a noncompetitive inhibitor of MEK, with a high specificity and affinity for MEK. In fact, its affinity seems to be higher than that of PD (Favata *et al.*, 1998). In our study, Western blot analysis of hearts treated with PD098059 in reperfusion showed three interesting effects of the drug in our model (figures 25 and 26).

First, in both postC and NonPostC groups treated with the drug there was a significant decrease in phosphorylated MAPK p38, compared to positive controls (postC without inhibitor). In fact, this decrease was more evident than the inhibition of ERK phosphorylation. This anomaly might be due to nonspecific inhibtion of p38, which is after all a MAPK analogue. Should this be the case, PD098059 administration also eliminated p38 MAPK as a functional role player in postC protection. However, in the light of the initial descriptions of PD (in which the specificity of the drug was highlighted), this explanation is unlikely.

The second and third surprises concern the phosphorylation of ERK p42/p44. Simultaneous statistical comparison of the NonPostC+PD, postC+PD and positive control (i.e. only postC) hearts (using ANOVA analysis) failed to indicate significant inhibition of ERK p44 by the inhibitor (fig. 26). This might be due to the small number of hearts in the positive control group (although this author thinks it is unlikely). It must be noted that leaving the NonPostC ERK p44 group out of statistical analysis (i.e. using an unpaired T test to compare phosphorylation levels of only the positive control and postC ERK p44

hearts) reveals a statistical difference between the positive control and postC ERK p44 groups (4.04 ± 0.17 AU vs 1.06 ± 0.16 AU; $p < 0.01$).

This observation highlights the third surprise, namely the absence of a significant reduction in ERK p42/p44 phosphorylation in the NonPostC group. In fact, NonPostC phospho-ERK44 was almost equal to the positive control (4.11 ± 1.28 AU and 4.04 ± 0.17 AU respectively). This unexpected observation might be due to the fact that PD098059 actually inhibits MEK 1/2, which is directly upstream of ERK p42/p44. The failure of PD to inhibit already activated MEK 1/2 (Alessi *et al.*, 1995) might also be a confounding effect. As described earlier, PD098059 has been used in various studies – including investigations to elucidate the signalling cascades involved in PostC. Darling *et al.* (2005) infused 10 µM of PD098059 for 30 minutes into rabbit hearts. In a study by Yang and colleagues (2004) they also administered 10 µM PD, for 20 minutes. This is the same concentration we utilised. In fact, it is the same concentration reported by Dudley *et al.* (1995), as the IC_{50} value of PD for the inhibition of either basal of partially activated MEK. It is therefore unlikely that the concentration we used was at fault. Currently we have no explanation for the failure of PD to inhibit ERK p42/p44 in NonPostC hearts.

A possible future route of investigation would be to use U0126 as an alternative inhibitor, and then compare it with our PD098059 data. Despite the unexpected observation that PD098059 did not inhibit ERK p42/p44 phosphorylation in NonPostC hearts, the inhibitor did inhibit the activation of ERK p42/p44 in postC hearts, i.e. the group of interest. For the purpose of this study, the dose and timing of PD administration was therefore adequate.

4.4.5. Summary

Two different timepoints in reperfusion (10 and 30 minutes), in both the working heart and Langendorff models, were investigated to assess the involvement of the RISK pathway (PKB/Akt and ERK p42/p44) and p38 MAPK in postC. We could only demonstrate a postC-associated increase in the phosphorylation of ERK p42 and PKB/Akt at 10 minutes reperfusion, in the retrogradely perfused Langendorff model. Inhibition studies revealed that it is only the increase in PKB/Akt phosphorylation that contributes to the infarct sparing effect of postC. Possible downstream components of the PI3-kinase – PKB/Akt pathway have been identified, in the setting of postC, as the mPTP (Bopassa *et al.,* 2006; Manintveld *et al.*, 2007), via GSK-3ß (Zhu *et al.*, 2006). Increased NO production has also

been implicated downstream of PKB/Akt, in PostC (Tsang *et al.*, 2004; Zhu *et al.*, 2006; Manintveld *et al.*, 2007).

Chapter 5: The effect of phosphatase inhibition in postconditioning and reperfusion

"The phosphorylation state of a protein is a dynamic process controlled by both protein kinases and protein phosphatases."

Wera S & Hemmings BA

***Biochem J* 1995; 311:17-29.**

Chapter 5: The effect of phosphatase inhibition in postconditioning and reperfusion

5.1. Background and motivation

Not much is known about the roles of the different phosphatases in the setting of ischaemia / reperfusion. The few studies that have been done, in general, demonstrate a pro-injury role for the phosphatases (Xiuhua *et al.*, 1997; Weinbrenner *et al.*, 1998; Armstrong *et al.*, 1998). This conclusion has been based on results obtained using pharmacological inhibition of some of the phosphatases, which caused protection against injury.

The precise mechanism of protection elicited by phosphatase inhibition is not known, but it seems obvious that it should be due to an increase in the phosphorylation state of proteins involved in protection. These proteins could be ion-channels and transporters (Carr *et al.*, 2002; Neumann *et al.,*1995), structural proteins (Fernandez *et al.,* 1990) or proteins involved in established protective pathways such as PKC or the mK^{+}_{ATP} – channel (Weinbrenner *et al.,* 1998; Armstrong *et al.,* 1997) or the reperfusion injury survival kinases (RISK).

It was therefore decided to investigate the possible role of the phosphatases in the mechanism of postC, by administering cantharidin, an inhibitor of protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A), in conjunction with our postC protocol. In view of the above, we hypothesise that inhibition of the phosphatases would be beneficial to the survival of ischaemic cardiac tissue, maybe even increasing the protective effects of postC. If indeed protein phosphatase inhibition were cardioprotective, the next question would be if it could be exerting its beneficial effects by increasing the activity of the protective reperfusion injury survival kinases that have been implicated in postC and IPC (i.e. PKB/Akt and ERK 1/2)?

5.2. Materials and methods

To investigate the possible role of the protein phosphatases in the settings of ischaemia / reperfusion and postC, cantharidin, a PP1 and PP2A inhibitor was administered at

different time points in the perfusion protocol. A stock solution of cantharidin was prepared by dissolving 5 mg of the inhibitor in 25 ml pure ethanol (AnalaR grade; Merck chemicals) to give a stock concentration of 102 μM. From this solution, 0.5 ml was diluted in 100 ml Krebs-Henseleit buffer to give a final concentration of 5 μM, with which the hearts were perfused.

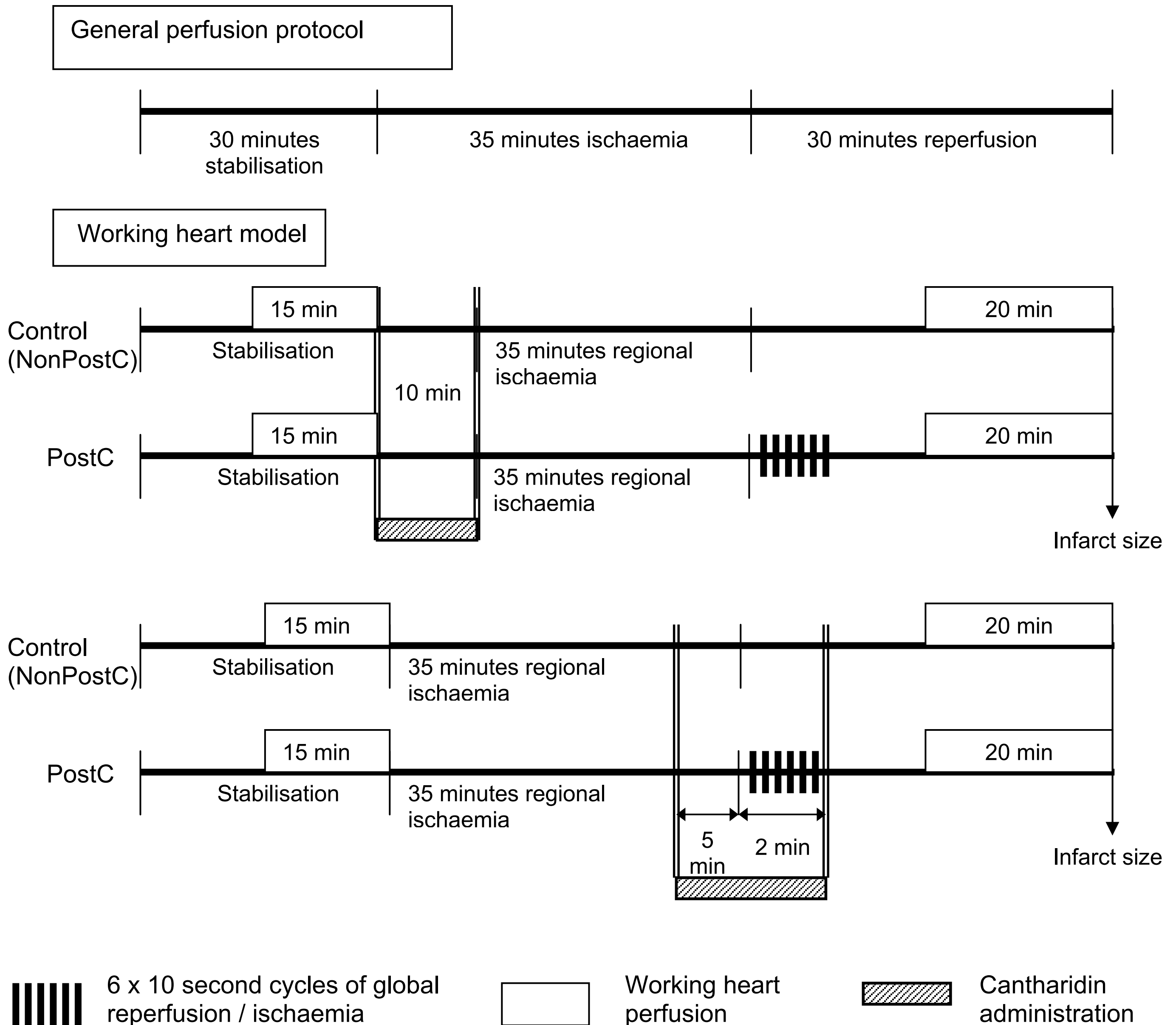


***Figure 32**: The study design used to investigate the effect of cantharidin on the infarct sparing effect of postC. Two different time points of cantharidin administration were investigated: either for a duration of 10 minutes directly before ischaemia, or during the final 5 minutes of ischaemia and the first 2 minutes of reperfusion; in each instance in the presence or absence of postC. Infarct size was used as the end-point of investigation.*

The same basic experimental protocol was followed, as previously described. Hearts were perfused in the working mode, with Cantharidin administered for a period of 10 minutes directly before sustained ischaemia (35 minutes regional ischaemia), or during the final 5 minutes of ischaemia and the first 2 minutes of reperfusion. This was done either in the presence or absence of the postC intervention (fig. 32). The postC intervention entailed 6 x 10 second cycles of global reperfusion / ischaemia, under strict thermal regulation during ischaemia (36.66 ± 0.11 °C) and the first 10 minutes of reperfusion (36.35 ± 0.09 °C). Infarct size (after 30 minutes reperfusion) was used as end-point. All other procedures are as described in chapter 2.

5.3. Results

5.3.1. Pre-treatment with cantharidin

For the investigation on the effect of Cantharidin pre-treatment (PreCanth) on infarct size, data from the NonPostC group that had been perfused at random with the postC group were pooled with data from a NonPostC group that had 10 minutes extra stabilisation (therefore directly comparable to the pre-treatment protocol). The end result was a NonPostC control group that comprised 17 hearts with a baseline AO of 40.29 ± 2.70 ml/min and a mean CO before ischaemia of 55.18 ± 2.70 ml/min, maintained during regional ischaemia at 36.65 ± 0.03 °C and during initial reperfusion at 36.62 ± 0.07 °C. The same postC-only group (n = 11; baseline AO = 40.00 ± 2.27 ml/min; baseline CO = 56.68 ± 2.87 ml/min) was used in the evaluation of the effects of cantharidin administration, both before ischaemia as well as in reperfusion. The effect of canthiridin pre-treatment on infarct size in the presence (PreCanth-postC: n = 7; AO = 45.14 ± 2.26 ml/min; CO = 63.86 ± 1.13 ml/min) and absence (PreCanth-NonPostC: n = 7; AO = 46.86 ± 2.13 ml/min; CO = 62.43 ± 2.26 ml/min) of postC is shown in figure 33.

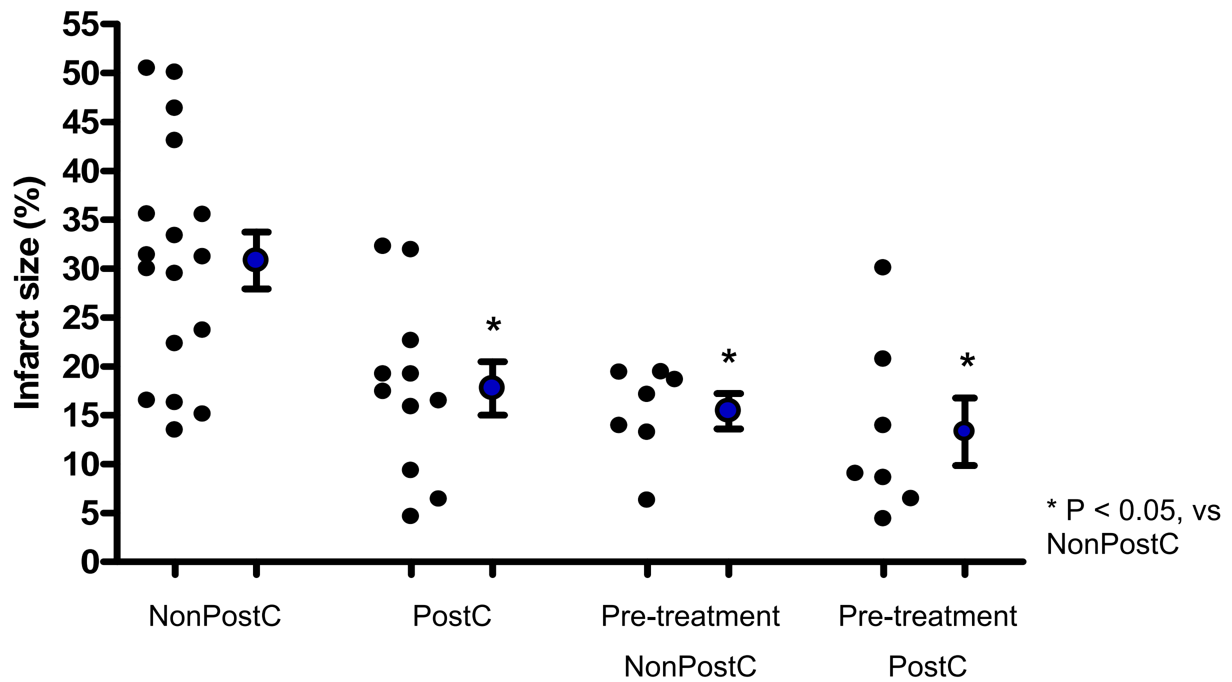


***Figure 33**: The effects of 10 minutes administration of a PP1 and PP2A inhibitor, cantharidin, immediately before sustained ischaemia on infarct size – in the presence and absence of a postC intervention.*

The significant reduction in infarct size elicited by postC (17.74 ± 2.72% vs NonPostC: 30.81 ± 2.90%, $p < 0.01$) has been described previously. Administration of cantharidin before sustained ischaemia also elicited the same degree of infarct size reduction (PreCanth-NonPostC: 15.42 ± 1.80% and PreCanth-postC: 13.30 ± 3.46%), irrespective of the presence or absence of postC. The total area at risk, in relation to the total cardiac surface area, could be a confounding factor in infarct size determination. The "area at risk" for the different groups are shown in figure 34.

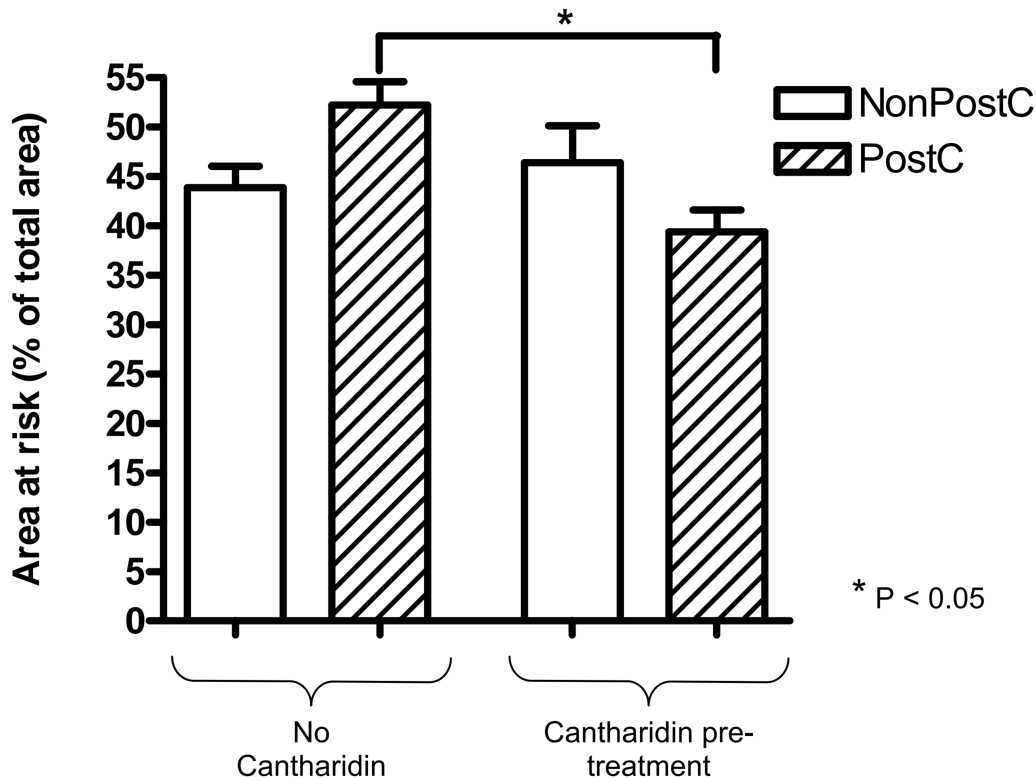


***Figure 34**: Area at risk (IFS + AR) in relation to the total area in the different treatment groups. There is a significant difference in the area at risk between the postC and cantharidin treated postC group. (NonPostC: n = 16; PostC: n = 9; PreCanth-NonPostC: n = 7; PreCanth-postC: n = 7).*

The area at risk in the postC group was found to be significantly higher than that of the postC group pre-treated with cantharidin (52.24 ± 2.38% vs 39.41 ± 2.21%). Since the infarct size of both these groups were similarly low (in comparison to NonPostC), it is unlikely that in this case, the measured area at risk is exerting a confounding effect. This was confirmed by the lack of significant correlation between the area at risk values and the infarct size data in both the postC and PreCanth-PostC groups (PostC: r-value = -0.31, p = 0.4133; PreCanth-PostC: r-value = -0.49, p = 0.3126).

Despite these significant reductions in infarct size associated with cantharidin pre-treatment and the postC intervention, these interventions could not induce a significant increase in functional recovery. Functional recovery of parameters after 30 minutes reperfusion, in the various experimental groups, are shown in table 12. Despite the lack of significant differences between the four groups (calculated using ANOVA), it does seem as if the PreCanth-NonPostC group tended to show weaker recovery. In fact, comparison of

only the pre-treated groups revealed a significant difference in aortic output recovery (PreCanth-NonPostC: 19.35±2.99% vs PreCanth-postC: 32.12±4.00%).

Table 12*: Recovery of functional parameters after 35 minutes regional ischaemia, in hearts exposed to combinations of postC, NonPostC and cantharidin pre-treatment. Although it seems as if hearts pre-treated with cantharidin, in the absence of postC, showed less recovery, it was not significant in an analysis taking all four groups into account.*

Intervention	Post-ischaemic function		Recovery (%)	
	AO (ml/min)	CO (ml/min)	AO (ml/min)	CO (ml/min)
NonPostC	5.42±1.60	20.04±2.67	9.11±2.97	37.11±4.49
PostC	2.40±1.36	20.90±2.36	5.30±2.80	37.06±4.04
PreCanth-NonPostC	1.00±1.00	12.07±1.88	2.27±2.27	19.35±2.99
PreCanth-PostC	5.57±2.53	20.71±2.90	11.36±5.09	32.12±4.00

As mentioned earlier, at certain concentrations cantharidin has been reported to elicit an inotropic effect. Neumann *et al.* (1995) reported that cantharidin increases the force of contraction of guinea-pig papillary muscles in a concentration dependent manner, reaching a maximum effect at 10 µM – although Boknik *et al.* (2001) found that 100 µM elevated force of contraction even further. To investigate the possibilty that the concentration of cantharidin we were using might induce vasoconstriction of the coronary arteries, coronary flow measurements were made at the end of Langendorff stabilisation, just before cantharidin administration, at the end of cantharidin perfusion and at the end of Langendorff reperfusion (i.e. after 10 minutes reperfusion; note that only measurements made during Langendorff perfusion were used). All hearts (both NonPostC and PostC) that received pre-treatment were included in this investigation, since the focus was on the period of cantharidin treatment, before the postC intervention. The effect of Cantharidin on coronary flow is shown in figure 35.

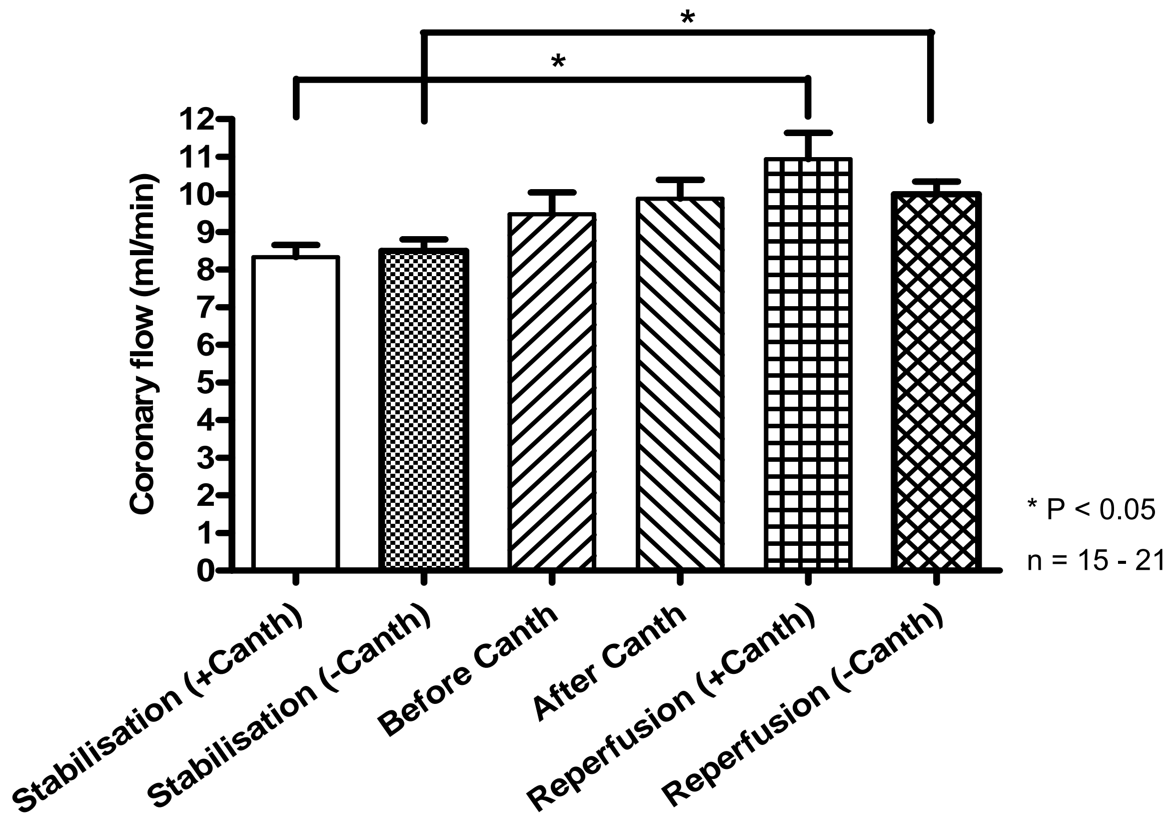


***Figure 35**: The effect of cantharidin administration on coronary flow. Measurements were taken at the end of Langendorff stabilisation, just before cantharidin pre-treatment, just after cantharidin administration and at the end of Langendorff reperfusion. This was done to investigate if cantharidin might have an effect of coronary artery tone. Coronary flow rates at the end of stabilisation and reperfusion, in hearts not treated with cantharidin, are also shown.*

Results show that a gradual increase in coronary flow occurs during the experimental protocol: at the end of Langendorff reperfusion the coronary flow rate is significantly higher than at the end of stabilisation (stabilisation: 8.33 ± 0.32 ml/min vs reperfusion: 10.93 ± 0.70 ml/min, $p < 0.01$). The same change in coronary flow rate was also observed in hearts which did not receive any canthadirin, but was also exposed to regional ischaemia and reperfusion (stabilisation: 8.50 ± 0.30 ml/min vs reperfusion: 10.00 ± 0.34 ml/min, $p < 0.01$) – ruling out a possible effect of canthadirin on the observed increase in coronary flow. This increase in coronary flow after ischaemia can probably be attributed to physical damage to the hearts, due to the tightening of the suture (i.e. the hearts tear slightly and therefore become more “leaky”). These results indicate that the cardioprotective effects we found to be associated with cantharidin, are not due to vasoconstriction.

5.3.2. Cantharidin treatment in reperfusion

In these studies administration of cantharidin (during the final 5 minutes of ischaemia and the first 2 minutes of reperfusion) was done in the presence (PostCanth-PostC: n = 13; baseline AO = 44.54 ± 2.27 ml/min; baseline CO = 61.77 ± 2.87 ml/min) or absence of postC (PostCanth-NonPostC: n = 10; baseline AO = 46.60 ± 2.58 ml/min; baseline CO = 64.75 ± 3.43 ml/min). Temperatures during ischaemia and the first 10 minutes of reperfusion were closely monitored and regulated in both groups, as described in chapter 3 (PostCanth-PostC: RI = 36.35 ± 0.15 °C; reperfusion = 36.24 ± 0.21 °C, and PostCanth-NonPostC: RI = 36.54 ± 0.12 °C; reperfusion = 36.44 ± 0.26 °C). Since these protocols did not employ an extra 10 minutes before ischaemia (contrary to the pre-treatment groups), the NonPostC hearts which were perfused with an extra 10 minutes stabilisation were excluded from the NonPostC goup – leaving 10 hearts with a baseline AO of 36.70 ± 4.22 ml/min and CO of 51.15 ± 4.62 ml/min. The same postC group as described in the above section (n = 11; baseline AO = 40.00 ± 2.27 ml/min; baseline CO = 56.68 ± 2.87 ml/min), was included as an experimental group.

As was the case with cantharidin pre-treatment, administration of the drug during reperfusion was associated with a decrease in infarct size in NonPostC hearts (NonPostC: 35.81 ± 3.67% vs PostCanth-NonPostC: 21.60 ± 2.79%, $p < 0.01$). Administration of cantharidin to postC hearts did not further reduce infarct size (postC: 17.74 ± 2.72% vs PostCanth-PostC: 15.39 ± 2.67%). Unlike the above described pre-treatment experiments, there were no significant differences between the area at risk values for the four groups. The area at risk values in the different groups are as follows: NonPostC: n = 9, 48.08 ± 2.96%; PostC: n = 9, 52.24 ± 2.38%; PostCanth-NonPostC: n = 10, 58.98 ± 1.50% and PostCanth-PostC: n = 13, 51.59 ± 3.31%. Infarct size data is shown in figure 36.

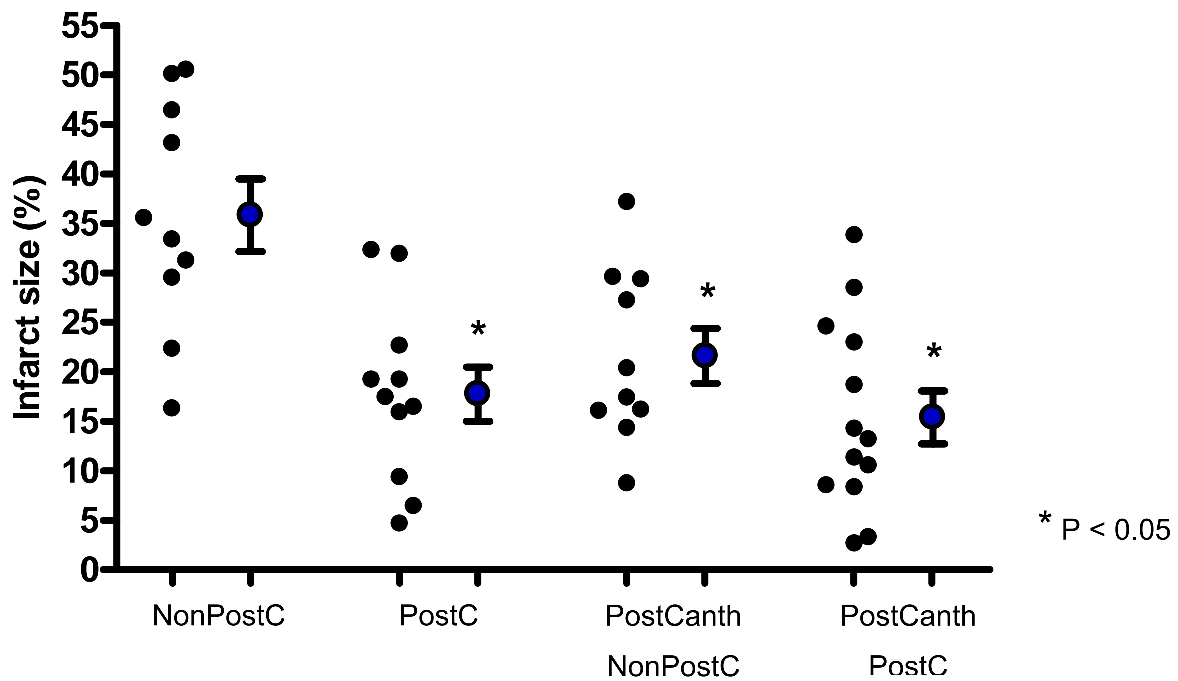


***Figure 36**: The effect of cantharidin administration during the final 5 minutes of ischaemia and the first 2 minutes of reperfusion, either with or without the application of a postC intervention, on infarct size. Cantharidin treatment reduced infarct size to the same extent as postconditioning.*

Just as observed in the pre-treatment groups, there was no significant increase in functional recovery in any of the treated hearts or in the postC group itself (table 13).

***Table 13:** Functional recovery in hearts exposed to 35 minutes regional ischaemia and 30 minutes reperfusion. Hearts were treated with cantharidin in reperfusion in the presence and absence of postC. No significant differences are present between the groups.*

Intervention	Post-ischaemic function		Recovery (%)	
	AO (ml/min)	CO (ml/min)	AO (ml/min)	CO (ml/min)
NonPostC	4.60±2.00	17.65±3.13	10.86±4.71	36.29±5.84
PostC	2.40±1.36	20.90±2.36	5.30±2.80	37.06±4.04
PostCanth-NonPostC	4.05±1.76	22.80±3.97	8.41±3.48	34.87±5.09
PostCanth-PostC	5.52±2.16	25.05±2.42	11.67±4.25	40.78±3.32

After 30 minutes reperfusion, the aortic output in the NonPostC group averaged 4.6 ± 2.00 ml/min, compared to 2.4 ± 1.36 ml/min in the the postC group. In hearts which received cantharidin in reperfusion, aortic output was 4.05 ± 1.76 ml/min in the NonPostC group and 5.52 ± 2.16 ml/min in the postC group. Cardiac output also declined to the same degree in all four groups: NonPostC = 17.65 ± 3.13 ml/min; postC = 20.90 ± 2.36 ml/min; PostCanth-NonPostC = 22.80 ± 3.97 ml/min and PostCanth-PostC = 25.05 ± 2.42 ml/min. The infarct sparing effect of cantharidin in our model, does therefore not seem to be associated with improved functional recovery during reperfusion.

5.3.3. Kinases and the infarct sparing effect of Cantharidin

In order to investigate the mechanism of cantharidin action in ischaemia / reperfusion, the drug was administered in the working heart model at the same time points as described above. Hearts were then freeze-clamped after 5 or 10 minutes of reperfusion. Western blotting (as described in chapter 2) was used to investigate the total and phosphorylated levels of p38 MAPK, ERK p42/p44 and PKB/Akt. Two different perfusion protocols were followed (fig. 37):

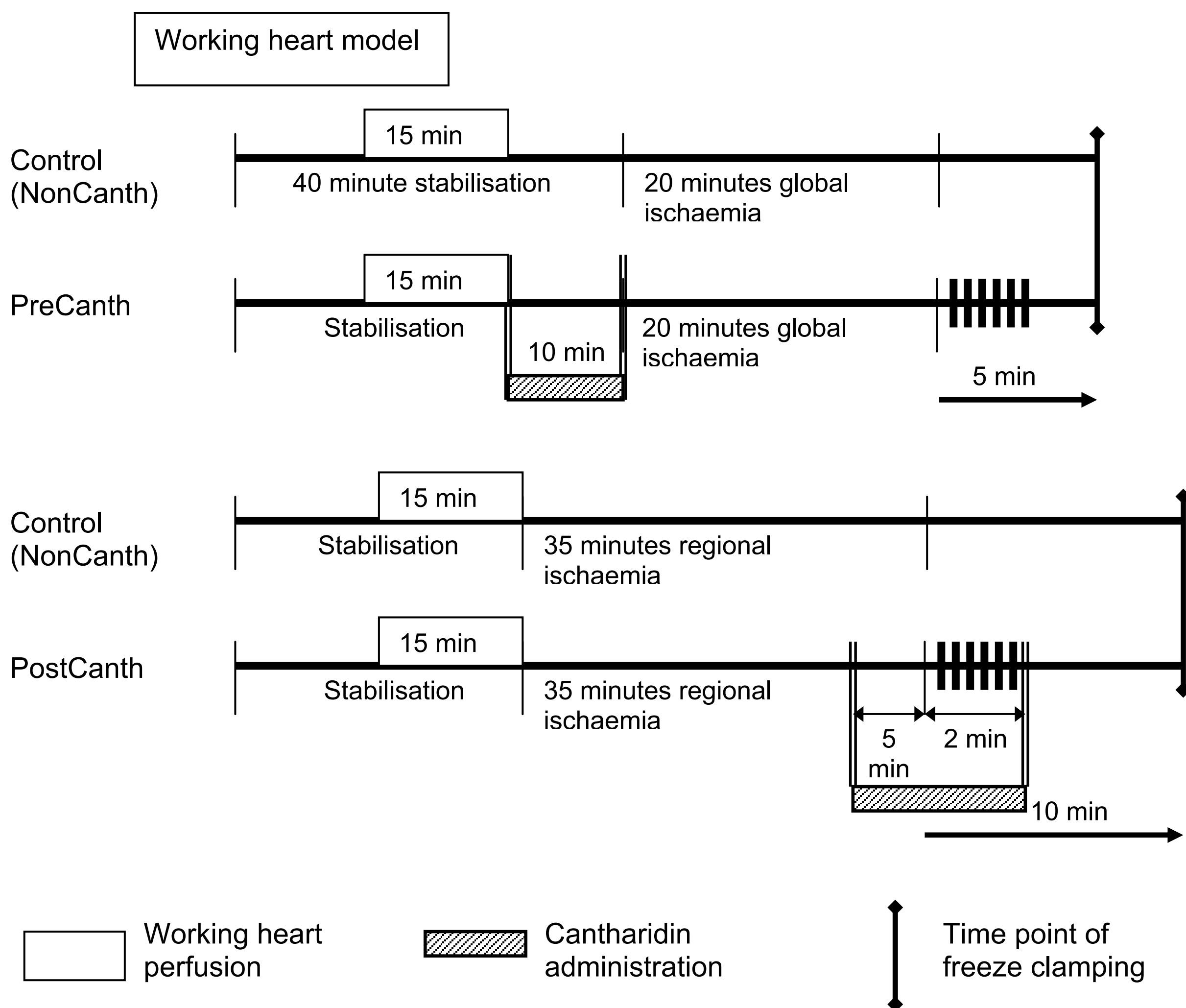


Figure 37*: Investigating the possible role of some of the signalling kinases in the mechanism of cantharidin cardioprotection. Cantharidin was administered either before ischaemia (PreCanth), or in the initial stage of reperfusion (PostCanth). Control hearts did not receive cantharidin (NonCanth). At this stage the postC protocol was not included in the experiments.*

5.3.3.1. Pre-treatment with Cantharidin

Two perfusion protocols were employed to investigate the effect of cantharidin administration before sustained ischaemia on the kinases of interest: In the PreCanth group (n = 5), cantharidin was administered for a period of 10 minutes directly before 20 minutes sustained global ischaemia and hearts were freeze-clamped after 5 minutes reperfusion. The NonCanth group (n = 5) followed the same protocol, but in the absence of any cantharidin (40 minutes stabilisation followed by 20 minutes global ischaemia and 5 minutes reperfusion) (fig. 37). Since we found thermal regulation during ischaemia and the first 10 minutes of reperfusion to be of importance in postC (see chapter 3), these hearts were also subjected to similar thermal regulation, i.e. approximately 36.5 – 37.3 °C during ischaemia / reperfusion. We were particularly interested in the effects on the kinase activities, it was therefore decided to use a global ischaemic model, which yields homogeneous tissue for analysis. It is important to note that, since the focus of this part of the investigation was solely on the activity of cantharidin, the postC-intervention was not applied in any experimental protocol. Baseline functional values of the two experimental groups are shown in table 14.

***Table 14**: Baseline functional values of hearts used for the investigation into the effect of cantharidin pre-treatment on the kinase profiles.Hearts were either treated with cantharidin before sustained ischaemia (PreCanth; n = 5) or perfused without intervention (NonCanth; n = 5).*

Intervention	Baseline values	
	AO (ml/min)	CO (ml/min)
NonCanth	46.40 ± 2.23	61.90 ± 2.68
PreCanth	51.20 ± 1.02	67.10 ± 1.56

Total protein and phosphorylation profiles associated with Cantharidin pre-treatment are shown in figure 38. No differences in total protein levels were observed, concerning any of the kinases of interest. This was not the case with the phosphorylation profiles. Interestingly Cantharidin pre-treatment was associated with a significant increase in the phosphorylation of p38 MAPK (PreCanth: 2.49 ± 0.17 AU vs NonCanth: 1.52 ± 0.26 AU, $p < 0.05$). Increased phosphorylation of ERK p42/p44 and PKB/Akt, in these experiments, were not recruited in the cardioprotection imparted by the administration of Cantharidin before sustained ischaemia.

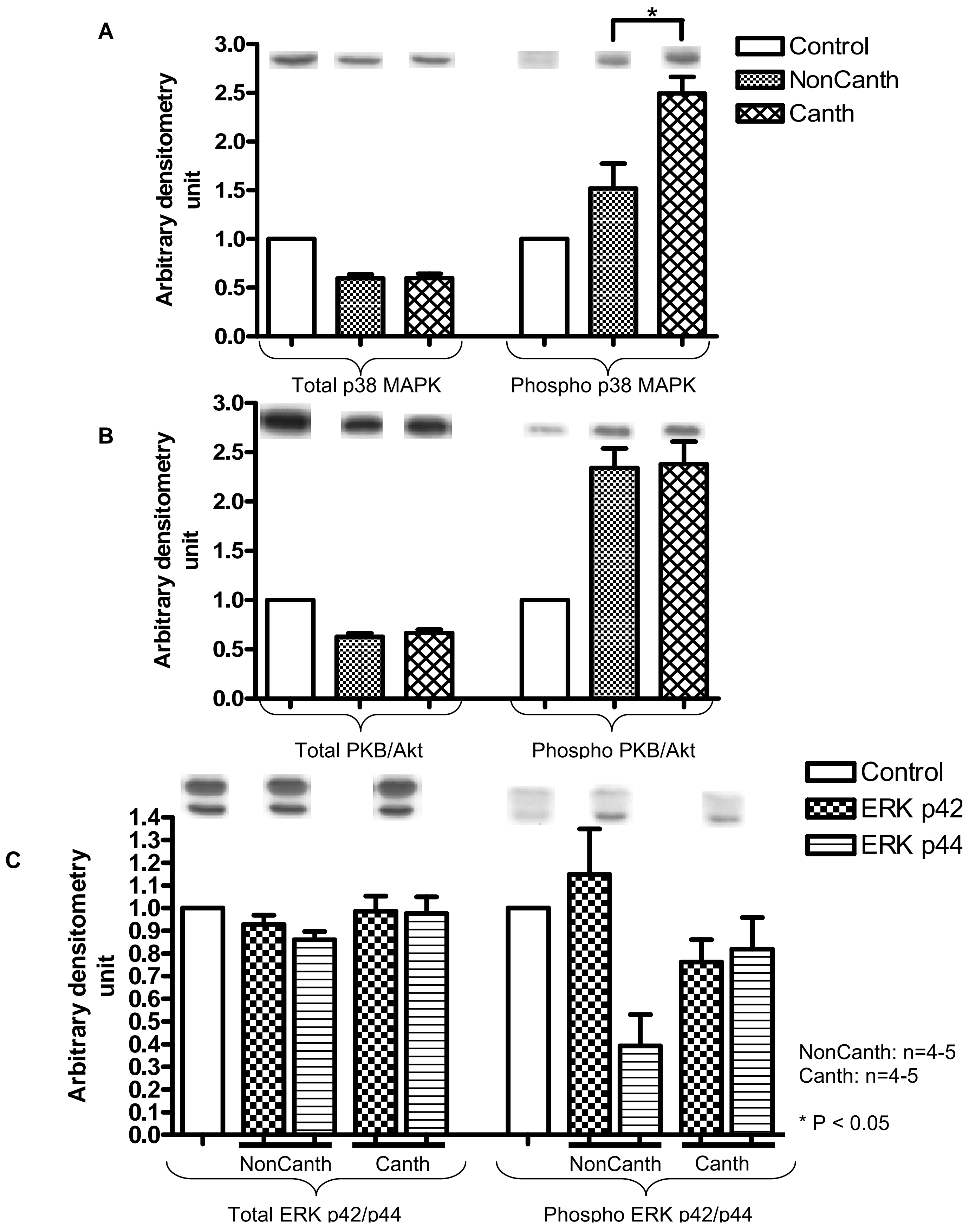


***Figure 38**: Levels of total and phosphorylated p38 MAPK (A), PKB/Akt (B) and ERK p42/p44 (C) after treatment with Cantharidin, prior to a 20 minute global ischaemic insult in the working heart model. Hearts were freeze-clamped at 5 minutes reperfusion. Cantharidin pre-treatment was not associated with any changes in the kinase profiles, except for a significant increase in phosphorylated p38 MAPK. Control hearts; n=2.*

5.3.3.2. Cantharidin treatment in reperfusion

To investigate cantharidin administered in reperfusion (Post-Canth), the same protocol was followed as was used in the investigation into the roles of the kinases in postC. Basically, hearts were stabilised and then subjected to 35 minutes of regional ischaemia, followed by 10 minutes of reperfusion. Two experimental groups were perfused: In the PostCanth-group (n = 6) cantharidin was administered in the final 5 minutes of ischaemia and the first 2 minutes of reperfusion, without the postC-intervention being co-administered. The second group served as a control group in which hearts did not receive any cantharidin (NonCanth, n = 6). Hearts were then freeze-clamped at 10 minutes reperfusion, since our study on the kinases in postC showed this time point to be important. All hearts were subjected to the same thermal regulation as described in chapter 3. Baseline functional data of the hearts used in these experimental groups are shown in table 15. It was decided, that in these experiments the ischaemic and non-ischaemic zones of the heart would be freeze-clamped separately. Since, our interest lay primarily with the ischaemic tissue, only these samples were analyzed using Western blotting (as described in chapter 2).

Intervention	Baseline values	
	AO (ml/min)	CO (ml/min)
NonCanth	48.00 ± 3.39	64.00 ± 3.22
PostCanth	39.67 ± 0.92	65.92 ± 0.91

Table 15*: Pre-ischaemic functional parameters as measured in the two experimental groups, i.e. either in the presence (PostCanth) or absence (NonCanth) of cantharidin during the final 5 minutes of ischaemia and the first 2 minutes of reperfusion. None of the experimental groups included a postC intervention.*

Results of the Western blotting analysis are shown in figure 39. As was the case with pre-treatment, cantharidin given during the final 5 minutes of regional ischaemia and the first 2 minutes of reperfusion did not alter the total levels of any of the kinases under investigation. Cantharidin administered in reperfusion did however exert an effect on the phosphorylation of some of the kinases (fig. 39). Similar to the effect of pre-treatment on p38 MAPK, cantharidin given in reperfusion was also associated with a significant increase in the phosphorylation of p38 MAPK (NonCanth: 5.64 ± 1.17 AU vs Post-Canth: 10.69 ± 1.29 AU, $P < 0.05$). In addition, administration of cantharidin in reperfusion was associated with a significant increase in the phosphorylated form of ERK p42 (NonCanth: 2.24 ± 0.21 AU vs Post-Canth: 3.34 ± 0.37 AU, $p < 0.05$), which was not the case with pre-treatment.

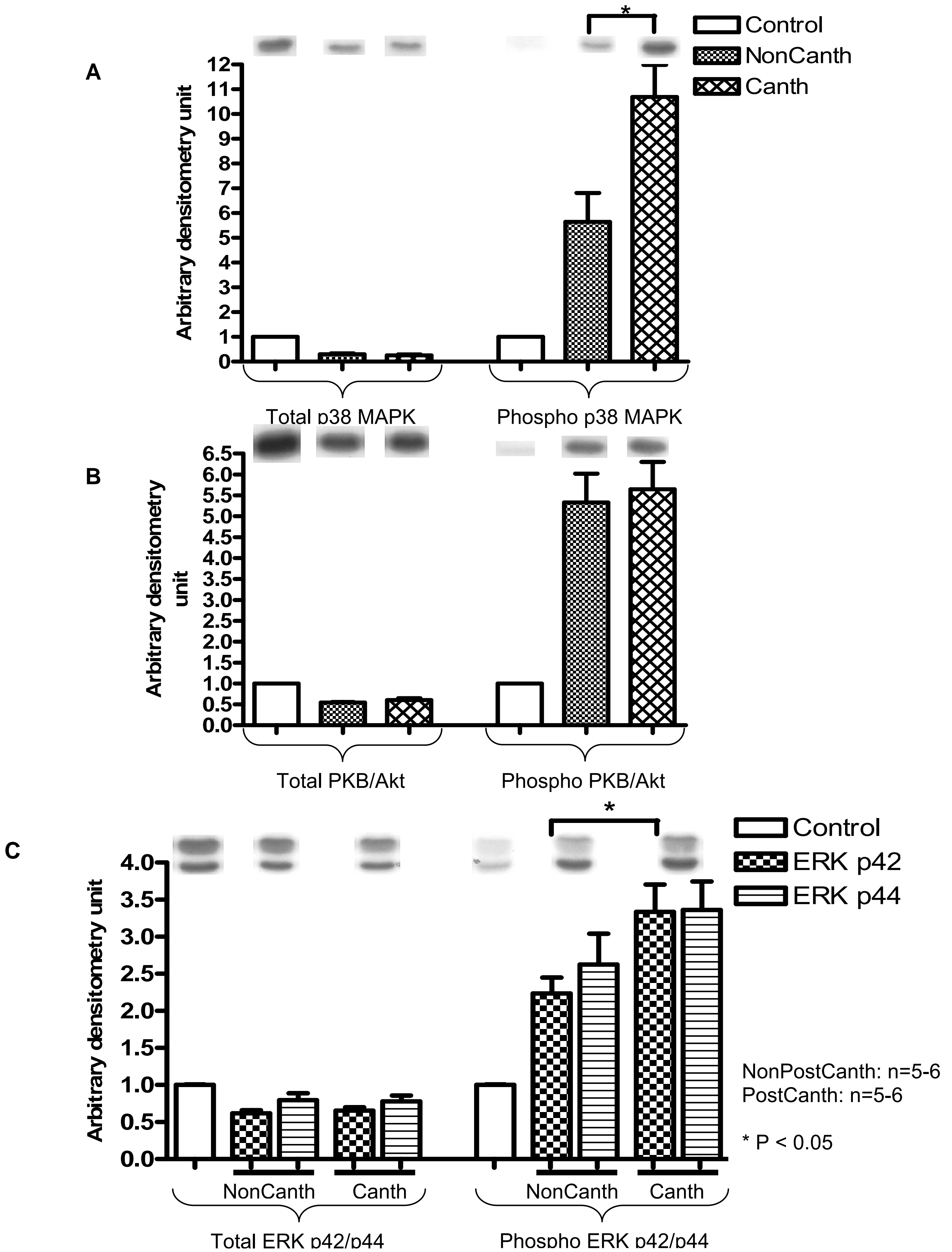


***Figure 39**: Total and phosphorylated p38 MAPK (A), PKB/Akt (B) and ERK p42/p44 (C) profiles at 10 minutes reperfusion, after 35 minutes regional ischaemia, in the presence or absence of Cantharidin during reperfusion. Cantharidin administration was associated with a significant increase in phosphorylated p38 MAPK and ERK p42. Control hearts; n=2.*

5.3.4. Vehicle controls

To assess the possible influence of the solvent used in the administration of cantharidin, hearts were treated with only 500 µL pure ethanol / 100 ml Krebs-Henseleit buffer, instead of cantharidin, in a pre-treatment protocol and a reperfusion protocol (as described for canthadirin in figure 37).

Ethanol administered for a duration of 10 minutes directly before 20 minutes global ischaemia, did not exert any effect on the phosphorylation of the kinases under investigation (n = 5), in comparison to hearts subjected to a similar protocol, but with no ethanol or drug administration (n = 5) (data not shown).

In the reperfusion protocol ethanol was administered for the final 5 minutes of regional ischaemia and the first 2 minutes of reperfusion. After 10 minutes reperfusion hearts were divided into ischaemic and non-ischaemic zones and both these segments freeze-clamped. As described in the cantharidin experiments, the tissue exposed to ischaemia was analysed using Western blotting. Hearts treated with ethanol (PostEtOH: n = 5, baseline AO = 48.80 ± 2.33 ml/min, baseline CO = 66.10 ± 2.86 ml/min) were compared to hearts exposed to a similar protocol, but in the absence of ethanol administration (NonEtOH: n = 4, AO = 46.50 ± 4.65 ml/min, CO = 62.13 ± 5.11 ml/min). Ethanol administered in reperfusion did not exert an effect on ERK p42/p44 (data not shown). There was however an association between ethanol reperfusion and a significant increase in the phosphorylation of p38 MAPK (NonEtOH: 0.31 ± 0.05 AU vs PostEtOH: 0.60 ± 0.07 AU; $p < 0.05$) and PKB/Akt (NonEtOH: 1.81 ± 0.24 AU vs PostEtOH: 2.88 ± 0.24 AU; $p < 0.05$) (figure 40).

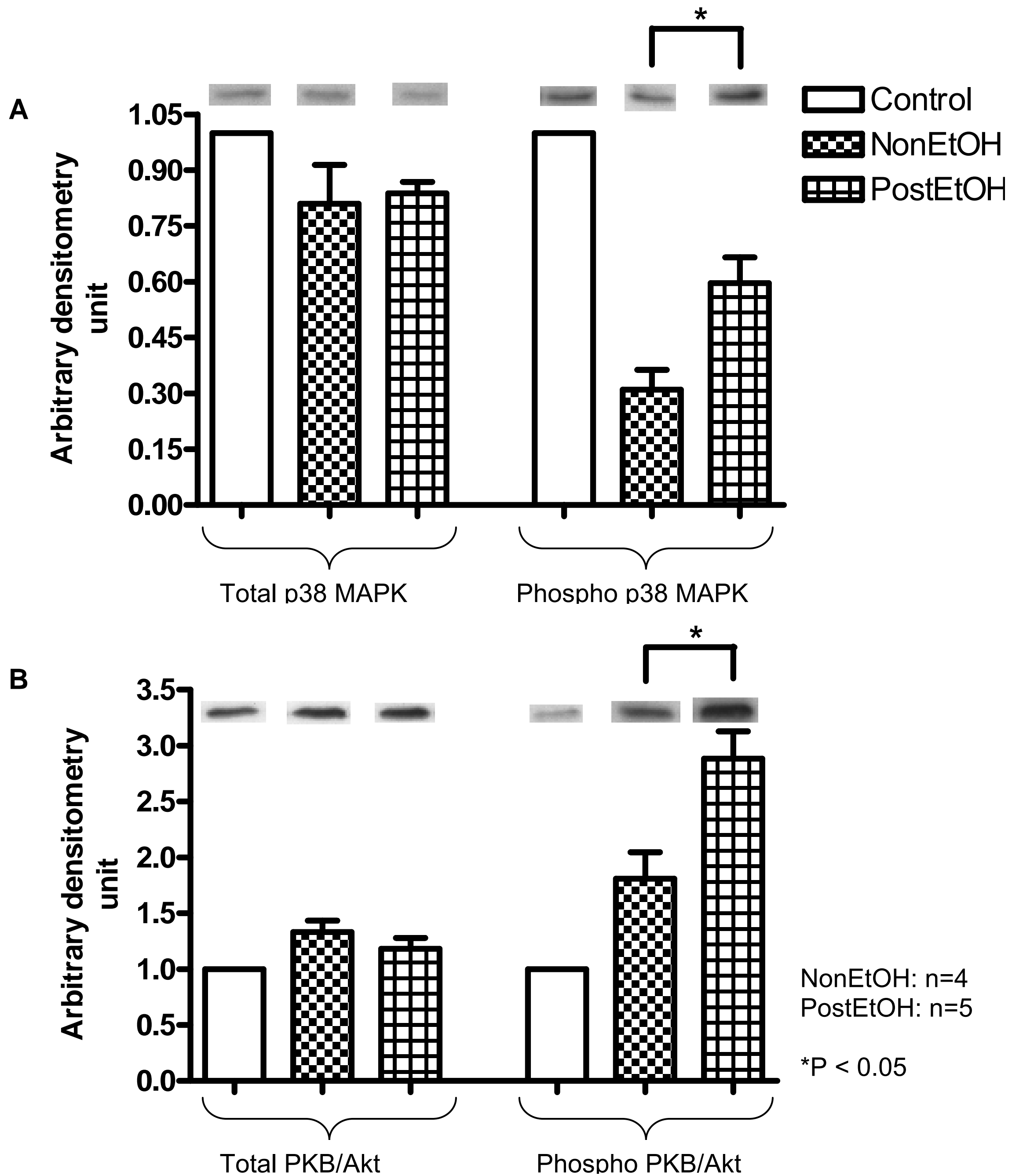


Figure 40*: The effect of ethanol administered in the final 5 minutes of ischaemia and the first 2 minutes of reperfusion on p38 MAPK and PKB/Akt in tissue exposed to ischaemia. Ethanol was associated with a significant increase in the phosphorylation of both these kinases, without exerting an effect of the total protein content.*

5.4. Discussion

5.4.1. Effect of cantharidin treatment on infarct size

Treatment with Cantharidin, either directly before ischaemia or in reperfusion, was associated with a decrease in infarct size (figures 33 and 36). This cantharidin-induced decrease in infarct size was observed in the presence, as well as the absence of the postC-intervention. Just as was the case with our initial studies on postC in the working heart model, we could not elicit any improvement in functional recovery in either the postC hearts, or hearts treated with cantharidin (again in the presence and absence of postC, given as a pre-treatment or in reperfusion). These results confirm the conclusions made in chapter 3, namely that a reduction in infarct size fails to show a relationship with an improvement in functional recovery in the *ex vivo* working heart model, and that eliciting functional recovery in the postconditioned working heart is quite a challenge.

The finding of a protective effect due to phosphatase inhibition, in the context of ischaemia / reperfusion is not a new one, and has been reported in the literature by several workers. Similar to our pre-treatment experiments: In 1997 Armstrong and co-workers reported that administration of a protein phosphatase inhibitor, fostriecin, for a period of 10 minutes directly before sustained ischaemia, protected isolated cardiomyocytes. These authors in fact suggested that it mimicked preconditioning. Xiuhua *et al.* (1997) similarly reported that, in vascular smooth muscle cells, 10 minutes incubation with okadaic acid (a protein phosphatase inhibitor) before a hypoxic preconditioning protocol, followed by hypoxia and reoxygenation, elicited a protective preconditioning-like effect. Armstrong *et al.* (1998) found that the administration of the protein phosphatase inhibitors, calyculin A and fostriecin, 75 minutes into ischaemia still protected rabbit cardiomyocytes – even to the same degree as pre-incubation before ischaemia. They speculated that this protection might be due to the increased phosphorylation of structural proteins (which then maintain cellular structural intergrity) and proteases (favouring inhibition of these enzymes).

Cardioprotection associated with phosphatase inhibition in reperfusion has been reported by Fenton *et al.* (2005), who investigated the effect of phosphatase inhibition on preconditioning in the aged rat heart. In agreement with our results, they reported that okadaic acid administered in reperfusion, in young rat hearts, was associated with a significant reduction in infarct size (31%). Interestingly, in this same model, when they

administered okadaic acid for 25 minutes before ischaemia, they did not observe a significant reduction in infarct size – contrary to our pre-treatment results. Contrary to our results and Fenton *et al.* (2005), Hausenloy and co-workers (2002) reported that administration of FK508 (an inhibitor of the phosphatase calcineurin) in reperfusion exerted no infarct sparing effect in the isolated rat heart.

Except for this latter study, it seems clear that phosphatase inhibition is associated with protection. The precise mechanisms by which phosphatase inhibition however protects cells, still have to be determined.

There was no additive cardioprotective effect observed with the combination of postC and cantharidin (either as a pre-treatment or administered in reperfusion). In fact the decrease in infarct size elicited by postC, cantharidin pre-treatment and postC combined with cantharidin were all of the same magnitude, with no significant differences between these groups (percentage decreases in infarct size are shown in table 4). This lack in additive infarct sparing effect might be due to a maximum degree of protection elicted by each of these treatment strategies. It could also be that these two strategies share the same mechanism, in which case postC protects by inhibiting phosphatases – this however seems unlikely, as will be discussed later in the text.

Table 4*: Percentage decreases in infarct size compared to the NonPostC group used in the cantharidin pre-treatment experiments (with an infarct size of 30.81 ± 2.90%). Decreases induced by postC and cantharidin treatment, either on its own or in combination with postC, are of the same magnitude. There does not seem to be a cumulative infarct sparing effect when Cantharidin is administered together with a postC intervention.*

	NonCanthadirin (%)		Cantharidin pre-treatment (%)		Cantharidin in reperfusion (%)	
NonPostC			49.95		29.90	
PostC		42.42		56.83		50.05

After the initial description of postconditioning, the possible additive protection that might be elicited by a combination of ischaemic preconditioning and postconditioning was investigated. Contradictory results were reported. Halkos *et al.* (2004) could not

demonstrate an additive effect when both these interventions were applied in their canine model. Both IPC and postC however elicited a comparable degree of protection (both IPC and postC in their model elicited an approximate 50% decrease in infarct size). Similar results were also reported by Tsang *et al.* (2004) in isolated perfused rat hearts. On the other hand, Yang and colleagues (2004) reported that IPC and postC both reduced IFS from 62% to between 35% and 40% in their rabbit model. However, co-administration of these interventions significantly reduced IFS even further to 22%, demonstrating an additive effect which argues against the possibility that postC on its own elicits a maximum degree of protection. More comparable to our work is a study done by Chiari *et al.* (2005). They found that co-administration of a non-protective postC intervention (3 x 10 seconds), together with a non-protective level of isoflurane in reperfusion (0.5 MAC administered three minutes before and two minutes after reperfusion) induced a decrease in infarct size from 41% to 17%, equal to a protective postC protocol (3 x 20 seconds, IFS: 20%) or a higher protective dose of isoflurane (1.0 MAC, IFS: 21%). Similarly, Weihrauch and coworkers (2005) found that the combination of non-protective concentrations of morphine and isoflurane during reperfusion (final 3 minutes of ischaemia and the first 2 minutes of reperfusion) elicited an infarct sparing effect equal to the effect exerted by any one of the two drugs, at double the concentration. In both these cases it could be argued that the drugs administered at the higher concentration in fact elicited a maximum degree of protection, with the co-administration of sub-protective interventions showing an additive effect – until the maximum protection was reached. In 2006 Tessier-Vetzel *et al.* reported that co-administration of isoflurane with an ischaemic postC protocol (4 x 30 seconds) enhanced the infarct sparing effects of the postC intervention. This was true with 2% isoflurane administered throughout their experiments, as well as only in reperfusion. Studies on the combination of a drug (a p53 inhibitor) and anesthetic postconditioning (Venkatapuram *et al.*, 2006), as well as combination of anesthetic preconditioning and anesthetic postcondtitioning (Obal *et al.*, 2005) all report enhancement of protection. However, Deyhimy *et al.* (2007) reported no additive effect with a combination of sevoflurane preconditioning and postconditioning.

It therefore seems possible that, in principle, an additive degree of protection can be be elicited when postC is combined with other interventions, although a maximum degree of protection can be reached. The relevant question therefore seems to be when is the applied postC protocol already eliciting a maximum degree of protection? Another closely related question would be whether the additional treatment acts via the same

cardioprotective mechanism as postC. If so, it makes even more sense that a maximum degree of protection can be reached, when all the components of the relevant mechanism have been maximally recruited. Since we saw a comparable degree of infarct size reduction between cantharidin treatment and postC, without an additive effect when the two are co-administered, we decided to investigate if cantharidin was indeed recruiting the same kinases as postC in its protection mechanism.

5.4.2. Cantharidin pre-treatment: effect on kinase profiles

The effect of cantharidin, administered directly before the onset of ischaemia, on the kinase profiles are shown in figure 38. Although there might have been enough time for protein synthesis (35 minutes, of which 20 minutes was global ischaemia) between the initiation of drug administration and eventual freeze-clamping, no differences in total protein levels were observed. This was not unexpected, since in all likelihood, cantharidin is conferring its cardioprotective effect through the modulation of existing proteins and kinases.

Interestingly, pre-treatment was associated with a significant increase in the phosphorylation of p38 MAPK. Again we are confronted with the problem whether p38 MAPK activation is protective. Interestingly, although PP2A has been implicated in p38 MAPK regulation (Doza *et al.*, 1995), it seems that this kinase is especially regulated by PP2C (Millward *et al.*, 1999). The observation that phosphatase inhibitors are associated with an increase in phosphorylated p38 MAPK is however not new. Armstrong *et al.* (1998) reported that administration of calyculin A before the onset of ischaemia increased the level of phosphorylated p38 MAPK during ischaemia. In a later paper they speculated (Armstrong *et al.*, 1999) that the protection associated with phosphatase inhibition might in fact be due to the increase in phosphorylated p38 MAPK, which then recruits the protective heat shock protein, HSP27 (they found that a p38 MAPK inhibitor, SB203580, was associated with an accelerated ischaemic dephosphorylation of HSP27 and development of injury in cardiomyocytes). On the other hand, Mackay & Mochly-Rosen (2000) found that administration of vanadate (a tyrosine phosphatase inhibitor) increased the phosphorylation of p38 MAPK during ischaemia, which then favoured an increase in cell damage. Inhibiting p38 MAPK (using SB203580) decreased cell damage, even in the presence of vanadate. These controversial observations, however, could be due to species differences (rabbits vs rats, respectively).

The question is therefore whether the increased p38 MAPK phosphorylation observed in our rat heart model, is of any significance in the increased cardioprotection induced by pre-treatment with cantharidin. Depending on the dominantly activated p38 MAPK isoform, this increase in phosphorylation might be protective. Saurin and co-workers (2000) however speculate that it might be the detrimental α-isoform which increases during ischaemia. This corresponds to the observation that protective ischaemic preconditioning is associated with an attenuated increase in phosphorylated p38 MAPK during ischaemia and reperfusion (Marais *et al.*, 2005). In fact, Moolman and colleagues (2006) report that the administration of a p38 MAPK inhibitor (SB203580) 10 minutes directly before ischaemia conferred protection against both infarct size development and apoptosis. This then leaves us with two options: either the increased phosphorylation due to phosphatase inhibition favours a protective isoform of p38 MAPK, or the observed increase is a cellular response associated with cantharidin which does not affect the cardioprotection elicited by the drug. Clearly, further investigation is required.

5.4.3. Cantharidin in reperfusion: effect on kinase profiles

As expected after a time lapse of just 8 minutes (between cantharidin administration and freeze-clamping), no significant differences in total protein levels were observed (fig. 39). These results indicate that, as with cantharidin pre-treatment, cantharidin in reperfusion is eliciting its cardioprotective effect through the modulation of existing proteins

The question is therefore: which proteins / kinases are being modulated by cantharidin in reperfusion? In our model we found that cantharidin administration in reperfusion was associated with significant increases in the phosphorylation of both p38 MAPK and ERK p42 (fig. 39).

As discussed in the pre-treatment group, the increase in p38 MAPK phosphorylation is probably due to the inhibitory effects of cantharidin on PP1 and PP2A activity, causing maintainance of phosphorylated p38 MAPK, which was generated during ischaemia and reperfusion. The question however remains whether this observed significant activation is actively contributing to protection, or not? As discussed above, it seems unlikely that increased p38 MAPK phosphorylation is cardioprotective in the rat heart.

Activation of ERK p42/p44 during reperfusion has been implicated in cardioprotection – both in ischaemic preconditioning (Hausenloy *et al.*, 2005) and postconditioning (Darling *et*

al., 2005; Yang *et al.*, 2004). In our experiments we found a slight increase in ERK p42/p44 phosphorylation in the first 10 minutes of reperfusion, compared to control hearts which were freeze-clamped directly after stabilisation (in ERK p42: NonCanth = 10611 ± 1006 total pixels vs control = 4750 ± 30.34 total pixels; and ERK p44: NonCanth = 4139 ± 659.2 total pixels vs control = 1577 ± 467.8 total pixels). It is therefore conceivable that this slight ischaemia / reperfusion-induced increase in phosphorylation is further enhanced and sustained in the presence of cantharidin during reperfusion. The phosphorylation of ERK p42/p44 is known to be regulated by amonst others PP2A (Anderson *et al.*, 1990; Sontag *et al.*, 1997; and Millward *et al.*, 1999), it is therefore not surprising that a PP2A inhibitor favours ERK p42/p44 phosphorylation.

Interestingly, cantharidin did not enhance PKB/Akt activation during reperfusion. PKB/Akt also presented with an increase in activation in the presence of only reperfusion (approximately a 5 fold increase in phospho-PKB/Akt in NonCanth hearts, compared to control hearts exposed to only 30 minutes perfusion). This absence of increased phosphorylation is difficult to explain, as cantharidin is an inhibitor of PP1 / PP2A and PP2A plays a role in the the regulation and dephosphorylation of PKB/Akt (Andjelkovic M *et al.*, 1996; Millward *et al.*, 1999; and Ugi *et al.*, 2004). We can only speculate that there might be other factors, beside the protein phosphatases, that are dephosphorylating PKB/Akt in our experimental setup. In this regard, PTEN (a protein tyrosine phosphatase) has also been identified as a negative regulator of the PI3-kinase – PKB/Akt pathway (Stambolic *et al.*, 1998). It must be remembered that the phosphorylation status of the kinases is the result of the balance between phosphorylating and dephosphorylating factors. Inclusion of cantharidin in our model definitely favours phosphorylation, but it could be, that for some proteins the equilibrium still remains shifted in the direction of dephosphorylation during reperfusion. Identifying all the factors involved in the determination of this balance is still an ongoing scientific pursuit.

5.4.4. Vehicle controls

Ethanol administered for a period of 10 minutes immediately before 20 minutes global ischaemia did not influence the expression or phosphorylation of any of the kinases under investigation. This confirms that the increased phosphorylation of p38 MAPK, associated with cantharidin pre-treatment, is indeed due to the action of the phosphatase inhibitor.

Administration of ethanol during reperfusion was however associated with signficant increases in the phosphorylation of both p38 MAPK and PKB/Akt (fig. 40). Not much work has been done on the effect of ethanol on the kinases in cardiac tissue. Studies that have been done on the effects of ethanol in other tissues have reported contradictory results: in the rat hippocampus ethanol increased the activation of MAPK p38 and ERK p42/p44 (Ku *et al.*, 2007); in rat testes (Koh *et al.*, 2007) and brain (Han *et al.*, 2006) ethanol was found to reduce the activation of PKB/Akt and ERK p42/p44, while Zhang *et al.* (2005) found that ethanol decreased phospho-ERK p42/p44 and exerted no effect on p38 MAPK in murine cardiomyocytes. Differences in ethanol administration and dosage, as well as model used and tissue studied, makes it virtually impossible to compare our results to those reported by others. The possible effects of ethanol in cardiomyocytes also fall outside the scope of this text.

Although it could be argued that the increase in phosphorylated p38 MAPK, associated with cantaridin administration during reperfusion, is actually due to the ethanol solvent present, it is also very likely that cantharidin at least contributed to this increase. Especially since we observed an ethanol-independent increase in p38 MAPK phosphorylation associated with cantharidin pre-treatment, confirming this stimulatory effect of phosphatase inhibition in our model.

The absence of increased PKB/Akt phosphorylation associated with cantharidin administration is even more surprising in view of the observed stimulatory effect of ethanol. Cantharidin has been associated with the increased phosphorylation of various proteins (Neumann *et al.*, 1995). It could therefore be speculated that cantharidin somehow interferes with the signalling cascade triggered by ethanol. At this stage, we are however unable to explain the absence of increased PKB/Akt phosphorylation in the presence of both ethanol and cantharidin (since both are expected to increase PKB/Akt phosphorylation).

It has been reported that transient exposure to ethanol before a period of sustained ischaemia, confers protection in both the intact heart, as well as cardiomyocytes (Chen *et al.*, 1999). For a review on the acute effects of ethanol administration, in the setting of ischaemia / reperfusion, see Krenz *et al.* (2004). Interestingly, Krenz *et al.* (2001) found that ethanol blocks its own protective effect, when it remains present during the sustained ischaemia. Although we did not investigate the effect of only ethanol on IFS, it is unlikely to

be a confounding factor in our model. In our pre-treatment experiments ethanol was still present during ischaemia, since there was no wash-out period between drug administration and ischaemia. Theoretically, the ethanol present would therefore not exert a protective effect. Other workers have reported the protective effects of phosphatase inhibition, confirming our results. In our lab phosphatase inhibition using okadaic acid, dissolved in DMSO, elicited the same protective profile as cantharidin. Although further investigation could be done on the effects of ethanol in reperfusion, we are confident that the infarct-sparing effects observed is attributable to cantharidin.

Despite the unexpected effects of the solvent on the kinase profiles, it seems reasonable to still conclude that cantharidin administered during reperfusion increases the phosphorylation of ERK p42 and p38 MAPK (fig. 39). The infarct sparing effect of cantharidin administered in reperfusion (fig. 36) remains independent of PKB/Akt activation.

5.4.5. Summary

In summary, we are now left with two interesting questions and one noteworthy observation. We found that p38 MAPK is significantly activated in reperfusion, in cantharidin treated hearts (pre-treatment, as well as in reperfusion). Cantharidin is also cardioprotecive and decreases infarct size. The question that therefore still needs to be adressed is whether p38 MAPK is important in this protection. To further investigate this problem infarct size could be monitored in the presence of a p38 MAPK inhibitor (such as SB203580), administered together with cantharidin – as a pre-treatment, but especially in reperfusion.

The same question can also be asked concerning the role of the upregulation of phosphorylated ERK p42 in the cardioprotection, observed with the administration of cantharidin in reperfusion. As with p38 MAPK, this question can possibly be answered by the administration of an ERK p42/p44 inhibitor such as PD098059 or U0126, together with cantharidin, during reperfusion.

Despite the above mentioned uncertainties and questions still to be investigated, we did observe an unexpected and definite lack of PKB/Akt activation in the presence of cantharidin. This is contrary to our observation in postconditioned hearts, in which we found a definite important role for the activation of PKB/Akt, during reperfusion, in the

cardioprotective mechanism of postconditioning. We can therefore conclude that cantharidin seems to elicit its cardioprotective effect through a different mechanism than postconditioning. However, despite these differences in protective mechanisms recruited, co-treatment of hearts with both a postC-intervention and cantharidin do not elicit a greater infarct sparing effect, than each on its own. Should it be the case that cantharidin is exerting its protective effect through a different mechanism than postC, it becomes even more difficult to explain why cantharidin and postC do not have additive protective effects. Another explanation for the lack of additive protection, despite separate signalling / mediator pathways, might be that both interventions recruit the same protection end-effector(s). However, in my view, it is most likely that each intervention on its own elicited a maximum degree of protection, which can not be increased further.

Another possibility to be considered is that cantharidin is eliciting its cardioprotective effect primarily through mechanisms that do not include the signalling kinases (discussed in chapter 1). Other possible cardioprotective candidates, that have been implicated in phosphatase inhibition, include an increased phosphorylation and stabilisation of structural proteins (Fernandez *et al.,* 1990) or more optimal handling of intracellular calcium (Neumann *et al.,* 1995; and Carr *et al.*, 2002) and sodium (Chen *et al.,* 1995).

Chapter 6: Conclusion

"A cheerful heart is good medicine…"

Proverbs 17:22

The Bible

Chapter 6: Conclusion

6.1. Developing a postconditioning protocol

In this study we endeavoured to develop a cardioprotective postconditioning protocol in the isolated perfused rat heart, using the retrogradely perfused Langendorff model, as well as the working heart model. The purpose of establishing these postC protocols was to characterise the kinases involved in postC cardioprotection, as well as to investigate the possible role of the protein phosphatases in the mechanism of postC.

In an effort to optimise postC protection, we focussed our attention on four different parameters:

I. The number of cycles of reperfusion and ischaemia: we applied 3, 4 and 6 cycles in different protocols.
II. The duration of each cycle: we experimented with 10, 15, 20 and 30 second cycles.
III. The mode of postC application: this refers to either manipulating the flow of perfusate to the heart as a whole, or manipulating flow to only the tissue exposed to the sustained ischaemic insult (by opening and closing the coronary artery ligature).
IV. The temperature of the hearts during ischaemia and early reperfusion, especially during the postC intervention itself.

We found that a 6 x 10 second global reperfusion / ischaemia protocol, applied at a temperature of approximately 37 °C, conferred an infarct sparing effect in both the working heart, as well as Langendorff models. We could however only demonstrate functional recovery in the Langendorff model. Our results suggest that although postC can confer cardioprotection, it is an intervention that is very sensitive to experimental conditions and it is not as robust in exerting protection as ischaemic preconditioning. It might be that the animal model we used (the rat) is particularly less sensitive to the cardioprotection conferred by postC. In the first postC experiments done in rats, it was noted that postC did not impart such a potent degree of protection, as observed in the dog and rabbit (Kin *et al.*, 2004). It is however a fact that postC protection has been reported to be subject to experimental variation in all laboratory animal species tested (Vinten-Johansen *et al.*, 2007). Nevertheless, it is encouraging that postC could limit infarct size in the energy

demanding working heart model. Although Sasaki *et al*. (2007) has also investigated postC in a working heart model, this study is the first to demonstrate the infarct sparing effect of a postC protocol in the working heart, similar to the protocols that have been successfully used in the Langendorff model. We could therefore show that postC can limit infarct size, even under conditions of hightened energy metabolism, as occurs in the working heart.

6.2. Signalling kinases involved in postconditioning

Characterisation of the kinase profiles (specifically PKB/Akt, p38 MAPK and ERK p42/p44) in our postC intervention in both *ex vivo* models, at 10 and 30 minutes reperfusion, yielded disappointing results: only at 10 minutes reperfusion in the Langendorff model an increase in phosphorylated ERK p42 and PKB/Akt, associated with postC, could be observed. This indicated that the kinase signalling cascase component of the postC mechanism is active only in the early minutes of reperfusion. Using pharmacological inhibition studies, we could demonstrate a functional role, in postC cardioprotection, for PKB/Akt, but not for ERK p42/p44. In our model, postconditioning is therefore associated with an increase in the phosphorylation of ERK p42 and PKB/Akt early in reperfusion, although only PKB/Akt is necessary in the mediation of the cardioprotective effect.

6.3. The role of protein phosphatases in postconditioning

For the investigation into the possible role of PP1 and PP2A in postC, an inhibitor of both (cantharidin) was administered. Inhibition of these serine / threonine phosphatases immediately before sustained ischaemia, as well as in reperfusion conferred an infarct sparing effect independent of postC, suggesting an alternative cardioprotective mechanism. Further investigation in the working heart revealed that cantharidin administration was associated with an increase in the phosphorylation of p38 MAPK and, when administered in reperfusion, also ERK p42. Although we did not determine the functional importance of these kinases in cantharidin protection, the difference in kinase activation profile indicates that cantharidin protects the hearts through a different signalling pathway, than postC. It therefore seems unlikely that postC is dependent on phosphatase inhibition in its mechanism.

6.4. Limitations and future directions

Although we found an elevated temperature to be important in eliciting postconditioning, it is a confounding factor which we only identified, but did not further investigate. It would be interesting and of experimental value to characterise the precise impact of temperature on postC efficacy. This could be done by systematically applying different temperatures during postconditioning. Such experimentation would however require even more strict thermal control and accurate temperature measurements than is currently the accepted standard.

The lack of phosphorylation of the kinases investigated in association with postC, observed in the working heart, might be due to the fact that tissue samples had to be divided into non-ischaemic and ischaemic zones. In the experimental setup we used this was a necessity. This procedure can however be avoided using a global ischaemia protocol. This would entail the development of a postC protocol in the working heart model that confers functional protection after global ischaemia – a feat we could not accomplish, or else measurement of infarct size in the globally ischaemic heart. The latter procedure has been found to be problematic, due to patchy necrosis which is difficult to quantify (data not shown).

Although we could not demonstrate functional importance for the postC-associated upregulation of ERK p42, this might be due to the inhibitor used. It would therefore be worthwhile to investigate the effect of another ERK p42/p44 inhibitor, U0126, on the infarct sparing effect of postC.

We found an increase in the phosphorylation of p38 MAPK associated with postC after 10 minutes reperfusion in tissue not exposed to prior sustained ischaemia, in the working heart model. This unexpected phenomenon could be addressed by investigating the effect of the postC protocol itself on healthy heart tissue *per se.* This would entail application of the brief postC cycles in hearts not exposed to a prior ischaemic insult, followed by freeze-clamping and analysis of the signalling kinases. It would be interesting to identify intracellular changes initiated by the protocol itself, independent of cells being damaged or exposed to stress.

The observation that postC and cantharidin cardioprotection is associated with different kinase profiles in reperfusion leaves us with two questions:

I. Is cantharidin mediating its protection through the observed increase in phosphorylated p38 MAPK and ERK p42? To investigate this, inhibitors of these kinases (SB203580 against p38 MAPK and PD098059 and / or U1026 against ERK p42/p44) could be co-administered with cantharidin, or immediately after cantharidin treatment.
II. If cantharidin and postC are indeed working through different mechanisms, why do the combination of these two interventions not shown an additive effect? It should however be ascertained if they are truly working through different pathways. We found that they recruit different signalling pathways, but end-effectors of importance in both interventions should also be identified. Especially the mPTP could be of importance. It has received a lot of attention in postC literature but less has been done in the context of phosphatase inhibition.

Another limitation of this study was that we only characterised the kinase profile associated with cantharidin in one experimental model, namely the working heart. Since we only observed kinase participation in the postC mechanism in the Langendorff model, it would be interesting to also characterise cantharidin in the Langendorff model.

We unexpectedly found that the solvent we used in our cantharidin experiments (ethanol) exerted an effect on the kinases, when administered during reperfusion. Although, it seems unlikely that this effect is a confounding factor in this study, it would be advisable to use a different vehicle in future studies with cantharidin. In this regard, Knapp *et al.* (1998) reported that they used DMSO in their cantharidin studies. DMSO was shown not to affect the kinase profile in our experimental setup.

It should be noted that one of the difficulties in investigating the phosphatases is the lack of truly specific inhibitors. Cantharidin is no exception, although it only inhibits PP1 and PP2A, it influences various intracellular proteins (Neumann *et al.*, 1995). A more specific inhibitor could therefore also be used, such as okadaic acid, which primarily inhibits PP2A at a concentration of 1 nM. Another approach to avoid the broad spectrum effects of the phosphatase inhibitors could be to investigate phosphatase activity localisation. This could be done by investigating only certain cellular fractions (for example Schaffer & Punna, 1993, who only focused on the sarcolemmal fraction of rat cardiomyocytes). It could be

possible that postC does recruit phosphatase inhibition, but only in certain cellular compartments – such as possibly the mitochondrial membrane or sarcolemma.

6.5. Summary

We confirmed that the application of brief cycles of reperfusion / ischaemia, directly at the onset of reperfusion, can reduce the infarct size associated with a sustained ischaemic episode, in the retrogradely perfused isolated rat heart. We also demonstrated that this phenomenon of postC can also be elicited in the more rigoruous working heart model. The cardioprotective effect of postC is mediated by the early reperfusion activation of PKB/Akt. We could not find evidence that postC recruits phosphatase inhibition in the signalling component of its cardioprotective mechanism. The inhibition of the protein phosphatases PP1 and PP2A however, does confer a powerful infarct sparing effect.

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