

*The role of protein  
phosphatases in myocardial  
ischaemia and reperfusion*

*WenJun Fan*



*Thesis presented in partial fulfillment  
of the requirements for the degree of  
Master of Medical Physiology at the  
University of Stellenbosch*

*Promotors: Prof Amanda Lochner  
Prof Johannes Moolman*

*December 2007*

## **Declaration**

**I, the undersigned, hereby declare that this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university in order to obtain an academic qualification.**

---

### **ABSTRACT**

Protein kinases and phosphatases play important roles in the phosphorylation state of intracellular proteins under both physiologic and pathophysiologic conditions. Compared to the large number of studies investigating the significance of kinases, in particular the mitogen-activated protein kinases (MAPKs) in myocardial ischaemia/reperfusion and ischaemic preconditioning, relatively few studies have been done on the protein phosphatases in this scenario. Although several role players in the signal transduction cascade of ischaemia/reperfusion and ischaemic preconditioning have been identified thus far, the exact mechanism of cardioprotection still remains unclear.

Previous studies from our laboratory have shown that the stress kinase, p38 MAPK, has a dual role in preconditioning: it acts as trigger of the process, while attenuation of its activation during sustained ischaemia and reperfusion is required for cardioprotection. Since the activation of p38 MAPK is dependent on both the upstream kinases for phosphorylation and phosphatases for dephosphorylation, we hypothesized that the balance between the activation state of the MAPKs and the induction of phosphatases may play a major role in determining the fate of cardiomyocytes exposed to ischaemic stress.

The objectives of this study were: (i) to assess the activity of the myocardial protein phosphatases (PSPs and PP1) during sustained ischaemia and during reperfusion of non-preconditioned and ischaemic preconditioned hearts; (ii) to evaluate the significance of these phosphatases in ischaemia/reperfusion as well as in ischaemic preconditioning using available appropriate inhibitors; (iii) to give particular attention to the role of the phosphatase, mitogen-activated protein kinase phosphatase-1 (MKP-1), in ischaemia/reperfusion. MKP-1 is upregulated by stress conditions and selectively inactivates p38 MAPK by dephosphorylation of the regulatory Thr and Tyr residues. The glucocorticoid, dexamethasone which increases MKP-1 expression, was used as agonist to upregulate MKP-1 experimentally.

The isolated perfused working rat heart was used as experimental model. After stabilization, hearts were subjected to either a one-cycle or multi-cycle ischaemic preconditioning protocol, followed by sustained global or regional ischaemia and reperfusion. Non-preconditioned hearts were subjected

to ischaemia/reperfusion only. For Western blot analysis of MAPKs, PKB/Akt and MKP-1, hearts were freeze-clamped at different times during the perfusion protocol. Endpoints were infarct size, functional recovery and phosphorylation of the MAPKs (ERK and p38 MAPK) and PKB/Akt during reperfusion. Expression of MKP-1 was monitored.

The results obtained showed that activation of PSPs and PP1 does not occur during sustained global ischaemia or reperfusion of non-preconditioned and preconditioned hearts. The role of the phosphatases was subsequently further investigated using two inhibitors namely cantharidin (5  $\mu$ M, a concentration which inhibits both PP1 and PP2A) and okadaic acid (7.5 nM, a concentration which inhibits PP2A selectively). Administration of cantharidin or okadaic acid during the preconditioning phase, completely abolished preconditioning induced cardioprotection as indicated by mechanical failure during reperfusion and increased infarct size, associated with increased phosphorylation of p38 MAPK and PKB/Akt and dephosphorylation of ERK42/44. These results suggest a role for PP2A in the trigger phase of preconditioning. Administration of cantharidin or okadaic acid during early reperfusion of preconditioned hearts improved functional recovery. This was associated with increased phosphorylation of ERK42/44 and PKB, but not p38 MAPK.

Dexamethasone, administered intraperitoneally to rats for 10 days (3mg/kg/day) or directly added to the perfusate (1  $\mu$ M) resulted in significant cardioprotection of hearts subjected to 20 min sustained global ischaemia, followed by 30 min reperfusion. This is associated with a marked upregulation of MKP-1 and dephosphorylation of p38 MAPK during reperfusion.

These studies suggest that the phosphatases are definitely involved in the phenomenon of ischaemia/reperfusion and ischaemic preconditioning. However, it also become clear that extensive further research is required to fully elucidate which phosphatases are involved and the mechanisms thereof. Due to the large size of the protein phosphatase family, this may prove to be a formidable task and far beyond the scope of this thesis. The results also suggested that pharmacological targetting of phosphatases involved in phosphorylation of the reperfusion injury salvage kinase (RISK) pathway (e.g. ERK42/44 and PKB/Akt) or dephosphorylation of pro-apoptotic kinases, such as p38 MAPK, may have significant clinical potential.

---

**ABSTRAK**

Proteïenkinases en fosfatases speel 'n belangrike rol in die fosforileringsstatus van intrasellulêre proteïene in beide fisiologiese en patofisiologiese toestande. In teenstelling met die groot aantal studies gedoen ten einde die rol van die kinases, veral die mitogeen-geaktiveerde proteïenkinases (MAPKs), in iskemie/herperfusie en iskemiese prekondisionering te ondersoek, is relatief min bekend aangaande die rol van die fosfatases in hierdie scenario. Hoewel verskeie rolspelers in die seintransduksieprosesse van iskemie/herperfusie en iskemiese prekondisionering reeds geïdentifiseer is, is die presiese meganisme van miokardiale beskerming steeds onbekend.

Vroeëre studies vanuit ons laboratorium het getoon dat die streskinase, p38 MAPK, 'n tweeledige rol in prekondisionering speel: dit is 'n sneller ("trigger") van die proses, terwyl verlaagde aktivering tydens volgehoue iskemie en herperfusie, noodsaaklik vir beskerming is. Ons hipotese is dus dat die balans tussen die aktiveringstatus van die MAPKs en induksie van fosfatases die oorlewing van kardiomyosiete blootgestel aan iskemiese stres, bepaal.

Die doelwitte van hierdie studie was: (1) bepaling van die aktiwiteit van miokardiale proteïen fosfatases (PSPs en PP1) tydens volgehoue iskemie en herperfusie van nie-geprekondisioneerde en iskemies-geprekondisioneerde harte; (ii) evaluering van die belang van fosfatases in iskemie/herperfusie beskadiging sowel as in iskemiese prekondisionering deur van geskikte inhibiteurs gebruik te maak; (iii) ondersoek na die rol van die fosfatase, mitogeen-geaktiveerde proteïen kinase fosfatase-1 (MPK-1) in iskemie/herperfusie beskadiging. Dit is bekend dat MKP-1 deur strestoestande opgereguleer word en p38 MAPK selektief deur defosforilasie van die regulatoriese Thr en Tyr residue inaktiveer word. Die glukokortikoïed, deksametasoon, wat MKP-1 uitdrukking stimuleer, is as agonis gebruik ten einde MKP-1 eksperimenteel op te reguleer.

Die geïsoleerde, geperfuseerde werkende rothart is as eksperimentele model gebruik. Na stabilisasie, is die harte aan 'n enkel- of veelvuldige siklus iskemiese prekondisioneringsprotokol onderwerp, gevolg deur volgehoue globale of streeksiskemie. Nie-geprekondisioneerde harte is slegs aan iskemie/herperfusie onderwerp. Harte is op verskillende tye tydens die perfusieprotokol

gevriesklamp vir Western blot analise van die MAPKs, PKB/Akt en MKP-1. Infarkt grootte en funksionele herstel tydens herperfusie is as indikatore van iskemiese beskadiging gebruik. Fosforilering van MAPKs en PKB/Akt sowel as uitdrukking van MKP-1 tydens vroeë herperfusie is gemonitor.

Die resultate toon dat aktivering van PSP en PP1 tydens volgehoue iskemie en herperfusie nie plaasvind nie. Die rol van die fosfatases is verder ondersoek deur van twee inhibiteurs gebruik te maak, naamlik cantharidin (5  $\mu$ M inhibeer beide PP1 en PP2A) en okadaic suur (7.5 nM inhibeer PP2A selektief). Toediening van of cantharidin of okadaic suur tydens die pre-kondisioneringsprotokol, hef pre-kondisionering-geïnduseerde beskerming totaal op, soos aangetoon deur hartversaking tydens herperfusie en 'n toename in infarkt grootte, tesame met 'n toename in die fosforilering van p38 MAPK en PKB/Akt en defosforilering van ERK42/44. Hierdie waarnemings dui op 'n rol vir PP2A as sneller in pre-kondisionering. Toediening van hierdie inhibiteurs tydens vroeë herperfusie het ook die miokardium beskerm, soos aangetoon deur 'n verbeterde meganiese herstel van gepre-kondisioneerde harte, tesame met 'n verhoogde fosforilering van ERK42/44 en PKB (maar nie p38 MAPK nie).

Deksametasoon, intraperitoneaal toegedien, vir 10 dae (3mg/kg/dag) of direk by die perfusaat gevoeg (1 $\mu$ M), het tot 'n hoogs beduidende beskerming teen iskemiese beskadiging gelei van harte blootgestel aan 20 min globale iskemie en 30 min herperfusie. Hierdie toename in funksionele herstel en afname in infarkt grootte het met 'n toename in MKP-1 uitdrukking en defosforilering van p38 MAPK gepaard gegaan.

Bogenoemde resultate dui op 'n definitiewe betrokkenheid van fosfatases in iskemie/herperfusie en iskemiese pre-kondisionering. Dit is egter ook duidelik dat intensiewe verdere navorsing benodig word om die presiese rol van die fosfatases te bepaal. Vanweë die grootte van die fosfatase familie, val dit egter buite die beskeik van hierdie studie. Ten slotte, die resultate toon dat farmakologiese manipulasie van fosfatases betrokke by die fosforileringstatus van anti-apoptotiese kinases soos ERK42/44 en PKB/Akt en defosforilering van pro-apoptotiese kinases, soos p38 MAPK, besondere kliniese toepassings mag hê.

## Acknowledgements

I would like to express my sincerest thanks to the following persons:

My **father** and **mother**, and **family** for their love and support.

Professor **Amanda Lochner** for her excellent supervision, constant support, encouragement and patience.

Professor **Johannes Moolman** for his supervision and support.

Professor **Johan Koeslag** for his excellent support and guidance.

**Colleagues** at the Department of Medical Physiology for their advice and support.

My **friends** for their constant encouragement and support during this study.

**INDEX**

	<b>Page No.</b>
<b>Declaration</b>	<b>II</b>
<b>Abstract</b>	<b>III</b>
<b>Abstrak</b>	<b>V</b>
<b>Acknowledgements</b>	<b>VII</b>
<b><u>CHAPTER 1</u> PROTEIN PHOSPHATASES.</b>	
1 General introduction	1
1.2 Literature Review	
1.2.1 Protein Phosphatases	2
1.2.1.1 Protein serine/threonine phosphatases	4
1.2.1.2 Dual specificity phosphatases	6
1.2.1.3 Protein tyrosine phosphatases	8
1.2.1.4 Protein histidine phosphatases	9
1.2.1.5 Phosphatase and tensin homolog deleted on chromosome ten	10
1.2.2 Introduction to preconditioning	
1.2.2.1 Ischaemic preconditioning: a brief description	11
1.2.2.2 Signalling in preconditioning	12
1.2.3 Protein phosphatases in the heart	
1.2.3.1 Effects on contractility and relaxation	14
1.2.3.2 Effects of ischaemia/reperfusion on phosphatase activity	16
1.2.3.3 Preconditioning and phosphatases	17
1.3 Aims of study	20
<b><u>CHAPTER 2</u> MATERIALS AND METHODS</b>	
2.1 Animals	21
2.2 Materials	21
2.3 Perfusion technique	21
2.4 Determination of infarct size	22
2.5 Assay of protein phosphatases	23
2.6 Western blots	23
2.7 Experimental protocols	24
2.8 Statistics	25
<b><u>CHAPTER 3</u> EFFECT OF ISCHAEMIA/REPERFUSION AND PRECONDITIONING ON MYOCARDIAL PSP AND PP1 ACTIVITIES</b>	
3.1 Introduction	26



---

3.2	Methods	27
3.3	Results	27
3.4	Discussion	30
<b>CHAPTER 4</b> MANIPULATION OF PP1 AND PP2A ACTIVITY IN ISCHAEMIC PRECONDITIONING: EFFECTS ON MECHANICAL RECOVERY AND KINASE ACTIVATION PATTERNS DURING REPERFUSION		
4.1	Introduction	32
4.2	Methods	32
4.3	Results	
4.3.1	Preliminary studies	35
4.3.2	Effect of preconditioning on cardiac mechanical recovery during reperfusion	35
4.3.3	Effects of cantharidin	
4.3.3.1	Post-ischaemic functional recovery of non-preconditioned hearts	38
4.3.3.2	Post-ischaemic functional recovery of preconditioned hearts	38
4.3.3.3	Effects of cantharidin on infarct size	45
4.3.3.4	Effect of cantharidin on kinase activation before sustained global ischaemia (GI)	47
4.3.3.5	Effect of cantharidin on kinase activation during reperfusion	50
4.3.4	Effect of okadaic acid	
4.3.4.1	Effect of okadaic acid on post-ischaemic functional recovery	57
4.3.4.2	Effects of okadaic acid on infarct size	60
4.3.4.3	Effect of okadaic acid on kinase activation during reperfusion	62
4.4	Discussion	65
<b>CHAPTER 5</b> ROLE OF MITOGEN-ACTIVATED PROTEIN KINASE PHOSPHATASES (MKP-1) ON RESPONSE OF HEART TO ISCHAEMIA/REPERFUSION: EFFECT OF DEXAMETHASONE		
5.1	Introduction	71
5.2	Methods	72
5.3	Results	
5.3.1	Effect of dexamethasone on cardiac mechanical recovery	74
5.3.2	Effect of dexamethasone on infarct size	74
5.3.3	Effects of dexamethasone on expression of MKP-1 and kinase activation	78
5.4	Discussion	83
<b>CHAPTER 6</b> CONCLUSION		87-88
<b>REFERENCES</b>		89

**Protocol List**

Page No

Chapter 3**Protocol 1**

28

Chapter 4**Protocol II**

34

Chapter 5**Protocol III**

73

**Table List**Chapter 3**Table 1** PSP and PP1 activities

29

Chapter 4**Table 2** Effect of ischaemia/reperfusion and preconditioning on mechanical performance

36

**Table 3** Effect of cantharidin on functional recovery

40, 41

**Table 4** Effect of okadaic acid on functional recovery

58

Chapter 5**Table 5** Effect of dexamethasone on functional recovery

75

**Figure List**Chapter 4**Figure 1** Functional recovery of N-PC and PC hearts

37

**Figure 2** Effects of cantharidin and okadaic acid pretreatment on functional recovery (15' GI)

42, 43

**Figure 3** Effect of cantharidin on functional recovery (20' GI)

44

**Figure 4** Effect of cantharidin on infarct size

46

**Figures 5 and 6** Effect of cantharidin on phosphorylation of p38 MAPK, ERK42/44 and PKB (without GI)

48, 49

**Figure 7** Effect of cantharidin on phosphorylation of p38 MAPK, ERK42/44 (15' GI)

51

---

<b>Figures 8, 9 and 10</b> Effect of cantharidin on phosphorylation of p38 MAPK, ERK42/44 and PKB (20' GI)	54-56
<b>Figure 11</b> Effect of okadaic acid on functional recovery (20' GI)	59
<b>Figure 12</b> Effect of okadaic acid on infarct size	61
<b>Figures 13 and 14</b> Effect of okadaic acid on phosphorylation of p38 MAPK, ERK42/44 and PKB (20' GI)	63, 64
 <u>Chapter 5</u>	
<b>Figure 15</b> Effect of dexamethasone on functional recovery (20' GI)	76
<b>Figure 16</b> Effect of dexamethasone on infarct size	77
<b>Figures 17, 18, 19 and 20</b> Effect of dexamethasone on expression of MKP-1 and phosphorylation of p38 MAPK, ERK42/44 and PKB (20' GI)	79-82

---

**CHAPTER 1****Protein Phosphatases****1.1 General introduction**

Protein phosphatases comprise several families of enzymes that catalyze the dephosphorylation of intracellular phosphoproteins, thereby reversing the action of protein kinases. Most phosphorylations are reversible, implying that the phosphorylation level of a protein reflects the balance between the activities of the involved protein phosphatases and protein kinases. Reversible protein phosphorylation is the basis for the regulation of many diverse cellular processes that include metabolism, contractility, transport, cell division, differentiation and development, learning and memory. About one-third of all eukaryotic proteins are controlled by phosphorylation of specific serine, threonine, and/or tyrosine residues (there is also evidence for histidine phosphorylation).

The importance of phosphorylation and dephosphorylation in intracellular signalling pathways has long been recognized, although attention has been focussed mainly on kinases because protein kinases have been relatively easy to study by measuring the incorporation of radioactive phosphate from [ $\gamma$ - $^{32}\text{P}$  ATP] into proteins or specific peptide substrates, but characterization of protein phosphatases is a more difficult task. Before one can assay activity of a protein phosphatase a suitably purified phosphorylated substrate must be prepared. This includes the commonly-used model protein substrate, Myelin Basic protein (MyBP), which can be used for assay of type 1 and type 2 protein serine/threonine phosphatases as well as dual specificity protein phosphatases.

The protein phosphatases that dephosphorylate protein kinases have not been well investigated. Future studies are required for identification of protein phosphatases and a better understanding of the cellular mechanisms that control the balance between protein phosphatases and protein kinases could lead to therapeutic strategies to limit many diseases including cardiovascular diseases, cancer and inflammatory diseases.

This literature review will therefore give a brief description of the major classes of phosphatases, with particular emphasis on the current knowledge of and insight in their roles in myocardial ischaemia/reperfusion damage and the phenomenon of preconditioning.

## **1.2 Literature Review**

### **1.2.1 Protein Phosphatases**

Protein phosphatases are classified into five independent protein families according to their substrate specificities, catalytic mechanisms and amino acid sequences, namely, PSPs: Ser/Thr phosphatases, DSPs (dual-specificity): Ser/Thr/Tyr phosphatases, PTPs: Tyr phosphatases, PHPs: Histidine (His) phosphatases and PTEN (phosphatase and tensin homolog deleted on chromosome ten): dual protein–lipid phosphatases.

It is well established that protein phosphorylation levels can be modulated by changes in the activities of protein kinases (enzymes that add phosphate groups to target proteins) and protein phosphatases (enzymes that hydrolyze phosphate esters and amidates). About one-third of all eukaryotic proteins are controlled by phosphorylation of specific serine, threonine, and/or tyrosine residues. Eukaryotic cells express a large variety of protein kinases and phosphatases, each with their own substrate specificity, subcellular localization, and regulation. There are 400 protein serine/threonine kinases and 100 protein tyrosine kinases encoded in mammalian genome, in contrast to only 25 protein serine/threonine phosphatases and 100 protein tyrosine phosphatases (1). This has been accounted for by distinct diversification strategies during evolution (2). Because a single phosphatase catalytic moiety often associates with several different regulatory or targeting subunits, the total number of functional phosphatase holoenzymes is expected to be similar to the number of protein kinases when holoenzymes are considered (see reference 3). In order to counteract the large number of protein kinases, protein phosphatases exhibit broad substrate specificity and interact with numerous regulatory and targeting proteins that control the activities of phosphatases by changing the conformation and location of these enzymes. Kinases and phosphatases may even be colocalized on the same protein but at different docking sites (4, 5). Since the discovery of protein phosphorylation–dephosphorylation as a possible regulatory device in the mid-1950s, there has been a steady expansion of the field.

In theory, one kinase and one phosphatase can operate a regulatory cycle. In practice, however, a multitude of cycles are intimately interrelated through overlapping substrates and, more importantly, through the mutual regulation of kinases and phosphatases by other kinases and phosphatases.

These regulatory cycles possess an enormous capacity for amplification, integration and processing of internal and external signals. It is probable that with the flurry of interest in genomics and proteomics, all of the protein kinase and phosphatase catalytic subunits or domains will soon be ascertained (see reference 6).

The phosphorylation–dephosphorylation state of a protein can profoundly affect its localization and redistribution. Almost any protein molecule involved and almost every single step of signalling cascades are associated with phosphorylation and dephosphorylation processes. For example, reversible phosphorylation and dephosphorylation by kinases and phosphatases respectively are the main mechanisms whereby apoptosis is regulated by the anti-apoptotic pro-survival kinase phosphatidylinositol-3-OH kinase (PI3K)-Akt (7, 7A, 8) and the Raf–mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (Raf–MEK1/2–ERk1/2) signalling cascades (9, 9A) ( the so-called reperfusion injury salvage kinase (RISK) pathway (10).

Phosphatase molecules which are capable of negatively regulating the stress activated protein kinase (SAPK) signalling pathway include members of four distinct groups: DSP, PP2C, PP2A and PTP, each mediating a distinct function. Differences in substrate specificities and regulatory mechanisms for these phosphatases form the molecular basis for the complex regulation of SAPK signalling. Regulation of a single substrate by multiple protein phosphatases suggests redundancy (11-14).

More insight in the regulation of protein phosphatases is required before they can be pursued as therapeutic targets (15). For example, studying the pathogenesis of signalling pathways often reveals an apoptotic component that contributes to disease progression. As the apoptotic program is fundamentally regulated by phosphorylation of specific regulatory proteins, influencing these phosphorylation processes offers the ability to modulate the life or death of cells. In particular, inhibitors of phosphatases could become promising candidates for therapy of many diseases.

### 1.2.1.1 Protein serine/threonine phosphatases.

Protein serine/threonine phosphatases (PSPs) are specific for phosphoserine/phosphothreonine-containing proteins and were first identified in classical biochemical studies. These protein phosphatases are divided in two large subfamilies by distinct amino acid sequences and crystal structures. They are metal-ion-dependent protein phosphatase (PPM) and the phosphoprotein phosphatase (PPP) subfamilies (16). The PPM family comprises  $Mg^{2+}$ -dependent enzymes, including protein phosphatase 2C (PP2C) and pyruvate dehydrogenase phosphatase. All other protein serine/threonine phosphatases belong to the PPP family, consisting of the subfamilies type-1 protein phosphatases (PP1), type-2 protein phosphatase A (PP2A) (including PP4 also known as PPX and PP6, a functional homologue of budding yeast Sit4), PP2B (also known as calcineurin), PP5 and PP7, which all have a structurally related core and a similar catalytic mechanism (17-19).

PP1 (35–38 kDa) is one of the most conserved eukaryotic proteins. This is nicely illustrated by the early branching eukaryote *Giardia lamblia*, which expresses an isoform of PP1 that is 72% identical to the mammalian PP1 isoforms (2). Also, the phenotypes associated with mutations of PP1 in fungi could be (partially) complemented by expression of mammalian PP1 (20, 21), indicating that PP1 is also functionally conserved. Mammals have three PP1 genes, encoding the isoforms PP1 $\alpha$ , PP1 $\gamma$ , and PP1 $\beta/\delta$ . PP1 may also regulate glycogen metabolism and the activation of glycogen synthase in vivo (22-24). PP1 is inhibited by heat-stable inhibitor proteins and preferentially dephosphorylate the  $\beta$ -subunit of phosphorylase kinase. In contrast, PP2 is insensitive to these inhibitors and preferentially dephosphorylate the  $\alpha$ -subunit of phosphorylase kinase. PP1 is also likely to function as a phosphorylase phosphatase in vivo, since alterations in the expression levels of PP1 or of specific G subunits resulted in corresponding changes in the activity of phosphorylase (25, 26).

PP1 promotes the dephosphorylation of phospho-CREB and attenuate CREB signalling (27-29), but the involved targeting subunit is unknown. Interestingly, it was recently reported that the histone deacetylase HDAC1 is part of a CREB-associated complex that also includes PP1 (30).

PP2A has been found to colocalize at the mitochondrial membrane with Bcl-2 and protects cells by opposing ERK-mediated Bcl-2 phosphorylation (31). PP2A is not only targeted to mitochondria by splice variants of its regulatory subunit (32), but also is sensitive to redox regulation (33).

Four phosphorylation sites on BAD have been identified, Ser112 (34), Ser136 (34), Ser155 (35,36)

and Ser170 (37). At least PP1 (38), PP2A (39) and PP2B (40) are responsible for the dephosphorylation of BAD. The mechanisms of the function of the various phosphorylation sites, however, are not the same for all positions. Phosphorylation of Ser112 and Ser136 creates binding sites for the interaction of BAD with 14-3-3 proteins (34), whereas phosphorylation of Ser155 triggers dissociation of BAD from Bcl-XL (36).

Activation of caspase-3 directly causes cleavage of the regulatory  $\alpha$  subunit of PP2A (41). This in turn increases PP2A activity, thus affecting the phosphorylation state of a cell dramatically. PP2A may function in the regulation of the mammalian MAPK pathway (42): this phosphatase regulate Raf1-MEK1/2-ERK1/2 signalling at multiple steps in this pathway (43), so inhibition of this PP2A would allow for the continual activation of both these kinases. It has been shown that the action of PP2A regulates ERK phosphorylation (44) and it appears that ERK is a downstream target of PP2A activity (45). The inhibition of ERKs by a cholesterol-regulated PP2A/HePTP complex has also been reported in the membrane (46). Phosphorylation of p38 MAPK also can increase the activity of PP2A upon stimulation with stress stimuli (47-49). Activation of p38 MAPK by adenosine A1 receptors also induces PP2A activation and translocation to the particulate fraction in cardiomyocytes, leading to inhibition of ERKs (49). The p38 MAPK $\alpha$  isoform mediates dephosphorylation and inhibition of PKB/Akt activity by inducing the targeting of the PP2A holoenzyme to caveolae through interaction with caveolin-1, which leads to survival attenuation upon cell adhesion (for review see 50, 51). However, regulation of PP2A by integrins can be mediated by p38 MAPK-independent mechanisms (52).

At least six distinct PP2C gene products (2C $\alpha$ , 2C $\beta$ , 2C $\gamma$ , 2C $\delta$ , Wip1 and Ca<sup>2+</sup>/calmodulin-dependent protein kinase phosphatase) operate in mammalian cells (53-55). PP2C $\alpha$ , PP2C $\beta$  and Wip1 in mammalian cells have been identified to be involved in the negative regulation of SAPK cascades (56, 57). In addition, PP2C $\alpha$  and PP2C $\beta$  may regulate cell cycle progression (58).

The immunosuppressant drug cyclosporine, which made organ transplantation possible, was the first drug discovered to exert its effect by inhibiting a protein phosphatase, although it was in use clinically before the mechanism of action was elucidated in 1990. Cyclosporine, in association with its cellular binding protein cyclophilin, is a potent and specific inhibitor of the Ca<sup>2+</sup>/calmodulin-dependent protein phosphatase PP2B. Inhibition of PP2B prevents dephosphorylation of an isoform of the nuclear factor of activated T-cells (NFAT). As a result, this



transcription factor cannot enter the nucleus, and production of interleukin-2 is suppressed and T-cell proliferation reduced (59).

### **1.2.1.2 Dual specificity phosphatases**

Dual specificity phosphatases (DSPs) dephosphorylate protein substrates on tyrosine, serine and threonine residues. DSP genes share two unique structural features; they contain a common active site sequence motif and two N-terminal CH2 domains, homologous to the cell cycle regulator Cdc25 (60).

Mitogen-activated protein kinase (MAPK) phosphatases (MKPs) are a subfamily of the DSPs which can remove phosphates from both the threonyl and tyrosyl residues in the MAPK activation motifs. Although the contribution of the MAPKs to cell growth and cell death has been examined extensively, much less is known about whether the MKPs play an essential role in the regulation of these processes. It is possible that MKPs may modulate the activity of the MAPKs response to a stimulus (14). Since both the magnitude and duration of MAPK activity dictate the outcome of numerous physiological responses, it is essential to understand the contribution of MKPs to MAPK regulation. MKPs have been implicated in the regulation of cell survival, proliferation, apoptosis, differentiation, and metabolism using overexpression approaches (14, 61).

The MKPs exhibit different specificities towards the various MAPKs (14, 61) and are critical negative regulators of MAPK-mediated signalling in a variety of biological processes (14, 62). Activation of MAPKs causes activation of dual specificity phosphatases, which in turn dephosphorylate the MAPKs and which are thus responsible for the temporal limitations in MAPK signalling.

To date, 11 genes encoding members of the classical MKP family have been isolated and characterized from the mammalian genome. They all share some common features, including an extended active-site motif with significant sequence similarity to the corresponding region of the VH-1 protein tyrosine phosphatase that was isolated from vaccinia virus (63). On the basis of structures predicted from genomic sequence, the MKPs can readily be divided into three subgroups in mammalian cells, namely, subgroup I: MKP-1, MKP-2, PAC-1 and hVH3; subgroup II: MKP-3, MKP-4, MKP-5 and MKP-10 and subgroup III: M3/6 (hVH5) and MKP-7. Their subcellular locations differ: some are expressed exclusively in the nucleus, for example, MKP-1, MKP-2, PAC-1 and hVH3 (64-66), some (MKP-3, MKP-7 and MKP-10) are predominantly expressed in the cytoplasm (89, 91), whereas others (M3/6, MKP-4 and MKP-5) show both cytoplasmic and nuclear

localizations (67-69).

MKP-1 (hVH-1, Erp, 3CH134, CL100) mRNA is ubiquitously expressed in various tissues, with the protein product localized preferentially to the cell nucleus (70). A plethora of reports have implicated MKP-1 in the regulation of a variety of physiological processes such as gene expression, cell growth, apoptosis, and immune responsiveness (14, 61). Although many studies demonstrate a role for MKP-1 in these biological pathways, a complete appreciation of whether MKP-1 is an essential physiological regulator has remained unclear.

In addition to oxidative stress and heat shock (71), MKP-1 is induced by various stimuli such as, osmotic shock, anisomycin, growth factors, UV light, 12-O-tetradecanoylphorbol 13-acetate (TPA), Ca<sup>2+</sup> ionophores and lipopolysaccharide (70, 72, 73). In Rat1 fibroblasts, MKP-1 is induced by Ca<sup>2+</sup> signalling (74), independently of MAPK activation (74, 75).

MKP-1 promotes cell survival by attenuating stress-responsive MAPK-mediated apoptosis. Overexpression of MKP-1 attenuates the activation of genes whose transcription is dependent upon MAPK activity. MKP-1 was identified as a critical negative regulator of the cAMP-mediated p38 MAPK pathway. MKP-1 preferentially dephosphorylates and inactivates p38 MAPK and JNK in the nucleus and to a lesser extent the growth factor-responsive MAPK extracellular-regulated kinases (ERKs) with a rank order of p38 MAPK > JNK > ERK (72, 76-78). The stability of MKP-1 is regulated by ERK-mediated phosphorylation of two C-terminal serine residues (79). MKP-1 binds to C-terminal region of p38 MAPK, that results in its activation (70). The details of the regulatory mechanism depend on the cell lineage. In vascular smooth muscle cells, mesangial cells and U937 cells, the activation of either ERK, JNK or p38 MAPK induces MKP-1; in NIH3T3 cells, the activation of JNK but not ERK up-regulates MKP-1 expression (72, 73, 77, 80). In addition, activation of p38 MAPK but not ERK or JNK enhances MKP-1 induction in H4IIE hepatoma cells. Ca<sup>2+</sup>/calmodulin-activated protein phosphatase (PP2B) participates in the induction of MKP-1 in cardiac myocytes (81).

MKP-2 (hVH2), a 42.6-kDa nuclear DSP, is widely expressed in various tissues (82) and induced by the nerve growth factor, TPA and hepatocyte growth factor in PC12 cells, peripheral blood T cells and MDCK cells, respectively (76, 82, 83). PAC-1, a DSP of 32 kDa, is found to be expressed in hematopoietic cells (66) and in hippocampus neurons following forebrain ischemia or kainic acid-induced seizure (84, 85, 86). Activation of ERK induces the enhanced expression of PAC-1 which then inactivates ERK in T cells (87). The dual specificity phosphatase VHR (hVH3) is a

low-molecular-weight DSP which lacks the amino-terminal domain (65, 86, 88). The amino-terminal noncatalytic domain of Pyst1 mediates the binding of ERK42 and loss of this domain abrogates substrate selectivity in vivo (89-92). PAC-1 and Pyst1 (MKP-3) dephosphorylate both ERK and p38 MAPK but not JNK (60, 76, 86, 89). MKP-4 is specificity for ERK over JNK or p38 MAPK (67). Expression of MKP-4 mRNA is highly restricted to substrate binding (67, 93, 94). VHR and MKP-10 have been shown to inactivate ERK in cells (65, 86, 88, 89, 91, 95). MKP-5, MKP-7 and M3/6 preferentially dephosphorylate both JNK and p38 MAPK, but not ERK (68, 69, 89, 60, 96, 97). It has been found that the phosphorylation of M3/6 does not regulate its half life (98). An internal motif, XILPXL(Y/F)LG, homologous to the SAPK binding site of c-Jun (delta domain), is important for M3/6 activity (98,99). MKP-2 and MKP-6 are highly specific for ERK and JNK, but not for p38 MAPK (76, 100). MKP-6 expression is up-regulated by CD28 costimulation of T cells. Binding of the expressed MKP-6 to CD28 is required for the feed back regulation of ERK and JNK by MKP-6 (100).

### **1.2.1.3 Protein tyrosine phosphatases**

It is well-established that protein-tyrosine phosphorylation is an important modulator of posttranslational modification affecting protein–protein interactions and enzymatic activities. Protein tyrosine phosphatases (PTPs) are specific for these phosphotyrosine-containing proteins and composed of transmembrane (receptor-like) and cytosolic (non-receptor) subfamilies. Currently, more PTPs have been characterized (101-103) , although the PC12 cell PTPs were not identified molecularly (104).

Receptor protein-tyrosine phosphatases (RPTPs) belong to the family of single-membrane-spanning PTPs, which act together with the antagonistically acting protein-tyrosine kinases (PTKs), to regulate the protein phosphotyrosine levels in cells (105). Thus, the cellular equilibrium of protein tyrosine phosphorylation is achieved through the actions of PTKs and PTPs. Disrupting the equilibrium of cellular tyrosine phosphorylation can cause a plethora of human diseases (see reference 106) and demonstrates the importance of tightly controlling the activities of both PTKs and PTPs. The PTKs were discovered almost a decade before PTPs, so the knowledge of the molecular actions of PTKs in normal cell signalling and human disease is much more than that of the PTPs. Recent studies demonstrated that RPTPs, like RPTKs, are regulated by dimerization. For example, it has been shown that oxidative stress regulates RPTP dimer formation (105). These phosphatases not only counterbalance the effects of receptor and cytosolic PTKs but

also transmit information through the plasma membrane. PTPs contain a highly conserved cysteine residue that is essential for the catalytic reaction. In contrast with the DSPs, the identification of substrates for the non-transmembrane tyrosine-specific PTPs has been much more problematic, now that all of the genes that comprise the PTP superfamily have been identified (107). The ability of PTP family members to differentiate between individual substrates can be attributed to the inherent specificity within the PTP catalytic domain. The catalytic domains of classical PTPs contain approx 280 residues and comprise 22 invariant and 42 highly conserved residues that fall within ten consensus motifs (108,109). A role for PTPases in regulating MAPK first came from genetic and biochemical studies of the osmoregulatory MAPK pathways in yeasts (110,111). PTPs were observed to inactivate ERKs (104,112). The Hog1p osmoregulatory MAPK in budding yeast is regulated by two tyrosine specific phosphatases encoded by PTP2 and PTP3 (110,111).

Three related PTP gene family members have been identified, namely, STEP (striatal enriched phosphatase), PTP-SL (STEP-like phosphatase, also known as PCPTP1) and HePTP (same as LC-PTP). STEP and PTP-SL are two homologous neuronal PTPs existing in both transmembrane and cytosolic forms that bind tightly as well as dephosphorylate and inactivate ERKs (112). HePTP is an additional cytosolic PTP present in lymphoid tissue also reported recently to bind and inactivate the MAPKs (12, 13, 113). PTP-SL, STEP, and HePTP appear to act as functional homologues of the tyrosine-specific PTPs Ptp2 and Ptp3 as well as Pyp1 and Pyp2, identified genetically as down-regulators of the MAPKs in budding and fission yeast, respectively (114, 115).

#### **1.2.1.4 Protein histidine phosphatases**

A unique protein phosphatase responsible for histidine dephosphorylation has been identified recently, named protein histidine phosphatases (PHPs) (see reference 6, 116). PHPs are unlike any of the known Ser/Thr or Tyr phosphatases. In contrast to the relatively well studied processes of Ser/Thr and Tyr phosphorylation, PHPs phosphorylation in eukaryotes is an emerging field.

There are important examples of phosphohistidine in mammalian proteins (e.g. G-proteins, P-selectin and annexin). Although corresponding mammalian His kinases still remain an enigma, a PHP has been discovered in vertebrates (see reference 6). Its primary structure and insensitivity to known inhibitors indicate that this is a novel protein.

Interestingly, PHP has been shown to be present in animals ranging from humans to nematodes but absent in bacteria. However, it became obvious that the role of His phosphorylation and dephosphorylation in mammalian cells is only just beginning to be explored (see reference 6).

### **1.2.1.5 Phosphatase and tensin homolog deleted on chromosome ten**

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a dual protein–lipid phosphatase which was discovered relatively recently (117, 118). PTEN dephosphorylates the second-messenger PtdIns(3,4,5)P<sub>3</sub> (PIP<sub>3</sub>) to the precursor PtdIns(4,5)P<sub>2</sub> (PIP<sub>2</sub>) and negatively regulates the PI3K/Akt pathway (119, 120).

PTEN also is called MMAC1 (mutated in multiple advanced cancers) or TEP-1 (TGF- regulated and epithelial cell-enriched phosphatase) and is present ubiquitously in cells and its activity is reflected by its cellular level, which can be modulated by transcription.

PTEN plays a significant role in regulating the balance between survival and death in many cell types, including cardiomyocytes. One of the mechanisms of its inactivation is via phosphorylation. It has been suggested that its activity can be upregulated by increased synthesis and downregulated by phosphorylation, oxidation and proteasomal degradation (121-123). Although PTEN downregulation may seem potentially harmful because it could promote unwanted growth and malignancies, the mechanisms through which its activity are regulated are complex and not yet elucidated completely (see reference 124).

It is also known that homozygous PTEN knockout mice are not viable whereas the heterozygous animals develop numerous tumors. In addition, in humans, many tumor types are characterized by deficient PTEN expression (125).

It has been demonstrated that PTEN can be inhibited by vanadium compounds (126, 127). Based on the homology of the active site between PTEN and protein tyrosine phosphatases (PTPs), it has been shown that PTP inhibitors such as bisperoxovanadium molecules can also inhibit PTEN. Sodium orthovanadate was shown to protect against cerebral ischaemia by increasing the tyrosine phosphorylation of PTEN (127). It was also documented that zinc ions downregulate PTEN expression in airway epithelial cells in a dose- and time-dependent fashion, via increased proteasome-mediated degradation and reduced PTEN messenger RNA expression (128).

PTEN may play a significant role in pathological conditions associated with the ischaemic heart disease (129). PTEN inhibition could ultimately prove to be significant in improving myocardial survival following ischaemia/reperfusion injury. A reversible inhibition of PTEN may be enough to upregulate the prosurvival PI3K/Akt pathway to reduce the cell death associated with such injury, without the negative hypertrophic consequences (see reference 130).

## **1.2.2 Introduction to preconditioning**

### **1.2.2.1 Ischaemic preconditioning: a brief description**

Myocardial ischaemic preconditioning is a phenomenon by whereby exposure to one or more short episodes of ischaemia/reperfusion increases the ability of the heart to tolerate a subsequent prolonged period of ischaemic injury (131-134). This phenomenon was first described by Murry et al (131) in the canine infarct model. In view of its possible clinical application, this finding evoked enormous interest from clinicians and researchers and several reviews on the topic have been published (for reviews see 135-137). In view of these, only a brief overview of the phenomenon will be given below.

Ischemic preconditioning represents a powerful procedure to protect ischaemic-reperfused myocardium in all animal species investigated thus far: dogs (131), rabbits (138-140), rats (141-144), pigs (145,146) as well as in human isolated myocytes (147), human muscle tissue (148).

Furthermore, and most promising, it has also been reported to occur in in-vivo human hearts (149-151).

Preconditioning induced cardioprotection is reduced in the aged as compared to the young adult rat heart (144, 152), also in adult and elderly human patients with myocardial infarction (152).

Not all time combinations and durations of ischaemia and reperfusion will trigger the preconditioning phenomenon and afford myocardial protection. In a preconditioning protocol, ischaemic episodes as short as 3–5 min followed by a minimal 5 min of reperfusion protects the myocardium (131), but a brief 1–2 min of preconditioning ischaemia followed by subsequent reperfusion has no protective effect (153-155). A single episode of ischaemia is essential to induce preconditioning (139, 156), but repetitive episodes of brief ischaemia are also effective (157, 158), and may enhance the cardioprotective effects. The duration of intermittent reperfusion is also meaningful. Typically, a 5-min period of ischaemia followed by up to 60 min of reperfusion prior to the sustained ischaemic insult results in salvage. One to four hours later protection is no longer evident (153, 159). However, if the time between the initial stimulus and sustained ischaemia is prolonged to 24–96 h, the protective effect may again be seen (157, 160).

The protection elicited by ischaemic preconditioning therefore appears in two separate phases or 'windows'. The term 'classic' or 'early preconditioning' refers to the effect of a brief bout(s) of

ischaemia (for example 5 min) followed shortly thereafter by a second insult which results in significant protection over 2–3 h following a preconditioning stimulus (131, 161, 162). If the time between the initial stimulus and sustained ischaemia is prolonged to 24–96 h, a second, but less significant, phase of cardioprotection was observed. This was initially labeled 'second window of protection', but is now termed 'delayed' or 'late preconditioning'.

Ischaemic preconditioning protects the myocardium against various deleterious effects of ischaemia such as slowing energy metabolism during the early stages of ischaemia (163), reducing the incidence and severity of reperfusion-induced arrhythmias (164-167), improving post-ischaemic recovery of function (168), preventing endothelial cell dysfunction (164-166), increasing the post-ischaemic developed tension in isolated atrial trabeculae muscles following simulated ischaemia (147, 148) and improving the resistance of isolated myocytes to hypoxic injury (169). It is also known as the most effective intervention to reduce infarct size during ischaemia/reperfusion and indicates that the heart has at its disposal a powerful endogenous protective mechanism, elicited by exposure to short periods of ischaemic stress. Amongst others, ischaemic preconditioning mediates protection through the recruitment of downstream antiapoptotic pathways of cellular survival (10, 170). These include the phosphorylation and inactivation of proapoptotic proteins such as BAD (171), Bax (172, 173), Bim (173) and caspases (174, 175), and the phosphorylation and activation of endothelial nitric oxide synthase (eNOS) (176), p70S6 kinase (177, 178), and protein kinase C (PKC) (179). It also attenuates release of cytochrome C from mitochondria (180, 181) and reduces neutrophil accumulation (182, 183).

#### **1.2.2.2 Signalling in preconditioning.**

It has become clear that numerous triggers and mediators can elicit this protective mechanism, and a major objective in recent years has been the identification of these triggers, mediators and also end effectors involved in the cardioprotection elicited by ischaemic preconditioning. A number of triggers are released during the short episodes of ischemia/reperfusion. Receptor dependent triggers are adenosine (138, 184, 185), opioids (186-188), bradykinin (189, 190), prostaglandins, norepinephrine, angiotensin and endothelin (191-194). This indicates that virtually all guanylyl inhibitory (Gi) protein coupled receptors in the heart can trigger the preconditioned phenotype. Receptor independent triggers are nitric oxide (NO) (193-195), reactive oxygen species (ROS) (196, 197) and calcium (198-201). ROS, in particular, which appear to be important in the triggering

process, are formed during the reperfusion phase of the preconditioning protocol (see reference 135).

The receptor dependent triggers activate very divergent pathways, although several converge again at PKC (for a review see 137, 202). Another important role player in triggering preconditioning is PI3-kinase activation, which occurs upstream of PKC. PI3-kinase activation in turn results in rapid phosphorylation, and hence activation of PKB/Akt (203).

PKB/Akt is known to stimulate nitric oxide synthase (NOS) to produce NO, which in turn, activates guanylyl cyclase, resulting in cGMP formation and PKG activation (204) during the triggering process. In 2005, Costa and coworkers (205) showed that PKG resulted in opening of the mitochondrial K<sup>+</sup>ATP channels, which has been proposed to be an end-effector of preconditioning. However, opening of these channels also appears to participate in the triggering process, since this is thought to lead to ROS production, resulting in PKC activation (136, 137, 206).

In 2004, a significant paradigm shift occurred: while it was previously generally accepted that protection occurred during the ischaemic period and that reperfusion allowed cells to recover from the damage caused by ischaemia, Hausenloy and coworkers (207) demonstrated that protection actually occurs during the early reperfusion period following ischaemia. Ischaemic preconditioning causes activation of PI3-K/Akt as well as the MEK1/2-ERK1/2 cascades during this stage. This appears to be very important, since pharmacological inhibition of these events abrogates cardioprotection.

The next step was to determine how the survival kinases are activated during reperfusion. Solenkova and coworkers (208) showed that blockade of the adenosine receptor during the first few min of reperfusion prevented activation of PKB/Akt as well as the anti-infarct effect of ischaemic preconditioning. Further studies by the same group suggested involvement of the adenosine A<sub>2</sub> $\beta$  receptor in particular.

The survival kinases, although important, are not the end-effectors of preconditioning: they are merely signalling molecules to transport the signal. It has also been shown that phosphorylation of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) (209) ( and inactivation of this particular kinase) by several kinases occurs in ischaemic preconditioning, preventing formation of the mitochondrial permeability transition pore (MPTP) (210). Hausenloy and his coworkers recently reported that the final step of the signal transduction pathway is inhibition of MPTP (211, 212). This pore is thought to be formed by alignment of the adenine nucleotide translocator on the inner mitochondrial membrane and the



voltage dependent anion channel on the outer membrane, with the small protein cyclophilin D playing an important role (212).

It can be concluded from the above that the focus thus far has been mainly on the large number of kinases involved in the process of preconditioning, evoking phosphorylation reactions which, in most cases, lead to activation of that particular kinase. However, very little indeed is known about the role of the phosphatases in this scenario.

### **1.2.3 Protein phosphatases in the heart**

#### **1.2.3.1 Effects on contractility and relaxation**

It is well-established that phosphatases and kinases are important role players in the regulation of contractility and relaxation of the heart. Increases in protein phosphorylation and enhanced cardiac function are reversed by protein dephosphorylation in a highly regulated manner.

Phosphatase expression in the heart is age-dependent and regionally different, with neonates having an about two-fold higher activity than adults (213), with the ventricles having a higher activity than the atria (214, 215). Phosphatase activity has been found to be increased in human heart failure (216). The activity of PP1 was found to be increased by 32% in ventricular membranes from infarcted hearts compared to control hearts (215).

Chronic beta-adrenergic stimulation has been found to enhance phosphatase (PP1 and PP2A) activity in the heart (217). The anti-adrenergic actions of adenosine can be attributed to PP2A activation. A recent study by Liu and Hofmann (49) showed that adenosine A1-receptor mediated PP2A activation occurs via a pertussis toxin-sensitive Gi protein-guanylyl cyclase-p38 MAPK pathway. This proposed novel pathway may play a role in acute modulation of cardiac function.

Recently, particular attention has been focused on phospholamban (PLB) which plays an important role in the activation of the SR Ca<sup>2+</sup> pump (SERCA). Phosphorylation of PLB occurs via PKA or a Ca<sup>2+</sup> calmodulin dependent protein kinase while it is dephosphorylated mainly by PP1 and PP2A (218). It has been shown that dephosphorylation of phospholamban reduces the activity of the associated SERCA pump (218), causing a reduced uptake of Ca<sup>2+</sup>. PKA, on the other hand, reduces the antagonistic downregulation of Ca<sup>2+</sup> by PP1.

The downregulated beta-adrenergic signaling in human heart failure correlated with decreased cAMP levels and hypophosphorylation of inhibitor-1 and phospholamban (219, 220). PP1 is regulated by two heat and acid stable proteins, inhibitors1 (I-1) and 2 (I-2). I-1 becomes active upon

phosphorylation on threonine 35 by PKA. This results in inhibition of PP1 and therefore enhanced PKA-mediated protein phosphorylation. Overexpression of PP1 led to a hypophosphorylation of phospholamban Ser-16, and the increased PP1 activity in inhibitor-1 null mice correlated with a hypophosphorylation of both Ser-16 and Thr-17. Not surprisingly, inhibitor-1 knock-out mice displayed a mildly depressed cardiac function (219). Moreover, a cardiomyocyte-restricted overexpression of PP1 was even associated with a severely impaired cardiac function, dilated cardiomyopathy, and increased mortality from heart failure (219). On the other hand, inhibition of PP1 and therefore enhanced PKA-mediated protein phosphorylation lead to amplification of the beta-response. Thus beta-adrenergic stimulation of the heart leads not only to PLB phosphorylation, but also inhibits PP1 activity which occurs in an indirect way (221).

In both excitable and nonexcitable cells, two distantly related types of tetrameric receptors, the ryanodine receptors (RyRs) and the inositol 1,4,5-trisphosphate receptors (IP3Rs), function as  $\text{Ca}^{2+}$  release channels of the sarcoplasmic reticulum (222). Unexpectedly, RyR2 channels in failing hearts were found to be hyperphosphorylated, and the levels of attached PP1 and PP2A were decreased (223). RyR-associated PP1 and/or PP2A are thought to counteract PKA-mediated protein phosphorylation. PKA increases RyR2-dependent  $\text{Ca}^{2+}$  release during contraction and indirectly promotes SERCA2a-mediated  $\text{Ca}^{2+}$  uptake during relaxation via the phosphorylation of phospholamban. Indeed, PKA-dependent phosphorylation of the related protein Neurabin-I has been shown to dissociate PP1 (224, 225). Recent studies (226) also demonstrated that the  $\text{Na}^+$  channel represents a target molecule for PP1.

PP1 also plays an important role in the dephosphorylation of the regulatory light chains associated with myosin heavy chains in muscle (for a review see 227). The organization and dynamics of the actin cytoskeleton is tightly controlled by reversible protein phosphorylation, and this regulation clearly involves PP1 (228). All known myosin phosphatases consist of PP1 (229-231) and has both a large and a small myosin phosphatase targeting (Mypt) subunit (229, 232, 233). The large Mypt targets PP1 to myosin and determines the substrate specificity of the phosphatase (234). The function of the small Mypt remains unclear, but it is known to interact directly with myosin and the large Mypt (235).

### **1.2.3.2 Effects of ischaemia/reperfusion on phosphatase activity**

The activation status of the myocardial phosphatases during ischaemia/reperfusion obviously should be important in determining the phosphorylation of kinases. PP1 and PP2A are present in the heart, with the amount of PP2A being  $\sim 3$  times higher than that of PP1 (236). PP2A activity is also present in isolated rat cardiomyocytes (237).

Most of these studies used phosphatase inhibitors to demonstrate involvement of these enzymes. However, interpretation of the results is complicated by the lack of specificity of many of these inhibitors. Armstrong, Ganote and coworkers (238) showed that protein phosphatase inhibitors calyculin A and fostriecin protected isolated rabbit cardiomyocytes from ischaemic damage, suggesting a role for PP2A activation in ischaemic damage. As expected, incubation with calyculin A induced high levels of p38 MAPK activity. Weinbrenner and coworkers (239) also reported that fostriecin, a potent inhibitor of PP2A, protects against ischaemia in rabbits.

PP2A regulates the phosphorylation of MAPK cascades (43, 44). A cholesterol-regulated PP2A/HePTP complex was also reported to inhibit ERK activity in the membrane (46). PP2A may function in the regulation of the mammalian SAPK pathway (42). Phosphorylation of p38 MAPK also can increase the activity of PP2A upon stimulation with stress stimuli (47-49). As stated previously, activation of p38 MAPK by adenosine A1 receptors can also induce PP2A activation and translocate to the particulate fraction in cardiomyocytes, leading to inhibition of ERKs (49). It has also been reported that the isoenzyme p38 MAPK $\alpha$  mediates dephosphorylation and inhibition of PKB/Akt activity by inducing the targeting of the PP2A holoenzyme to caveolae through interaction with caveolin-1. This leads to survival attenuation upon cell adhesion (for reviews see 50, 51). However, regulation of PP2A by integrins can be mediated by p38 MAPK-independent mechanisms (52).

It should be pointed out that the effects of phosphatase inhibitors on kinase activity during ischaemia differ between the species. The p38 MAPK activation during sustained ischaemia in rabbits was suggested to be cardioprotective (239A), which is in agreement with the effects observed using phosphatase inhibitors (238-240). However, in contrast to the results obtained in rabbit cardiomyocytes, Mackay and Mochley-Rosen (241) showed in rat neonatal cardiomyocytes that the tyrosine phosphatase inhibitor, vanadate, extended the strength and length of p38 MAPK activation during ischaemia, resulting in a higher susceptibility to cell death. They concluded that a tyrosine phosphatase is inactivated during ischaemia resulting in prolonged p38 MAPK activation

and cell death.

Thus inhibition of dephosphorylation rates or stimulation of kinase activity may protect or damage cells by maintaining protein phosphorylation during ischaemia, depending on the animal species. Clearly, in view of these controversial results, the role of phosphatases in ischaemia/reperfusion warrants further investigation.

### **1.2.3.3 Preconditioning and phosphatases**

As in the case of ischaemia/reperfusion, little information is available regarding the role of protein phosphatases in preconditioning-induced cardioprotection. However, in view of the marked changes in the activation state of several kinases during an ischaemic preconditioning protocol (242, 243), it is to be expected that the protein phosphatases also play an important role in this protocol. As far as we know, measurement of protein phosphatase activity per se during sustained ischaemia of preconditioned and non-preconditioned hearts or isolated cardiomyocytes was determined in one study only (239)

A number of reports showed that inhibitors of protein phosphatases (PP) imitated ischaemic preconditioning in some experimental models (238, 239, 240). For example, the aging-induced increase in the dephosphorylation of proteins can be reversed by the inhibition of endogenous protein phosphatases associated with preconditioning signalling, while enhancing the phosphorylation state of cellular proteins by the inhibition of protein phosphatase activity in the young adult cardiac preparations mimics preconditioning and limits cell death occurring in response to ischaemia (238, 239, 244, 245). Armstrong and Ganote (240) reported that phosphatase inhibitors, such as fostriecin, could mimic the cardioprotective effects of preconditioning. Additional evidence for the possible involvement of PP2A in triggering preconditioning, is the finding that adenosine, a well-established trigger of preconditioning, causes PP2A activation in ventricular myocytes via a pertussis toxin-sensitive Gi protein-guanylyl cyclase-p38 MAPK pathway (49). Indications are that elevations in tissue cAMP and cGMP, which occur during short episodes of ischaemia (5 min, as in ischaemic preconditioning), leading to activation of p38 MAPK (242, 246) may in turn cause activation of the phosphatase. This is in agreement with the finding that p38 MAPK activation may act as a trigger of ischaemic preconditioning (242) and could explain the transient nature of p38 MAPK activation during a multi-cycle preconditioning phase. Stimulation of MAPKs activities by phosphatase inhibitors such as okadaic acid exerted cardioprotective effects

associated with stimulation of JNK and p38 MAPK (244, 247) and mimicked the anti-infarct effects of preconditioning in several animal models (248,249). These results, however, are in contrast to those reported by Weinbrenner et al (239) who could not demonstrate that inhibition of PP1 or PP2A was involved in the mechanism of preconditioning.

Ladilov and coworkers (see reference 250) recently suggested that PP1 was a mediator of hypoxic preconditioning (HP) in isolated heart since the inhibitor cantharidin (used at 20  $\mu$ M which inhibits both PP1 and PP2A) abolishes hypoxic preconditioning, while okadaic acid (at 5 nM, which inhibits PP2A only) enhanced protection. Armstrong et al also reported that PP1 activation represents a mediator of HP protection in a rat model, whereas PP2A activation has the opposite effect (238, 240). They demonstrated that hypoxic preconditioning led to a significant reduction of  $Ca^{2+}$  overload in anoxic myocytes and suggested that this was the cellular basis of hypoxic preconditioning.  $Ca^{2+}$  accumulation in ischaemia, in turn is linked to intracellular  $Na^+$  overload (251). It was recently demonstrated that the  $Na^+$  channel represents a target molecule for PP1 (226) and it was suggested that  $Na^+$  overload in hypoxic cardiomyocytes subjected to phosphatase inhibition are due to changes in the phosphorylation of  $Na^+$  channels (see reference 250). These results suggested that HP provides protection of isolated hearts and cardiomyocytes against ischaemic injury by a mechanism endogenously antagonized by PP2A activation and supported by activation of PP1.

It is noteworthy that the phosphatase-dependent HP effect is found in the same experimental model (isolated cardiomyocytes from the adult rat) as the PKC-dependent mechanism of protection(252). It also was reported in some biological models that PP2A inhibition in ischaemic preconditioning can provide myocardial protection against ischaemia independent of PKC activation (158, 192, 253, 254).

An increase in tyrosine residue phosphorylation via increased tyrosine kinase activity has been implicated in the signal transduction pathway of ischemic preconditioning (255, 256). Vanadate has been shown to enhance tyrosine residue phosphorylation by inhibition of tyrosine phosphatase (257, 258). Liem and coworkers (see reference 259) have shown that administration of vanadate to rats caused a significant reduction in infarct size, when administered before coronary artery ligation, suggesting that the tyrosine phosphorylation state is an important determinant of ischaemia/reperfusion damage. These workers also demonstrated that the cardioprotective actions of vanadate could be abolished by the  $K^+$ ATP channel blocker, glibenclamide, suggesting that

opening of the K<sup>+</sup>ATP channel is involved in the actions of vanadate. Thus phosphatase inhibition might play an important role in preconditioning. These findings however are in direct contrast to those reported by Mackay and Mochly-Rosen (241) who showed that vanadate caused a higher susceptibility to cell death. It is obvious that these contradictory observations need to be further evaluated.

It is clear that our knowledge regarding the phosphatases in the heart, particularly during ischaemia/reperfusion and preconditioning-induced cardioprotection, is incomplete. The phosphatases may be of enormous significance in this regard, but our insight is severely hampered by the lack of basic information regarding the behaviour of these enzymes in the heart. In view of the putative significance of kinases in cardiac pathology, pharmacological manipulation of the phosphatases may become a significant new therapeutic target. In order to do so, our knowledge regarding the roles of the phosphatases in ischaemia/reperfusion injury should be expanded.

### **1.3 Aims of study**

As stated in the literature survey, the phosphorylation status of proteins is determined by the actions of both kinases and phosphatases. The mitogen activated protein kinase (MAPK) signalling systems have been suggested to play a pivotal role in the outcome of ischaemia/reperfusion. However, little is known about the putative regulatory role of the protein phosphatases in ischaemia/reperfusion as well as in preconditioning. We thus hypothesized that the balance between the activation state of the kinases and the induction of phosphatases may play a major role in determining the fate of cardiomyocytes exposed to ischaemic stress. In addition, it is proposed that the dual specific phosphatase, MAPK phosphatase 1 (MKP-1), is of particular significance in this regard since it is expressed by exposure to oxidative stress and selectively dephosphorylates p38 MAPK.

The broad objective of this study is therefore to evaluate the role of the myocardial protein phosphatases in establishing the activation status of the MAPKs in ischaemia/reperfusion as well as in ischaemic preconditioning and to determine their contribution to cardioprotection.

The specific aims are the following:

1. Assessment of PSP and PP1 activities during sustained ischaemia and during reperfusion of non-preconditioned and ischaemic preconditioned hearts.
2. Investigation into the significance of phosphatase activation in ischaemia/reperfusion and ischaemic preconditioning. This was done by the use of appropriate pharmacological agents administered at specific times during the experimental protocol to allow distinction between trigger and mediator actions. Cantharidin and okadaic acid were used as inhibitors of PP1 and PP2A, while dexamethasone was applied to induce MKP-1 expression. Endpoints were infarct size and functional recovery of isolated perfused hearts, which were correlated with activation of the MAPKs (ERK42/44 and p38 MAPK) and PKB/Akt, and expression of MKP-1.

---

**CHAPTER 2****Materials and Methods****2.1 Animals**

Male Wistar rats (220-250g body weight) were used for all studies and allowed free access to food and water until the time of experimentation. Rats were anaesthetised by intraperitoneal injection of pentobarbital (100mg/kg). In one series of experiments dexamethasone or an equivalent volume of saline was administered intraperitoneally for 10 days (3mg/kg/day) before experimentation. The project was approved by the Ethics committee of the University of Stellenbosch (Faculty of Medicine) and the investigation conforms with the Guide for the Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**2.2 Materials**

The protein Serine/Threonine Phosphatase (PSP) Assay System was obtained from New England Biolabs. The primary antibodies for p38 MAPK, ERK42/44 and PKB/Akt as well as phospho-p38 MAPK (Thr180/ Tyr 182), phospho-ERK42/44 (Thr 202/ Tyr 204) and phospho-PKB/Akt (Ser 473) were purchased from Cell Signaling Technology; the antibody MKP-1(M-18):sc-1102 was obtained from Santa Cruz Biotechnology. Horseradish peroxidase-labelled secondary antibody, hyperflow ECL and the ECL detection reagents were obtained from Amersham Biosciences. Routine chemicals were of Analar grade and obtained from Merck, RSA. Cantharidin, okadaic acid and dexamethasone were purchased from Sigma Chemical Co. Decasone Injection (4mg/ml) was obtained from Pharmicare Limited, Port Elizabeth, RSA.

**2.3 Perfusion technique**

The hearts were rapidly excised and arrested in ice cold (4°C) Krebs-Henseleit bicarbonate buffer (KHB) containing (in mM): NaCl 119; NaHCO<sub>3</sub> 25; KCl 4.75; KH<sub>2</sub>PO<sub>4</sub> 1.2; MgSO<sub>4</sub> 0.6; NaSO<sub>4</sub> 0.6; CaCl<sub>2</sub> 1.25; Glucose 10; pH 7.4. Perfusion with KHB was initiated via the aorta within 1 min after removal of the heart from the animal by the Langendorff technique in a retrograde, non-recirculating manner. The buffer was oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (37 °C, pH 7.4). The left atrium was cannulated to allow arterial perfusion. A temperature probe was inserted into the pulmonary artery for constant monitoring of myocardial temperature. The temperature was maintained at 36.5°C during sustained global or regional ischaemia. Global ischaemia was induced by simultaneous



clamping of both the aortic and left arterial cannula. Normothermic, zero-flow global ischaemia was utilized for the induction of ischaemic preconditioning (1x5 min or 3x5 min, alternated with 5 min reperfusion) as well as sustained ischaemia (20 min). Reperfusion was initiated by unclamping of the tube to the aortic cannula, allowing retrograde perfusion. Working rat hearts were perfused at a preload of 15 cm H<sub>2</sub>O and an afterload of 100 cm H<sub>2</sub>O (not electrically stimulated). After sustained ischaemia, hearts were subsequently reperfused for 30 min (10 min retrograde, 20 min working heart). Retrograde perfusion during the first 10 min of reperfusion was found to be essential, since it allows the hearts to stabilize after the ischaemic incident.

Drugs were administered through a side-arm into the aortic cannula, while the heart was retrogradely perfused.

Intra-aortic pressure and heart rate were monitored via a pressure transducer ( Viggo Spectromed ) inserted into the aortic cannula, while the coronary and aortic flow rates were measured manually. Mechanical activity was monitored before and after sustained ischaemia. Work performance was calculated according to the formula described by Kannengieser et al (260): Kannengieser formula  $0.002222 \times (\text{Aortic pressure} - 11.25) \times \text{Cardiac output}$

#### **2.4 Determination of infarct size**

A suture was passed around the main branch of left coronary artery, and the end was pulled through a small vinyl tube to form a snare. The coronary artery was occluded by tightening the snare. Ischaemia was confirmed by a reduction in coronary flow. Infarction was induced by 35 minutes of regional ischaemia which was then followed by 30 min reperfusion. Previous studies from our laboratory showed that shorting of the reperfusion period from 120 to 30 min had no effect on the determination of infarct size (260A).

At the end of the experiment the silk suture around the coronary artery was securely tied and ~1 ml of a 0.5% Evans Blue suspension slowly injected via the aorta cannula. Hearts were frozen overnight before being cut into 2 mm thick slices. After defrosting, the slices were stained with 1% w/v triphenyltetrazolium chloride in phosphate buffer pH 7.4 at 37°C for 15 min. Slices were fixed in 10% v/v formaldehyde solution to enhance the contrast between stained viable tissue and unstained necrotic tissue. The left ventricle area at risk (R) and the area of infarct (I) tissue were determined using computerised planimetry (Summa Sketch II; Summa Graphics). The infarct size was expressed as a percentage of the risk zone (I/R%).

## **2.5 Assay of protein phosphatases**

Activities of myocardial protein serine/threonine phosphatases (PSP) and protein phosphatase 1 (PP1) were determined using the commercial assay system of New England Biolabs according to the manufacturer's instructions. Briefly, tissues were homogenized using a buffer containing (in mM): Tris-HCl 50, pH7.0; Na<sub>2</sub>EDTA 0.1; DTT 5. The homogenate was centrifuged for 10 min at 3500 rpm and the supernatant used for the measurement of phosphatase activity. The substrate for the assay, Myelin Basic Protein (MyBP), was prepared by phosphorylation of serine and threonine residues with cAMP dependent protein kinase A in the presence of [<sup>33</sup>P] ATP. The reaction was terminated by adding trichloroacetic acid to precipitate the phosphoprotein, inactivate the protein kinase and remove the excess ATP. The PSP activity of tissue samples was then determined by measuring the release of inorganic phosphate from the labelled substrate in medium containing (in mM): Tris-HCl 50, pH 7.0; Na<sub>2</sub>EDTA 0.1; DTT 5; Brij 35, 0.01%. Brij (polyoxyethylene 23 lauryl ether) is a non-ionic detergent which is used for the extraction of membrane proteins. Results are expressed as nmol phosphatase activity/min/mg protein (one unit of protein phosphatase activity releases 1 nmol phosphate from MyBP per min in the standard assay of 50 ul). PP1 activity was determined as described above, except that the PP1 assay buffer contained 1 mM MnCl<sub>2</sub>, in addition to the components described above. The protein content of the supernatant was determined using the Bradford technique (261).

## **2.6 Western blots**

Hearts were freeze-clamped with pre-cooled Wollenberger tongs at different times during the perfusion protocol and plunged into liquid nitrogen. The tissue was pulverized and homogenized with a Polytron homogenizer in 800 µl lysis buffer. For phospho-p38 MAPK (Thr 180/Tyr 182), phospho-ERK42/44 (Thr 202/ Tyr 204) and phospho-PKB/Akt (Ser 473) as well as total p38 MAPK, ERK42/44 and PKB/Akt, the lysis buffer contained (in mM): Tris 20 (pH 7.5); HCl 20; EGTA 1; EDTA 1; sodium orthovanadate 1; sodium pyrophosphate 2.5; NaCl 150; β-glycerophosphate 1; 1% Triton X-100; phenylmethyl sulphonyl fluoride (PMSF) 0.3; aprotinin 10 µg/ml and leupeptin 10 µg/ml. For MKP-1, the lysis buffer contained (in mM): Hepes 50 (pH 7.5); EGTA 10; EDTA 10; 0.5% Triton X-100; phenylmethyl sulphonyl fluoride (PMSF) 1; aprotinin 2.5 µg/ml and leupeptin 2.5µg/ml, pH 7.4. Samples were centrifuged at 1000 g for 10 min to obtain the supernatant which was used for

Western blotting. The protein content was determined using the Bradford technique. The tissue lysates were diluted in Laemmli sample buffer, boiled for 5 min. For each sample, 10 µg of protein for p38 MAPK and ERK42/44; 30µg for PKB/Akt; 50 µg for MKP-1 were loaded separately onto polyacrylamide gels (12% for p38 MAPK, ERK42/44 and MKP-1; 10% for PKB/Akt) using the Bio-RAD Mini-PROTEAN II System. The separated proteins were transferred to a PVDF membrane (Immobilon® P, Millipore) and stained with Ponceau Red for visualization of proteins. To assess the quality and quantity of the transfer, the membranes were laser-scanned and densitometrically analyzed (UN-SCAN-IT, Silkscience). Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline- 0.1% Tween 20 (TBST). The amount of protein as well as activated enzyme were visualized with the appropriate primary antibody. The membranes were probed with polyclonal primary antibodies: 1:1000 dilution for phospho-p38 MAPK (Thr 180/Tyr 182), phospho-ERK42/44 (Thr 202/ Tyr 204) and phospho-PKB/AKT (Ser 473) as well as total p38 MAPK,ERK42/44 and PKB/Akt; 1:500 dilution for MKP-1(M-18):sc-1102. Membranes were subsequently washed with large volumes of TBST (2×1 min and then 3×5 min) and the immobilized primary antibody conjugated with a diluted horseradish peroxidase-conjugated antirabbit antibody (1:4000). After thorough washing with TBST, membranes were covered with ECL™ detection reagents and exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission via a non-radioactive method. Films were densitometrically analyzed (UN-SCAN-IT, Silkscience) and the values obtained normalized to the corresponding controls.

## **2.7 Experimental protocols**

In all groups, hearts were stabilized for 15 min retrograde perfusion, and then perfused in the working heart mode for 15 min. Hearts were preconditioned with one or three cycles of 5 min global ischaemia interspersed with 5 min reperfusion (preconditioning, 1xPC and 3xPC) or perfused retrogradely for 30 min (non-preconditioning, N-PC), equal in duration to the total time needed to precondition hearts with ischaemia. All hearts were then subjected to 20 min sustained global ischaemia or 35 min regional ischaemia followed by 30 min reperfusion (10 min retrograde, 20 min working heart ) (see protocol I, page 28). In addition, one group was subjected to 15 min sustained global ischaemia followed by 30 min reperfusion. Protocols for drugs administration will be described in Chapter IV and V.

## **2.8 Statistics**

All values are expressed as mean  $\pm$  standard error (S.E). Multiple comparisons were made by one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. When two groups were compared, Student's t-test was used. Statistical significance was set at  $p < 0.05$ . A minimum of 6 rats were used per experimental group.

---

**CHAPTER 3****Effect of Ischaemia/Reperfusion and Preconditioning  
on Myocardial PSP and PP1 Activities****3.1 Introduction**

As stated in the literature survey (chapter I), the mitogen activated protein kinase (MAPK) signalling systems have been suggested to play a pivotal role in the outcome of ischaemia/reperfusion. However, little is known about the putative regulatory role of the protein phosphatases in ischaemia/reperfusion as well as in preconditioning. It was thus hypothesized that the balance between the activation state of the MAPK and the induction of phosphatases may play a major role in determining the fate of cardiomyocytes exposed to ischaemic stress.

Relatively few studies have been done on phosphatases in this scenario. PP1 and PP2A are present in the heart, with the amount of PP2A being ~3 times higher than that of PP1 (236). PP2A is also present in isolated rat cardiomyocytes (237). Most, if not all, studies on phosphatase involvement in ischaemia/reperfusion, employed phosphatase inhibitors. The interpretation of these results is often complicated by the lack of specificity of many of these inhibitors. However, as far as we know, measurement of phosphatase activity during sustained ischaemia of preconditioned and non-preconditioned hearts was only done in one study (239), showing a decline in the activity of both PP1 and PP2A during ischaemia, but no difference between the groups. Although MAPKs and PKB/Akt activities during a preconditioning protocol were studied by several workers (207, 208, 262, 263), including ourselves (242, 246), only PTEN activation in phosphatases was studied during a preconditioning phase (see reference 130).

The aim of our first experiment was therefore to assess PSP and PP1 activities during sustained ischaemia and during reperfusion of non-preconditioned (N-PC) and 3 x 5 min preconditioned hearts (3 x PC).

### **3.2 Methods**

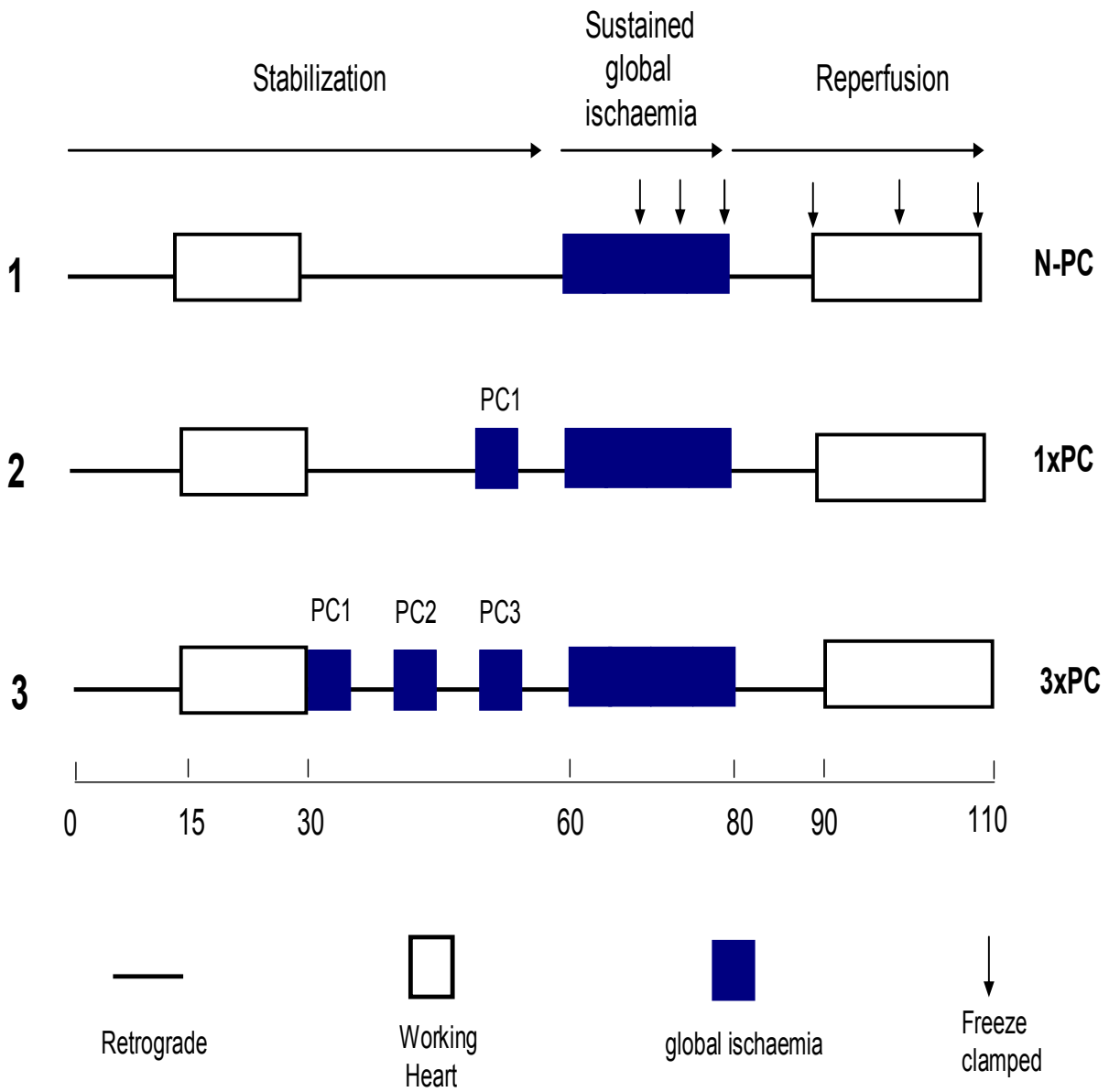
Three series of hearts were perfused, namely controls, non-preconditioned (N-PC) and hearts preconditioned with 3 x 5 min global ischaemia (3 x PC). Control hearts were perfused for a total of 30 min. N-PC and 3xPC hearts were subjected to 20 min global ischaemia, followed by 30 min reperfusion (see protocol I). Hearts were freeze-clamped at 10, 15 and 20 min of sustained ischaemia and at 10, 20 and 30 min of reperfusion.

### **3.3 Results**

Table 1 shows there were no differences in PSP and PP1 activities after 10, 15, 20 minutes sustained ischaemia or after 10, 20, 30 minutes reperfusion ( $p > 0.05$ ) in both N-PC and 3 x PC hearts. In addition, PSP and PP1 activities in N-PC and 3 x PC hearts at all these time intervals did not differ significantly from their respective control values.

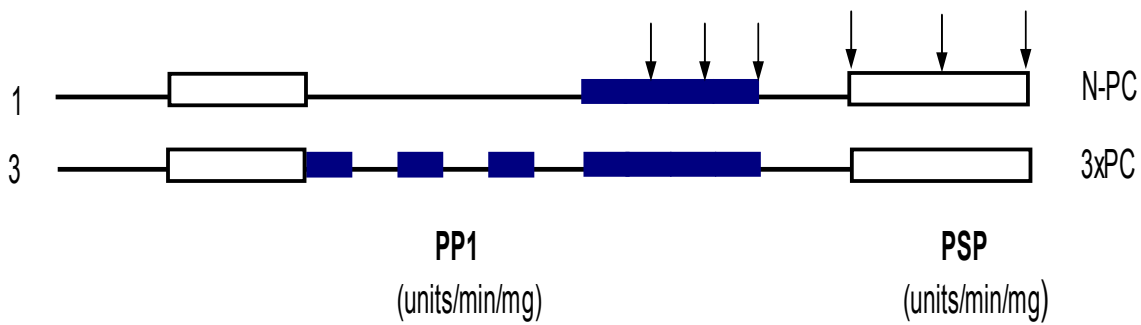
In summary, the data showed that the PSP and PP1 activities did not differ between non-preconditioned and 3 x 5 min preconditioned hearts during both sustained ischaemia and during reperfusion.

## Protocol I



**Table 1**

**PSP and PP1 activities of non-preconditioned and preconditioned hearts during sustained global ischaemia and reperfusion.**



Time(min)	Control 4.26±0.28	N-PC	PC	Control 3.79±0.34	N-PC	PC
		(n=6)		(n=8)		
GI	10'	4.73±0.66	4.64±0.57	3.87±0.50	3.48±0.28	
	15'	4.31±0.63	4.21±0.75	3.36±0.40	3.21±0.38	
	20'	4.28±0.67	4.05±0.47	3.48±0.50	3.69±0.60	
		(n=6)		(n=2)		
Rep	10'	4.93±0.45	4.87±0.29	4.19	4.50	
	20'	4.87±0.60	5.05±0.63	3.34	2.82	
	30'	4.56±0.47	4.79±0.70	3.90	4.33	

PSP: protein serine/threonine phosphatases; PP1: protein phosphatase 1, GI: sustained global ischaemia; Rep: reperfusion; PSP and PP1 activities were expressed as units/min/mg (1 unit of phosphatase activity releases 1 nmol phosphate from MyBP per min in a standard assay). Data are means ± SE. For more information about protocols, see protocol I.



### 3.4 Discussion

Previous studies from our laboratory (242, 243, 246) and as well as those from others (262-264) showed that the major subgroups of the MAPK signalling pathway, viz JNK (265, 266), ERK42/44 (262, 264, 267) and p38 MAPK (242, 263, 266) are activated by short episodes of ischaemia-reperfusion, as occurs during an ischaemic preconditioning protocol. In particular, p38 MAPK has been studied as a candidate kinase in the cardioprotection elicited by preconditioning. Our own results indicated attenuation of p38 MAPK activation during both sustained ischaemia and reperfusion to be associated with cardioprotection (242). However, the role of p38 MAPK as a mediator of protection during sustained ischaemia, is still controversial, since increased activation of the kinase during sustained ischaemia of preconditioned hearts has also been reported (239A). The reason(s) for this discrepancy need to be established, but species differences (rat vs. rabbit) may play a role.

Should our postulated hypothesis hold true, it is to be expected that upregulation of phosphatase activity during ischaemia/reperfusion may contribute to attenuation of p38 MAPK activation, seen in previous experiments (242). The results presented in Table 1 shows that it is unlikely that upregulation of the protein serine/threonine phosphatase (PSP) and more particularly PP1 are involved in the changes seen. In fact, neither ischaemia nor reperfusion, for 20 and 30 min respectively, had any effect on the activities of these phosphatases, and values obtained were similar to those of control hearts.

The possibility exists that the reperfusion times used in this study were too short and that changes may occur after longer periods of reperfusion. This, however, remains to be determined.

As far as we are aware, the effect of ischaemic preconditioning on PP1 and PP2A activity during sustained ischaemia but not reperfusion was studied by only one other group, namely Weinbrenner *et al* (239), using isolated rabbit hearts as well as cardiomyocytes. In their model, a time-dependent significant decline in PP1 activity was observed during 60 min sustained ischaemia, with very little change in PP2A activity. As was also reported by Weinbrenner and coworkers (239), we did not observe differences between the phosphatase activity of non-preconditioned control and

preconditioned hearts subjected to 20 min sustained global ischaemia (Table 1). Phosphatase activity has not been studied during reperfusion after sustained ischaemia and remains to be established.

In contrast to these negative findings, Ladilov and coworkers (see reference 250) suggested that PP1 was a mediator of hypoxic preconditioning of the isolated heart, since the inhibitor cantharidin (used at a concentration which inhibits both PP1 and PP2A) was found to abolish hypoxic preconditioning-induced protection.

Finally, although our study thus far could not provide evidence for increased phosphatase activation by ischaemic preconditioning, it has to be acknowledged that only two phosphatase groups were evaluated in this study. In view of the size, structural diversity and complexity of the phosphatase family, it is possible that other phosphatases may be involved in the response of the myocardium to ischaemia. In addition, in view of Ladilov's (see reference 250) observations, the role of at least PP1 needs to be reinvestigated.

---

**CHAPTER 4****Manipulation of PP1 and PP2A Activity in Ischaemic Preconditioning: Effects on Mechanical Recovery and Kinase Activation Patterns during Reperfusion****4.1 Introduction**

In view of the negative results obtained thus far (see Chapter III) as well as the size of the phosphatase family, which makes identification of the involvement of a specific phosphatase extremely difficult (in view of the sheer volume of experimental work involved), it was decided to focus on two phosphatase inhibitors to further elucidate the putative roles of PP1 and PP2A in ischaemia/reperfusion and preconditioning. PP1 and PP2A account for >90% of all serine/threonine dephosphorylation reactions (268) and are expressed in all tissue types, participate in a large variety of cellular activities and are involved in numerous cell signalling pathways, also in cardiac myocytes (237).

In this study, we used cantharidin and okadaic acid to differentiate between these two phosphatases. Cantharidin has an  $IC_{50}$  for PP1 of  $10^{-6}M$ , for PP2A of  $10^{-7}M$ . To inhibit both PP1 and PP2A, we used the drug at a concentration of 5  $\mu M$ . Okadaic acid has an  $IC_{50}$  for PP1 of  $10^{-7}M$ , for PP2A of  $10^{-9}M$ . To inhibit PP2A only, we used okadaic acid at a concentration of 7.5 nM. The effects of these drugs on cardioprotection were evaluated by administration of the drugs in N-PC and PC hearts. Mechanical recovery during reperfusion, infarct size as well as the activation patterns of PKB/Akt, p38 MAPK and ERK42/44 during reperfusion were used as endpoints.

**4.2 Methods**

For studies on functional recovery during reperfusion and kinase activation, hearts were subjected to 20 min global ischaemia followed by 30 min reperfusion. For evaluation of infarct size, hearts were subjected to 35 min coronary ligation (to obtain a reproducible infarct) and 30 min reperfusion. For Western blotting, hearts were freeze-clamped as follows: (1) During the PC protocol: at the end of 5 min ischaemia and at the end of 5 min reperfusion (before onset of 20 min sustained ischaemia); (2) during reperfusion: 5 and 15 min after 20 min sustained ischaemia.

**Drug treatments (protocol II)**

Isolated rat hearts were randomly assigned to one of the following treatment groups ( $n \geq 6$  per group). Drugs used were cantharidin (5  $\mu\text{M}$ ) and okadaic acid (7.5 nM). Cantharidin was dissolved in absolute ethanol (final concentration 0.5% in perfusate), while okadaic acid was dissolved in distilled water. N-PC and PC protocols are described before (see protocol I)

**(1) Drugs administered before 15 min or 20 min sustained global ischaemia in N-PC hearts.**

N-PC hearts were treated with a drug for 10 minutes before 15 or 20 min sustained global ischaemia (without washout), followed by 30 min reperfusion (protocols 2,4).

**(2) Drugs administered during PC phase before 20 min sustained global ischaemia.**

1xPC hearts received the drug for 5 min before and 5 min after one 5-min period of global ischemia, then subjected to 20 min sustained global ischaemia followed by 30 min reperfusion (Drug+1PC+Drug, protocol 7); in some hearts the drug was washed out after the preconditioning ischaemia, before being subjected to 20 min sustained global ischaemia and 30 min reperfusion (Drug+1PC, protocol 8). 3xPC hearts were treated with drugs for 5 min before each three 5-min periods of global ischemia, then subjected to 20 min sustained global ischaemia followed by 30 min reperfusion (Drug+3PC, protocol 12).

**(3) Drugs administered during reperfusion after 20 min sustained global ischaemia.**

N-PC, 1xPC or 3xPC hearts received the drug during the first 10 minutes of reperfusion after 20 min sustained global ischaemia, followed by 20 min reperfusion in the working mode (N-PC R+Drug, 1PC R+Drug and 3PC R+Drug, protocols 5, 9, 11).

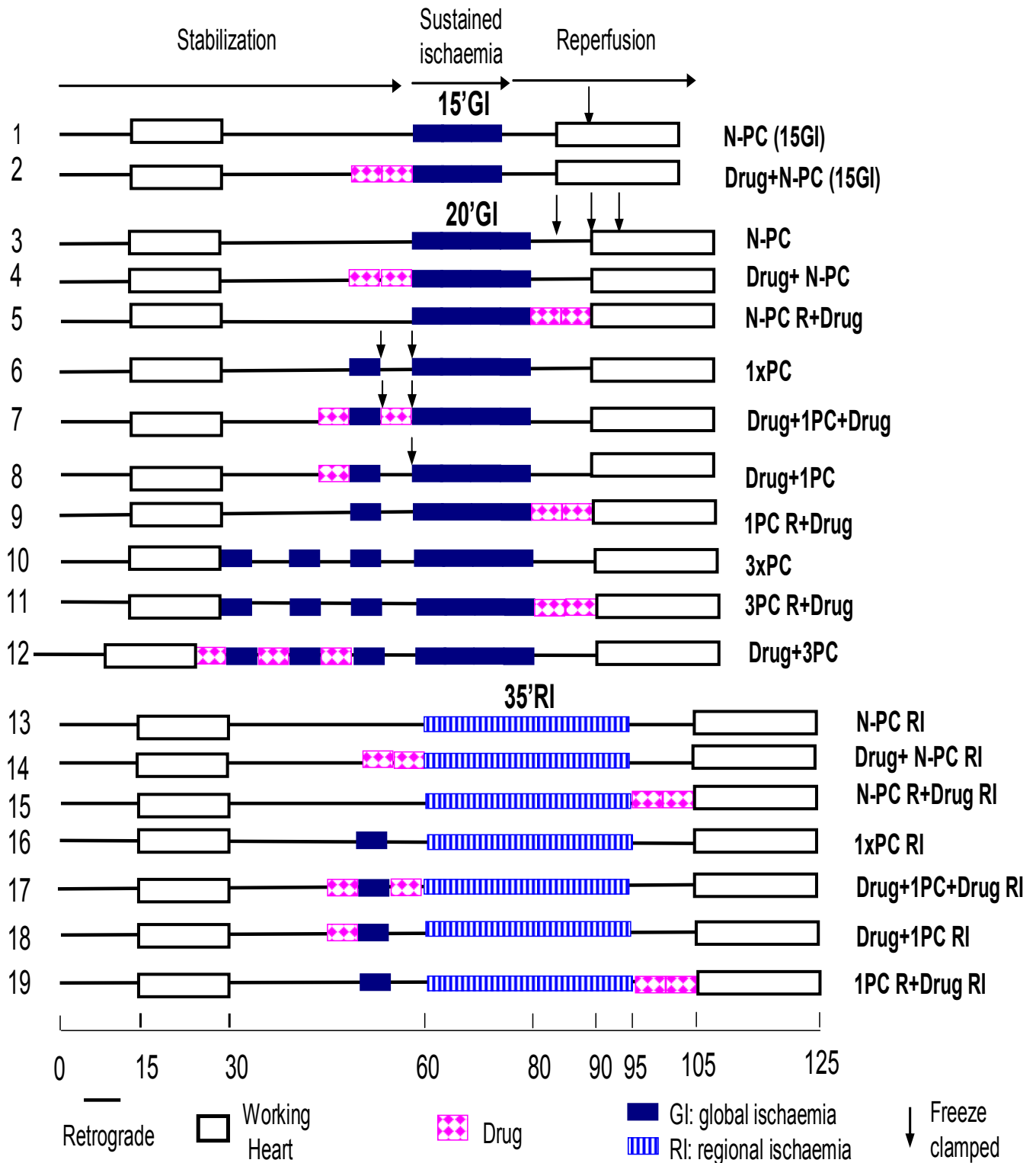
**(4) Drugs administered in N-PC protocol or during PC phase before 35 min sustained regional ischaemia.**

N-PC hearts were treated with a drug for 10 minutes before 35 min sustained regional ischaemia (without washout), followed by 30 min reperfusion (protocol 14). In 1xPC hearts, drugs were given for 5 min before and 5 min after one 5-min period of global ischemia (protocol 17), in some hearts the drug was washed out after the preconditioning ischaemia (protocol 18) and then subjected to 35 min sustained regional ischaemia and 30 min reperfusion.

**(5) Drugs administered during reperfusion after 35 min sustained regional ischaemia.**

N-PC or 1xPC hearts were given the drugs during first 10 minutes of reperfusion after 35 min sustained regional ischaemia, followed by 20 min reperfusion in the working mode (protocols 15 and 19).

## Protocol II



## 4.3 Results

### 4.3.1 Preliminary studies

In preliminary studies, when hearts were subjected to **15 min** sustained global ischaemia in N-PC protocol, all hearts recovered during 30 min reperfusion (see Table 3); when hearts were subjected to **25 min** sustained global ischaemia in 3xPC protocol, almost all hearts were failed during 30 min reperfusion (data not shown).

In view of these results, it was decided to limit the period of sustained global ischaemia to 20 min.

### 4.3.2 Effect of ischaemia/reperfusion and ischaemic preconditioning on cardiac mechanical recovery during reperfusion

Table 2 and Fig 1 show that exposure of non-preconditioned hearts to 20 minutes sustained global ischaemia caused a significant reduction in all parameters of mechanical function measured during 30 min reperfusion, compared to values obtained before ischaemia ( $p < 0.05$ ). A significant decrease in AO, CO and TW compared to values obtained before ischaemia ( $p < 0.05$ ) was also seen in preconditioned (1xPC and 3xPC) hearts. In these series Psp, HR and CF remained unchanged ( $p > 0.05$ ).

However, comparison of values obtained during reperfusion of N-PC and PC hearts, showed that both 1xPC and 3xPC caused a significant increase in CF, AO, CO, Psp, HR and TW when compared with non-preconditioned hearts ( $p < 0.05$ ). The post-ischaemic functional recovery of AO, CO and TW was also significantly higher in 3xPC than in 1xPC hearts ( $p < 0.05$ ).

**Table 2****Effects of ischaemia/reperfusion and ischaemic preconditioning on mechanical performance of the isolated rat heart**

	Protocol	CF	AO	CO	Psp	HR	Tw
Before ischaemia	All (61)	15.1±0.3	44.4±0.5	59.6±0.7	92±1	303±3	12.43±0.18
After ischaemia	N-pc (22)	5.6±1.4 †	3.9±1.3 †	9.5±2.5 †	39±10 †	128±32 †	1.67±0.45 †
	1xpc (17)	12.5±0.7*	20.6±2.3*†	33.2±2.5*†	79±1*	276±11*	5.80±0.49*†
	3xpc (22)	13.3±0.9#	29.3±1.1#&†	42.7±1.5#&†	89±3#	295±15#	8.52±0.57#&†

\* P &lt; 0.05 1xPC vs N-PC

# p &lt; 0.05 3xPC vs N-PC

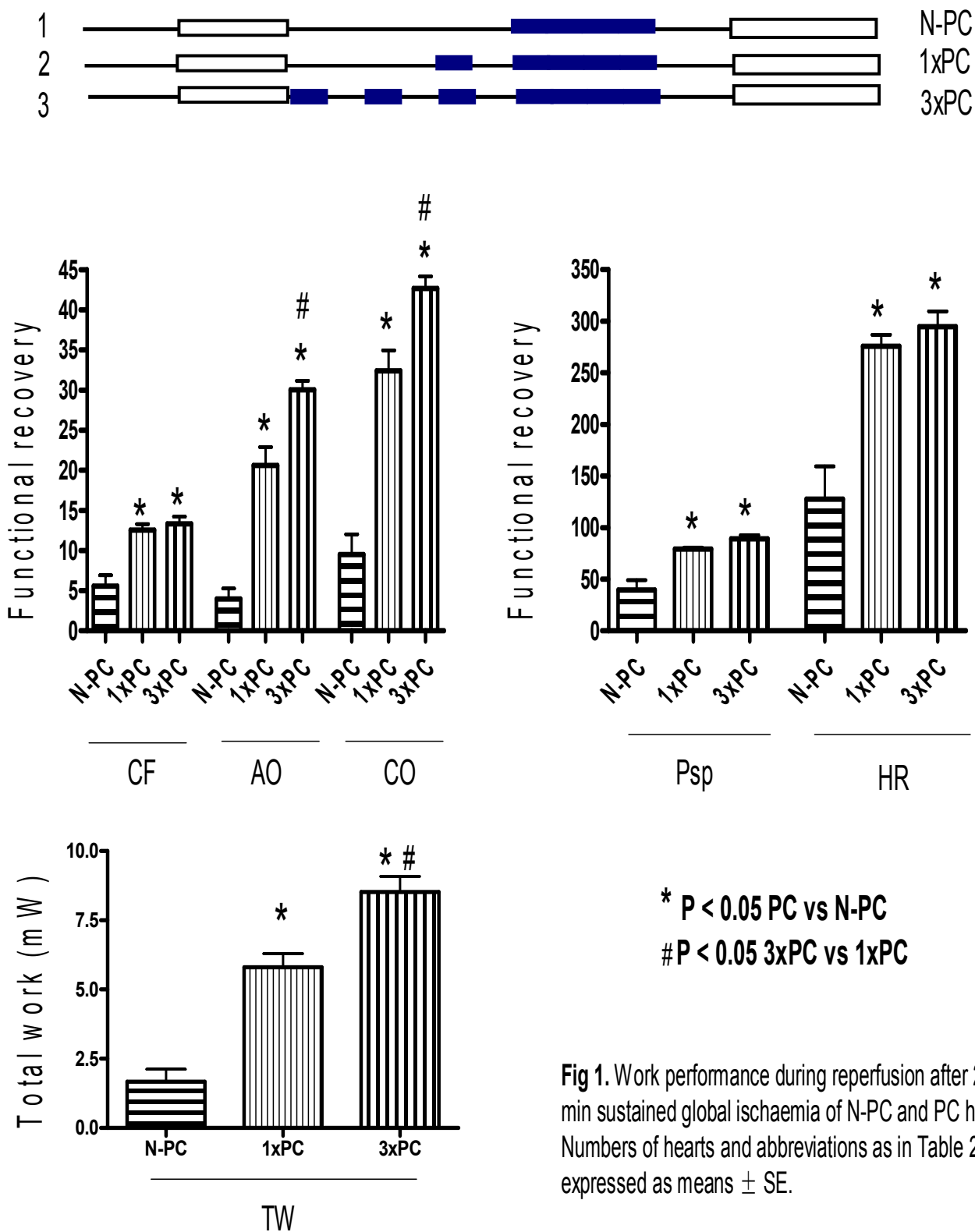
&amp;p &lt; 0.05 3xPC vs 1xPC

† p &lt; 0.05 vs Before ischaemia

Data are means ± SE; numbers in parentheses indicate number of hearts.

CF: coronary flow (ml/min) AO: aortic output (ml/min) CO: cardiac output (ml/min)

Psp: peak systolic pressure (mmHg) HR: heart rate (beats/min) TW: total work (mW)



**Fig 1.** Work performance during reperfusion after 20 min sustained global ischaemia of N-PC and PC hearts. Numbers of hearts and abbreviations as in Table 2. Data expressed as means  $\pm$  SE.



### **4.3.3 Effects of cantharidin**

Cantharidin (5  $\mu$ M) was added to the perfusate before and after sustained global ischaemia of N-PC and PC hearts (see protocol II).

#### **4.3.3.1 Post-ischaemic functional recovery of non-preconditioned hearts**

In a preliminary study, hearts were pretreated for 10 min with cantharidin (5  $\mu$ M) or okadaic acid (7.5 nM) before being subjected to **15 min** sustained global ischaemia (Drug+N-PC, 15'GI, see protocol II 2). This caused a significant reduction in AO, CO, and TW ( $p < 0.05$ , Table 3, Fig 2.1,2.2) during reperfusion when compared with untreated N-PC(15'GI) hearts. CF, Psp and HR were unaltered ( $p > 0.05$ ).

In follow-up studies, cantharidin (5 $\mu$ M) administered for 10 min before 20 min sustained global ischaemia (Can+N-PC, protocol II 4), caused complete mechanical failure during reperfusion and no measurements could be made. When cantharidin was added during first 10 min of reperfusion after 20 min sustained global ischaemia (N-PC R+Can, protocol II 5), there was no effect on any of the parameters of mechanical activity during reperfusion when compared with untreated N-PC hearts ( $p > 0.05$ , Table 3).

#### **4.3.3.2 Post-ischaemic functional recovery of preconditioned hearts.**

In these studies, cantharidin (5  $\mu$ M) was added to the perfusate during the preconditioning phase and during reperfusion of preconditioned hearts (see protocol II: 7-12). Except for CF, HR and Psp in 1xPC, 3xPC and 1xPC R+Can as well as all values in 3xPC R+Can hearts, all parameters of mechanical function during reperfusion were significantly reduced in all groups when compared with values obtained before induction of ischaemia.

Table 3 shows that cantharidin (5  $\mu$ M) added 5 min before and 5 min after one ischaemic episode (Can+1PC+Can, see protocol 7) or 5 min before each of three 5-min periods of global ischemia (Can+3PC, protocol 12), significantly abolished cardiac mechanical recovery (CF, AO, CO, Psp, HR, TW) during reperfusion after 20 min sustained global ischaemia compared to preconditioned hearts without cantharidin pretreatment (protocol 6, 10). However when cantharidin (5  $\mu$ M) was administered only 5 min before the 5 min global ischaemia and washed out (Can+1PC, protocol 8),

---

only the aortic output was significantly reduced (Can+1PC:  $10.4 \pm 3.8$  vs. 1xPC:  $20.6 \pm 2.3$ ,  $p < 0.05$ , Fig. 3), but no marked changes in CF, CO, Psp, HR, TW compared to 1xPC hearts without cantharidin pretreatment (protocol 6). This indicated that the effects of cantharidin can be attenuated by washing out the drug before the onset of sustained global ischaemia.

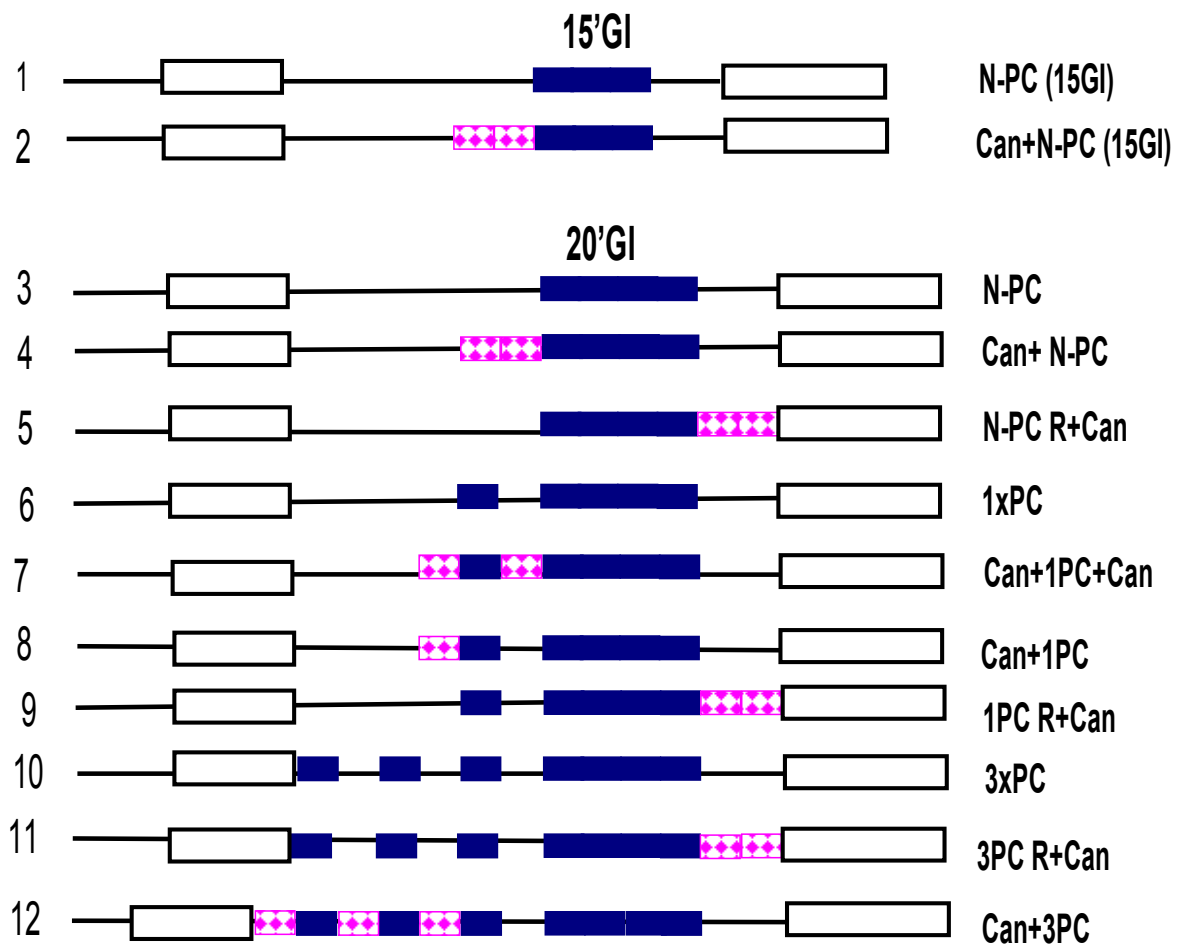
Cantharidin ( $5 \mu\text{M}$ ) added during first 10 min of reperfusion in 1xPC hearts (1PC R+Can, see protocol 9), had no effect on cardiac mechanical recovery (CF, AO, CO, Psp, HR, TW) compared with 1xPC hearts without cantharidin treatment, respectively ( $p > 0.05$ ), but cantharidin ( $5 \mu\text{M}$ ), when added during the first 10 min of reperfusion in 3xPC hearts (3PC R+Can), significantly increased postischaemic CF, AO, CO ( $p < 0.05$ ) and TW ( $0.05 < p < 0.1$ ) compared with 3xPC hearts without cantharidin treatment.

In summary, cantharidin added during preconditioning phase abolished functional recovery during reperfusion induced by both 1xPC and 3xPC, while cantharidin administered during reperfusion only, had no deleterious effects. In fact, an increased functional recovery was seen in 3xPC hearts which received the drug during early reperfusion.

## Table 3

### (1) Protocols

Effect of cantharidin (5µM) on functional recovery after sustained global ischaemia of N-PC and PC hearts



For more information about protocols, see protocol II.

**Table 3****(2) Functional recovery**

	Protocols	CF	AO	CO	Psp	HR	Tw
Before ischaemia	All	15.1±0.3	44.4±0.5	59.6±0.7	92±1	303±3	12.43±0.18
	N-PC(15'GI)	12.3±0.8	25.3±1.2†	37.7±1.7†	80±1	291±13	6.75±0.33†
	Can+N-PC(15'GI)	10.1±1.2†	3.6±2.3 *†	13.7±3.1*†	49±14†	162±59†	1.82±0.82 *†
	N-PC	5.6±1.4†	3.9±1.3 †	9.5±2.5 †	39±10†	128±32†	1.67±0.45†
	Can+N-PC ‡	0.00 †	0.00 †	0.00 †	0.00 †\$	0.00 †\$	0.00 †
	N-PC R+Can	7.0±3.4†	3.8±2.7 †	10.8±4.9†	38±17†	146±66†	1.85±0.85†
After ischaemia	1xPC	12.5±0.7	20.6±2.3†	33.2±2.5†	79±1	276±11	5.80±0.49†
	Can+1PC+Can	9.0±0.4#†	3.4±1.6#†	12.4±1.9#†	45±12#†	120±57#†	1.55±0.52#†
	Can+1PC	10.0±1.6†	10.4±3.8#†	20.4±5.3†	79±2	269±22	3.76±0.98†
	1PC R+Can	15.0±1.3	25.7±3.2†	40.7±3.9†	82±1	297±8	7.53±0.85†
	3xPC	13.3±0.9	29.3±1.1†	42.7±1.5†	89±3	295±15	8.52±0.57†
	Can+3PC	6.4±0.9 •†	0 •†	6.4±0.9 •†	9±9 •†	0 •†	0.20±0.20 •†
	3PC R+Can	17.8±1.3&	36.8±0.9&♦	54.7±1.8&♦	82±2	324±15	10.14±0.45♦*

† p &lt; 0.05 vs Before ischaemia

\* P &lt; 0.05 vs N-PC(15'GI)

\$ P &lt; 0.05 vs N-PC(20'GI)

# P &lt; 0.05 vs 1xPC

• P &lt; 0.05 vs 3xPC

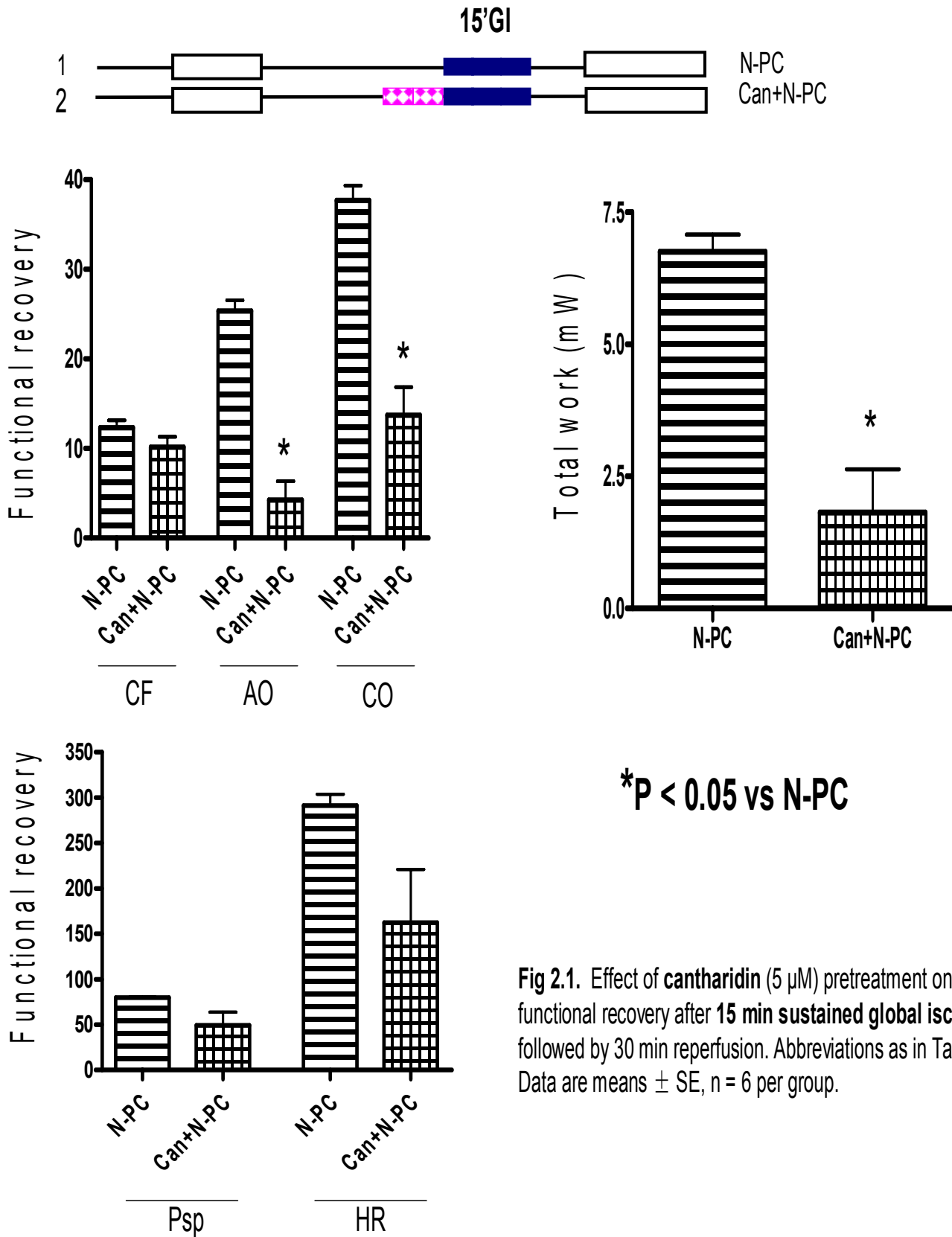
&amp;P &lt; 0.05 vs 3xPC

\* 0.05 &lt; P &lt; 0.1 vs 3xPC

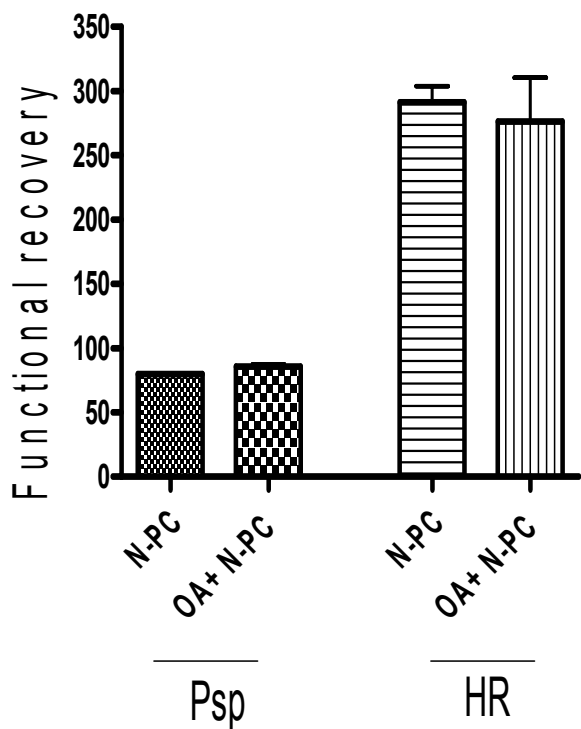
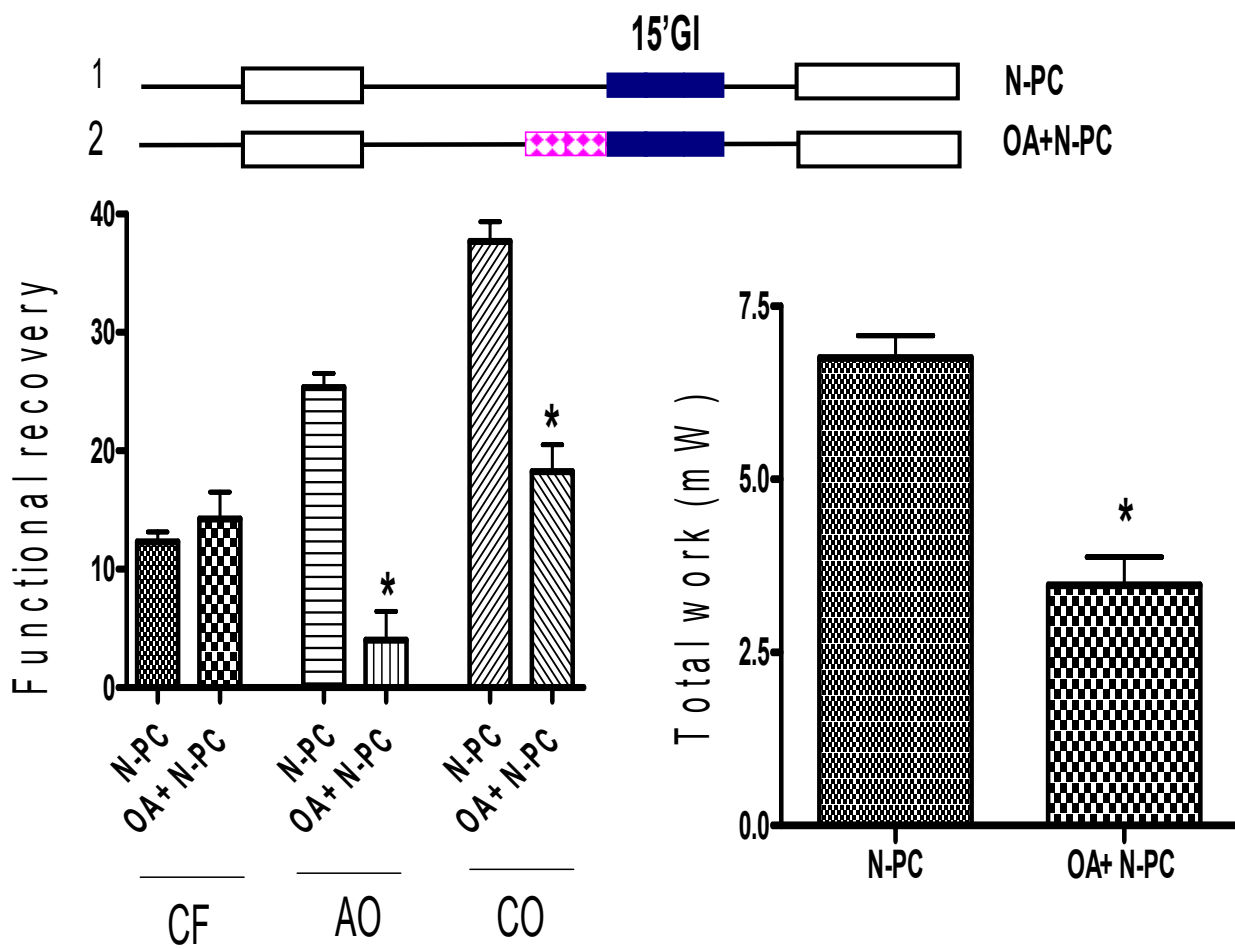
♦ P &lt; 0.05 vs 1xPC R+Can

Abbreviations: as in Table 2. Data are means ± SE. n = 6 per group.

‡ Cantharidin added to the perfusate before 20 min GI caused complete mechanical failure during reperfusion and no measurements could be made.

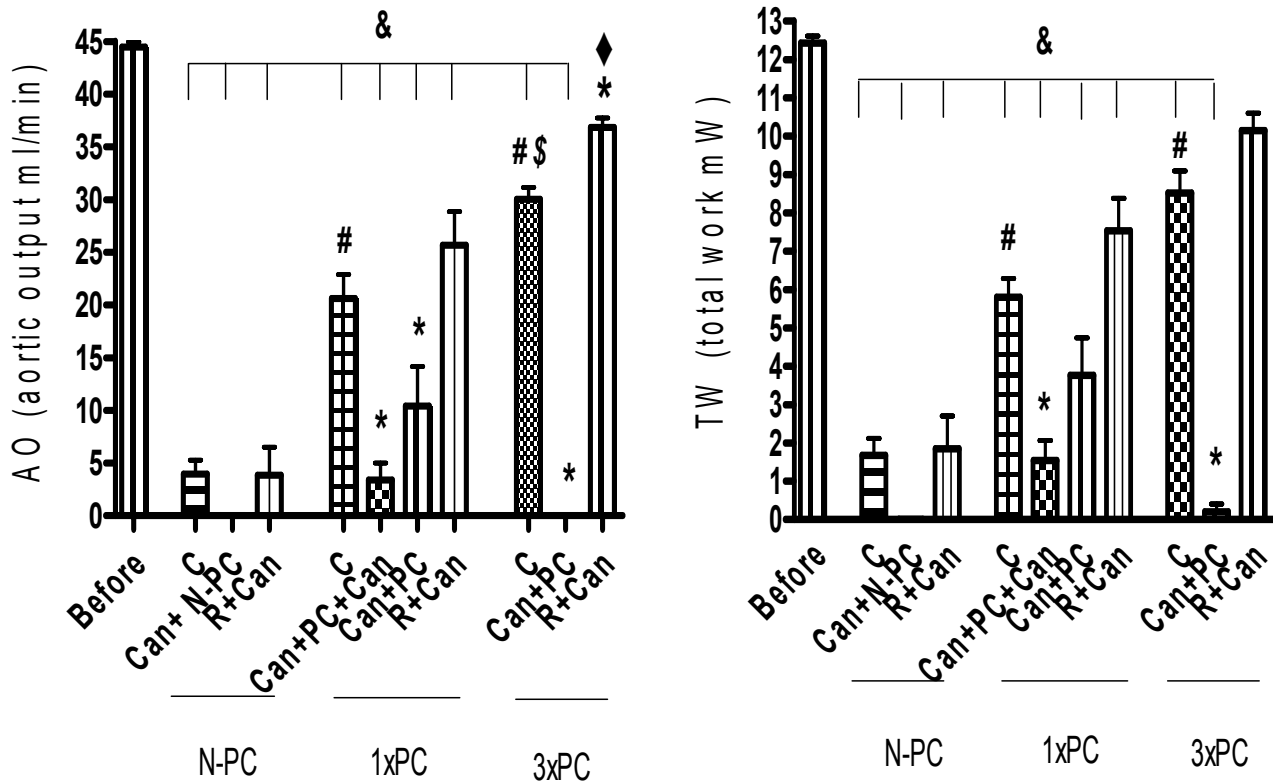


**Fig 2.1.** Effect of cantharidin (5  $\mu$ M) pretreatment on functional recovery after 15 min sustained global ischaemia followed by 30 min reperfusion. Abbreviations as in Table 2. Data are means  $\pm$  SE, n = 6 per group.



\*P < 0.05 vs N-PC

**Fig 2.2.** Effect of *okadaic acid* (7.5 nM) pretreatment on functional recovery after 15 min sustained global ischaemia followed by 30 min reperfusion. Abbreviations as in Table 2. Data are means  $\pm$  SE, n = 5 per group.



\* P < 0.05 vs corresponding C value of group  
 # P < 0.05 (1xPC)C and (3xPC)C vs (N-PC)C  
 \$ P < 0.05 (3xPC)C vs (1xPC)C  
 ♦ P < 0.05 vs 1xPC R+C  
 & P < 0.05 vs Before

**Fig 3.** Effect of **cantharidin** (5  $\mu$ M) treatment on cardiac function during 30 min reperfusion after 20 min global ischaemia. Abbreviations as in Table 2. C= Untreated. Data expressed as means  $\pm$  SE, n= 6 per group. see protocol II.

#### **4.3.3.3 Effects of cantharidin on infarct size.**

Infarct size is expressed as a percentage of the area at risk in rat hearts. For these studies, hearts were subjected to 35 min regional ischaemia, followed by 30 min reperfusion (protocol II 13-19).

When cantharidin (5  $\mu$ M) was administered for 10 min before 35 min sustained regional ischaemia in N-PC hearts (protocol II 14), there was no change in infarct size versus untreated N-PC hearts.

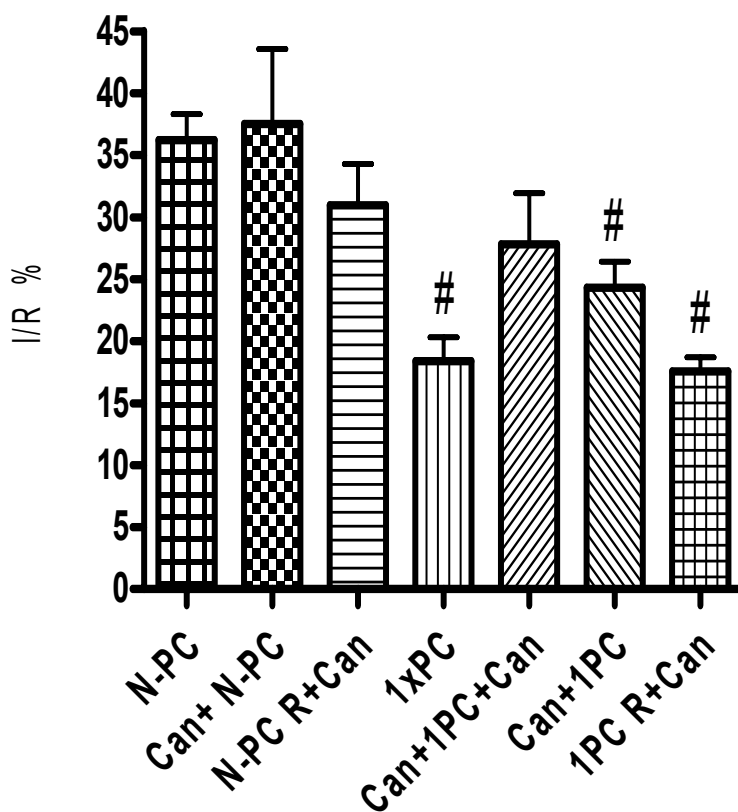
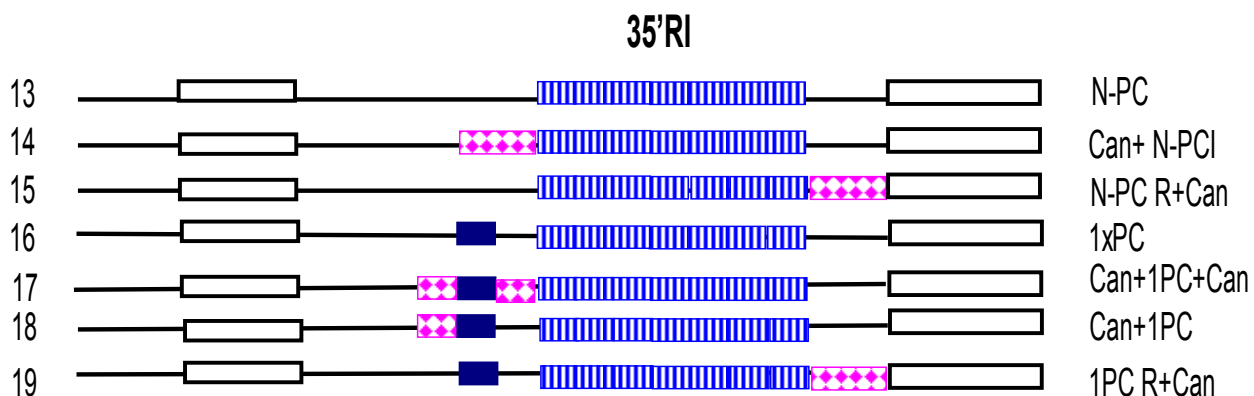
In 1xPC hearts, infarct size was significantly reduced to  $18.4 \pm 1.9\%$  from  $36.2 \pm 2.1\%$  in N-PC hearts ( $p < 0.05$ , Fig. 4). Cantharidin (5  $\mu$ M) added 5 min before and 5 min after one 5-min ischaemic preconditioning protocol (Can+1PC+Can, protocol 17), showed a significant increase in infarct size (Can+1PC+Can:  $27.8 \pm 4.1\%$  vs. 1xPC:  $18.4 \pm 1.9\%$ ,  $p < 0.05$ , Fig 4), which did not differ from N-PC hearts ( $36.2 \pm 2.1\%$ ,  $p > 0.05$ ). Washout of cantharidin during a 1xPC protocol (Can+1PC) retained the beneficial effect of 1xPC (Can+1PC:  $24.3 \pm 2.1\%$  vs. 1xPC:  $18.4 \pm 1.9\%$ ,  $p > 0.05$ ).

Cantharidin (5  $\mu$ M) added during the first 10 min of reperfusion (N-PC R+Can or 1PC R+Can, protocols 15, 19), had no influence on infarct size of N-PC or PC hearts (N-PC R+Can:  $31.0 \pm 3.3\%$  vs. N-PC:  $36.2 \pm 2.1$  and 1PC R+C:  $17.6 \pm 1.1\%$  vs. 1xPC:  $18.4 \pm 1.9\%$ ). Thus the presence of cantharidin (5  $\mu$ M) for the first 10 min of reperfusion did not abrogate the PC-induced reduction in infarct size. Cantharidin was also without effect on infarct size of N-PC hearts when added before or during reperfusion.

In these studies, the area at risk did not differ between the groups. The averaged value was  $46.8 \pm 4.3\%$ .

In summary, administration of cantharidin during the preconditioning phase, abolished the beneficial effects of this intervention on infarct size, while administration during reperfusion was without effect on infarct size in both N-PC and PC hearts.





# P < 0.05 vs N-PC

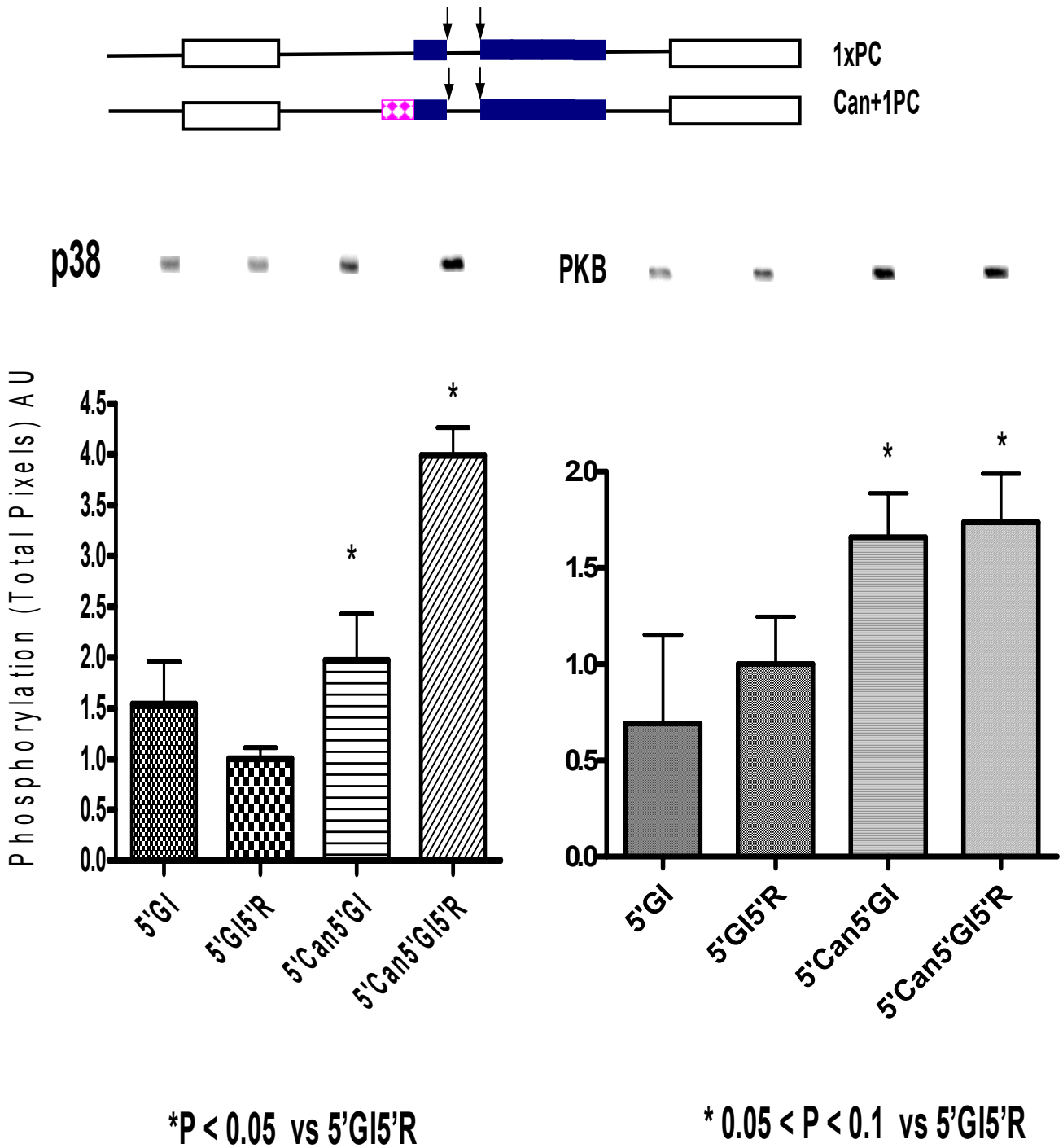
**Fig 4.** Effect of **cantharidin** (5µM) on **infarct size** (IS) of hearts subjected to 35 min coronary ligation and 30 min reperfusion. Infarct size expressed as a percentage of the ischaemic (risk) zone. Data are means ± SE, n ≥ 6 per group, see protocol II.

#### **4.3.3.4 Effect of cantharidin on kinase activation before sustained global ischaemia (GI).**

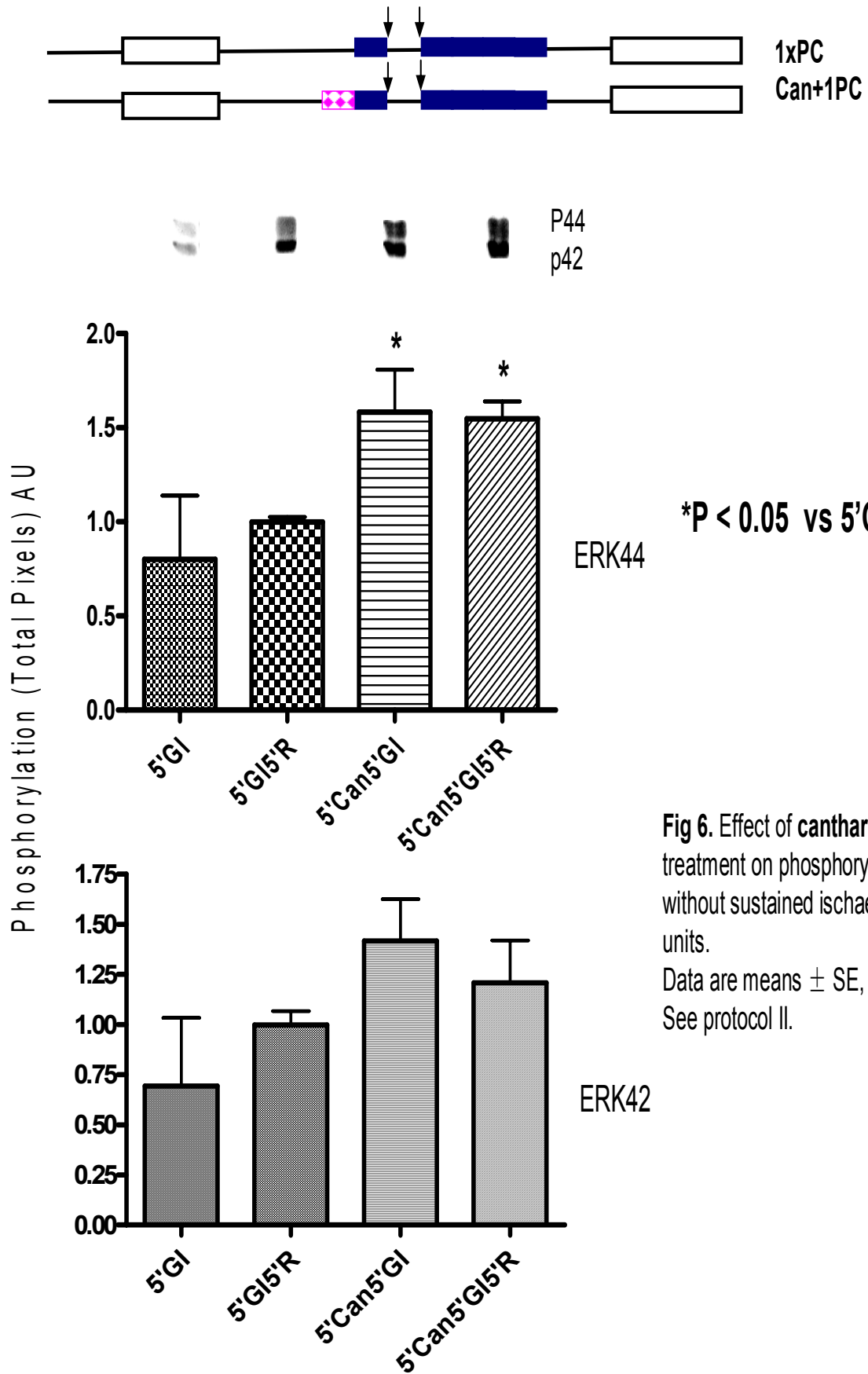
To evaluate events before the onset of sustained global ischaemia, rat hearts were exposed only to 1 x 5 min global ischaemia (1xPC), then freeze-clamped before sustained ischaemia in the presence or absence of cantharidin. All values obtained were normalized with untreated hearts. The results showed that 1x 5 min ischaemia (5'GI) increased p38 MAPK activity, but when reperfused for 5 min (5'GI5'R), the activation of p38 MAPK is reduced (au. 5'GI 1.54+0.11 vs. 5'GI5'R 1.00+0.11). There were no changes in ERK42/44 and PKB activities in 5'GI5'R versus 5'GI without reperfusion.

Since cantharidin was dissolved in ethanol, vehicle control studies were performed where ethanol (0.5%) alone was administered for 10 min before or after 20 min sustained ischaemia and hearts freeze-clamped after 5 and 10 min reperfusion. Assay of kinase activities showed that ethanol has no effect on kinase activation when administered before sustained ischaemia. However, when ethanol added during reperfusion, a slight but significant PKB activation was observed, while p38 MAPK and ERK42/44 were not affected (Van Vuuren and Lochner unpublished data).

It is important to note that administration of cantharidin (5  $\mu$ M) before 5 min ischaemia was sufficient to significantly activate all the kinases involved (Figs 5 and 6) and that they remained activated until the time that the heart goes into long ischaemia (au. p38 MAPK: 5'Can5'GI 1.97+0.46, 5'Can5'GI5'R 3.99+0.28 vs. 5'GI5'R 1.00+0.11,  $p < 0.05$ , respectively; ERK44: 5'Can5'GI 1.58+0.23, 5'Can5'GI5'R 1.55+0.09 vs. 5'GI5'R 1.00+0.03,  $p < 0.05$ , respectively; PKB: 5'Can5'GI 1.66+0.23, 5'Can5'GI5'R 1.74+0.25 vs. 5'GI5'R 1.00+0.25,  $0.05 < p < 0.1$ , respectively). Thus, the activations of these kinases were sustained even after 5 min reperfusion. It was concluded that cantharidin pretreatment (i.e. during the preconditioning phase) caused increased phosphorylation of all three kinases which persisted until the onset of sustained ischaemia.



**Fig 5.** Effect of cantharidin (5  $\mu$ M) treatment on phosphorylation of p38 MAPK and PKB without sustained ischaemia. AU: arbitrary units. Data are means  $\pm$  SE, n = 6 per group. See protocol II.



**Fig 6.** Effect of **cantharidin** (5  $\mu$ M) treatment on phosphorylation of ERK42/44 without sustained ischaemia. AU: arbitrary units. Data are means  $\pm$  SE, n = 6 per group. See protocol II.

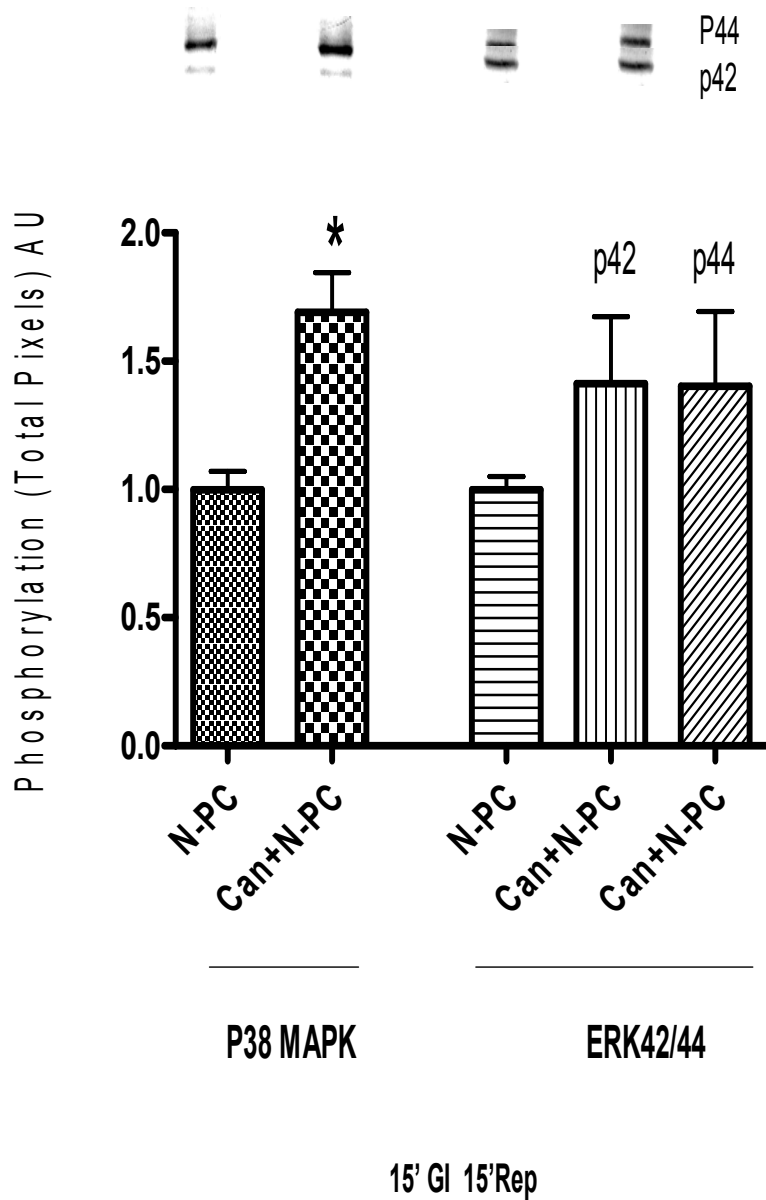
#### **4.3.3.5 Effect of cantharidin on kinase activation during reperfusion.**

##### **Preliminary studies**

To determine the optimal time of reperfusion for assessment of kinase activity, a preliminary series of experiments was done. These hearts were freeze-clamped at 5, 10, 15, 20 or 30 min of reperfusion (n= 3 hearts/series). Subsequent Western blotting showed that PKB/Akt, in particular, was maximally activated at 5 min reperfusion, while no changes were seen after 20 min. It was therefore decided to freeze-clamp hearts after 5 and/or 15 min reperfusion.

Total p38 MAPK, ERK42/44 and PKB were evaluated at 5, 15 and 30 min reperfusion, and were found to be unchanged (data not shown). In view of the unchanged total values for the above kinases, the corresponding blots were not included in the results section.

Also in a preliminary study, pretreatment of N-PC hearts with cantharidin before 15 min sustained global ischaemia (Can+N-PC 15'GI) caused a significantly increased phosphorylation of p38 MAPK after 15 min reperfusion (a.u.  $1.69 \pm 0.15$  vs. 1.00 in N-PC 15'GI,  $p < 0.05$ , Fig. 7), but no marked difference in ERK42/44 phosphorylation (a.u.  $1.41 \pm 0.26/1.40 \pm 0.29$  vs. 1.00 in N-PC 15'GI, respectively,  $p > 0.05$ ).



\* P < 0.05 vs N-PC

**Fig 7.** Effect of **cantharidin** (5  $\mu$ M) treatment on phosphorylation of p38 and ERK42/44 MAPK after **15 min sustained global ischaemia** during 15min reperfusion. AU: arbitrary units; GI: sustained global ischaemia. Data are means  $\pm$  SE, n = 6 per group. See protocol II 1,2.

### **Effect of cantharidin on phosphorylation of p38 MAPK.**

When cantharidin (5  $\mu$ M) was administered during the preconditioning protocol (1x5 min or 3x5 min) before 20 min sustained global ischaemia, there was a twofold increase in p38 MAPK phosphorylation at 5 min and 15 min reperfusion (a.u Can+1PC+Can: 5 min  $2.30 \pm 0.17$  and 15 min  $1.98 \pm 0.25$ ; Can+3PC: 5 min  $2.08 \pm 0.40$  and 15 min  $2.36 \pm 0.20$ ,  $p < 0.05$ , respectively, vs. untreated control, Fig. 8).

Cantharidin (5  $\mu$ M) administered during 5 min reperfusion in preconditioned (PC R+Can) and non-preconditioned (N-PC R+Can) hearts, did not change phosphorylation of p38 MAPK compared to untreated N-PC or PC hearts, respectively (a.u 1PC R+Can:  $0.97 \pm 0.07$ ; 3PC R+Can:  $1.29 \pm 0.18$ ; N-PC R+Can:  $0.88 \pm 0.13$ , vs. untreated hearts 1.00,  $p > 0.05$ ); after 15 min reperfusion, there were slight but no significant increases in p38 MAPK activity (a.u 1PC R+Can:  $1.35 \pm 0.26$ ; 3PC R+Can:  $1.45 \pm 0.27$ ; N-PC R+Can:  $1.66 \pm 0.29$ ,  $p > 0.05$ , Fig. 8).

### **Effect of cantharidin on phosphorylation of ERK42/44.**

Cantharidin (5  $\mu$ M) administered during the 1xPC phase (Can+1PC+Can, protocol 7) before 20 min sustained global ischaemia, reduced phosphorylation of ERK42/44 during 5 min reperfusion (a.u  $0.34 \pm 0.10/0.71 \pm 0.12$ ) compared to untreated 1xPC hearts, but only ERK42 activity was markedly different ( $p < 0.05$ , Fig. 9). After 15 min reperfusion, no changes were observed. Cantharidin (5  $\mu$ M) added during 3xPC protocol (Can+3PC, protocol 12), had no significant effects on ERK42/44 activity during 5 and 15 min reperfusion.

When cantharidin (5  $\mu$ M) was administered during reperfusion (PC R+Can and N-PC R+Can), there were slight but not significant increases of ERK42/44 phosphorylation after 5 min reperfusion (a.u 1PC R+Can:  $1.34 \pm 0.12/1.49 \pm 0.19$ ; 3PC R+Can:  $1.22 \pm 0.03/1.23 \pm 0.07$ ; N-PC R+Can:  $1.44 \pm 0.20/1.46 \pm 0.09$ ,  $p > 0.05$ , Fig. 9) compared to untreated N-PC or PC hearts, respectively. After 15' min reperfusion, ERK42/44 activity had significantly increased in 3PC R+Can (a.u  $1.94 \pm 0.32/2.08 \pm 0.54$ ) and in N-PC R+Can (a.u  $1.83 \pm 0.16/2.33 \pm 0.11$ ),  $p < 0.05$ , respectively. During 10 min reperfusion, in 1PC R+Can also increased ERK42/44 activity (a.u  $1.58 \pm 0.24/1.60 \pm 0.23$ ), but

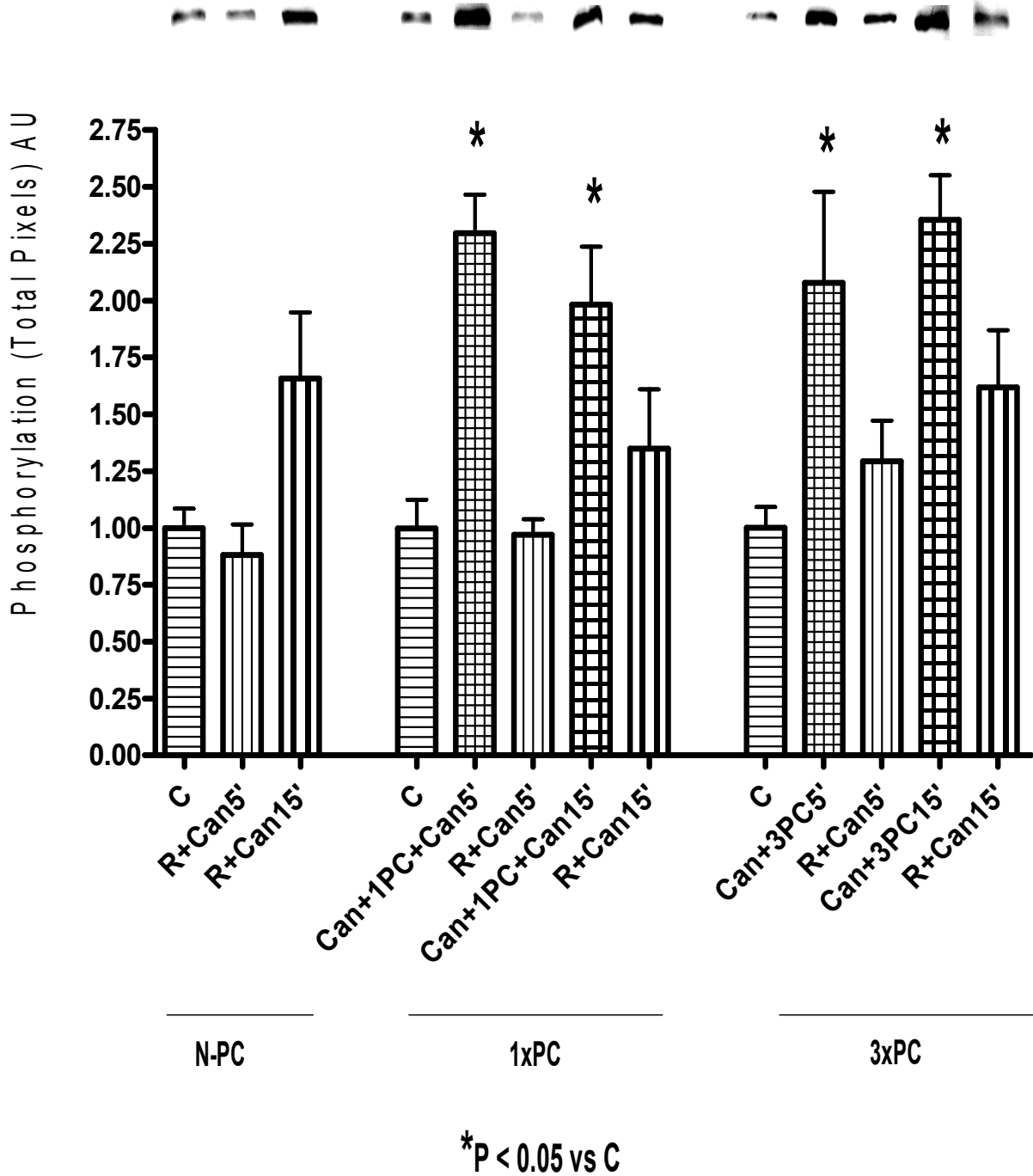
only ERK44 activity (a.u.  $1.60 \pm 0.23$ ) was markedly increased versus untreated 1xPC hearts ( $p < 0.05$ , Fig 9).

#### **Effect of cantharidin on phosphorylation of PKB.**

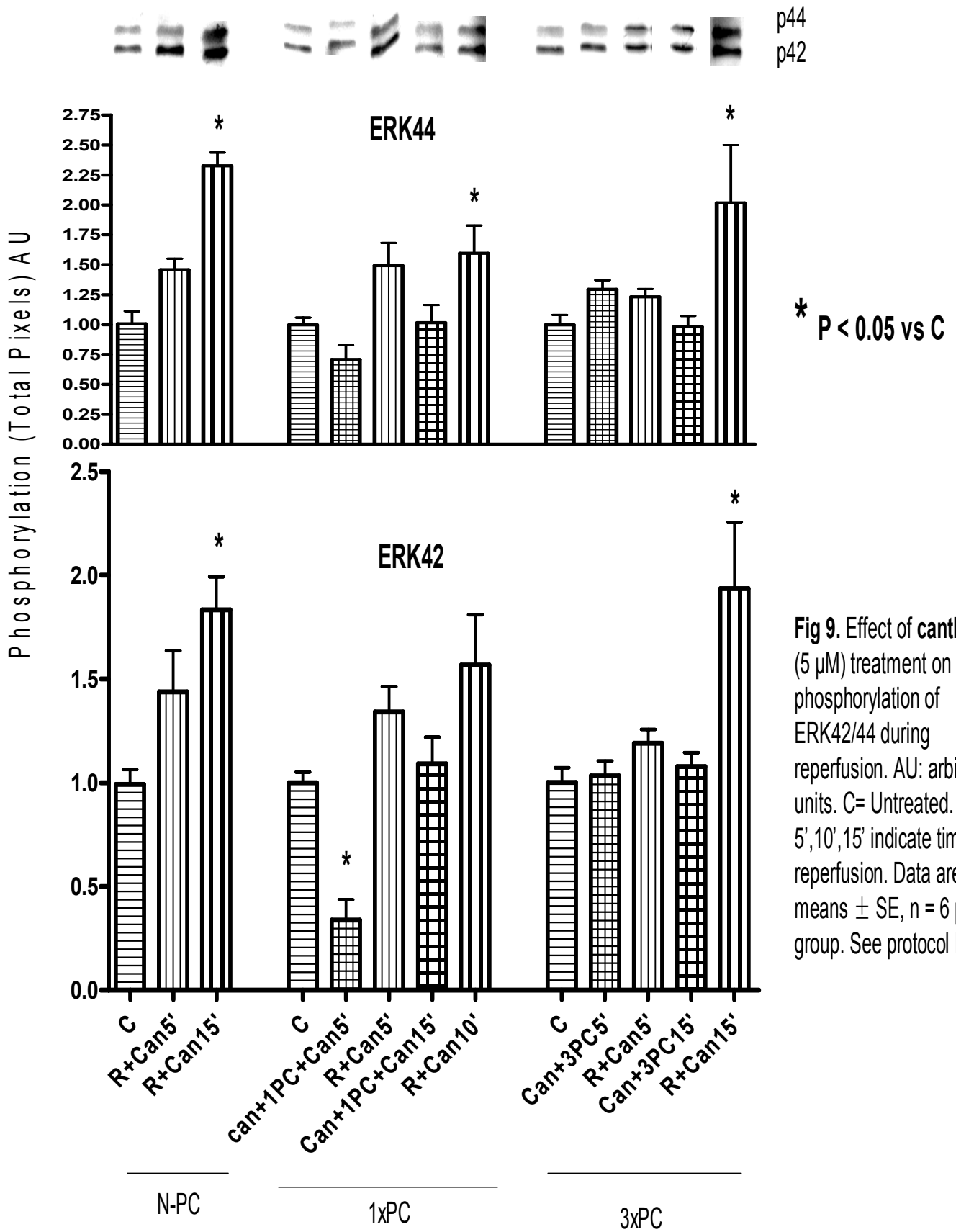
When cantharidin ( $5 \mu\text{M}$ ) was administered during reperfusion or during the preconditioning protocol, there were significant increases in PKB phosphorylation during 5 min reperfusion (a.u. 1PC R+Can:  $1.85 \pm 0.16$  ; Can+1PC+Can:  $1.80 \pm 0.49$  ; Can+3PC:  $1.68 \pm 0.30$ ,  $p < 0.05$  vs untreated hearts, respectively, Fig. 10). After 15 min reperfusion, only 3PC R+Can showed a marked increase in PKB activity (a.u.  $2.23 \pm 0.35$ ,  $p < 0.05$ ). There was no change in PKB phosphorylation in N-PC R+Can protocol after 5 and 15 min reperfusion.



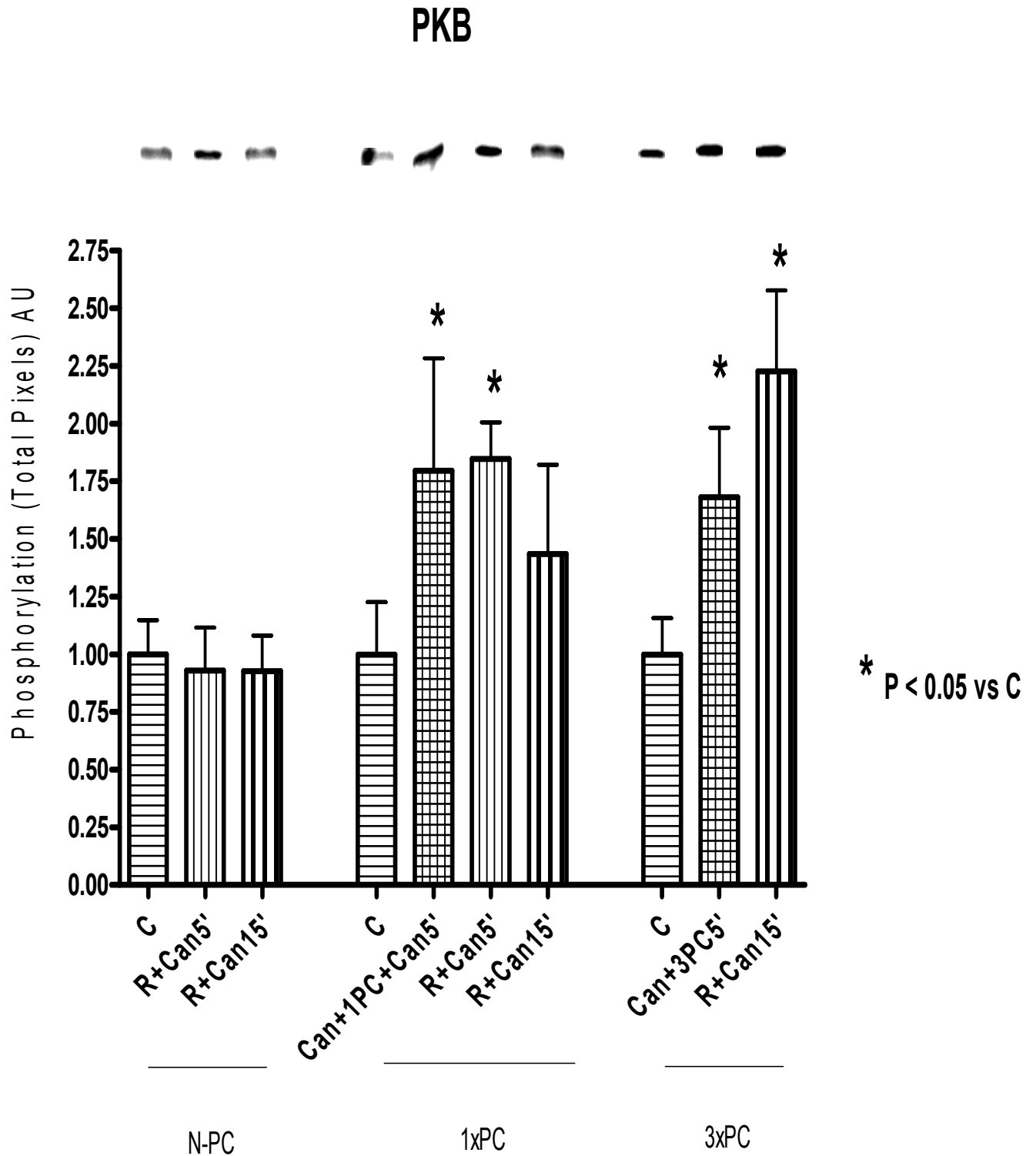
### P38 MAPK



**Fig 8.** Effect of **cantharidin** (5  $\mu$ M) treatment on phosphorylation of **p38 MAPK** during reperfusion. AU: arbitrary units. C= Untreated. 5' and 15' indicate time of reperfusion. Data are means  $\pm$  SE, n = 6 per group. See protocol II.



**Fig 9.** Effect of cantharidin (5  $\mu$ M) treatment on phosphorylation of ERK42/44 during reperfusion. AU: arbitrary units. C= Untreated. 5', 10', 15' indicate time of reperfusion. Data are means  $\pm$  SE, n = 6 per group. See protocol II.



**Fig 10.** Effect of **cantharidin** (5  $\mu$ M) treatment on phosphorylation of **PKB** during reperfusion. AU: arbitrary units. C= Untreated. Data are means  $\pm$  SE, n = 6 per group. See protocol II.

#### **4.3.4 Effect of okadaic acid**

In view of the fact that very similar results were obtained in one and three cycle preconditioned hearts when treated with cantharidin, it was decided to study the effects of okadaic acid (7.5 nM) in a single cycle preconditioning protocol only.

##### **4.3.4.1 Effect of okadaic acid on post-ischaemic functional recovery**

As observed above, when hearts were treated with okadaic acid (7.5 nM), except for Psp, HR and CF in 1PC R+OA protocol, all parameters of mechanical function measured during reperfusion were significantly less than the values obtained before induction of ischaemia (Table 4, see protocol II. 5, 7 and 9).

As also described before in a preliminary study, okadaic acid (7.5 nM) administered for 10 min before **15 min** sustained global ischaemia (OA+N-PC, 15'GI, protocol II 2), caused a significant reduction in AO, CO, and TW ( $p < 0.05$ , Fig 2.2) during reperfusion when compared with untreated N-PC(15'GI) hearts. CF, Psp and HR were unaltered ( $p > 0.05$ ).

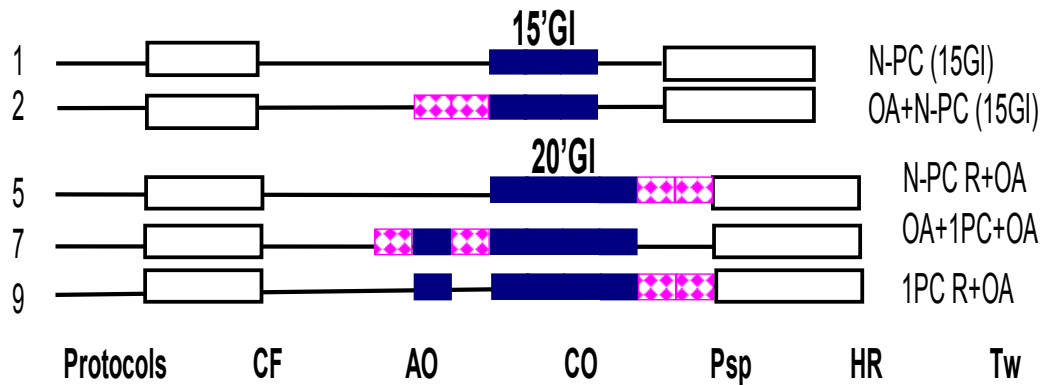
In follow-up study, okadaic acid (7.5 nM) added 5 min before and 5 min after one ischaemic episode (OA+1PC+OA, protocol 7), significantly abolished cardiac mechanical recovery (CF, AO, CO, Psp, HR, Tw) during reperfusion after 20 min sustained global ischaemia, compared with untreated 1xPC hearts.

Okadaic acid (7.5 nM) added during first 10 min of reperfusion in N-PC protocol (N-PC R+OA, protocol 5), had no effect on cardiac mechanical recovery (CF, AO, CO, Psp, HR, TW) compared with N-PC hearts without okadaic acid treatment ( $p > 0.05$ , Table 4); in 1xPC R+OA protocol, okadaic acid had significantly increased post-ischaemic AO and TW versus 1xPC untreated hearts ( $p < 0.05$ , Fig 11).

In summary, in 1xPC hearts, okadaic acid added during preconditioning phase abolished preconditioning-induced functional recovery during reperfusion, while okadaic acid administered during reperfusion only, improved mechanical performance during 30 min reperfusion. In contrast to the preconditioned hearts, okadaic acid, when administered during the reperfusion of non-preconditioning hearts, was without effect on functional recovery.

**Table 4**

Effect of okadaic acid (7.5nM) on functional recovery after 20 min sustained global ischaemia of N-PC and PC hearts.



	Protocols	CF	AO	CO	Psp	HR	Tw
Before ischaemia	All	15.1±0.3	44.4±0.5	59.6±0.7	92±1	303±3	12.43±0.18
After ischaemia	N-PC(15GI)	12.3±0.8	25.3±1.2†	37.7±1.7†	80±1	291±13	6.75±0.33†
	OA+N-PC(15GI)	14.3±2.3	3.2±2.1†&	18.3±2.3†&	86±2	276±34	3.47±0.41†&
	N-PC	5.6±1.4†	3.9±1.3†	9.5±2.5†	39±10†	128±32†	1.67±0.45†
	N-PC R+OA	6.8±3.1†	9.2±4.1†	16.0±7.2†	38±17†	154±69†	2.73±1.22†
	1xPC	12.5±0.7	20.6±2.3†	33.2±2.5†	79±1	276±11	5.80±0.49†
	OA+1PC+OA	7.3±3.3*†	3.0±1.8*†	10.3±4.8*†	36±16*†	120±75*†	1.54±0.79*†
	1PC R+OA	14.3±1.1	30.8±2.7#†	45.2±3.5†	90±5	284±20	9.01±0.63#†

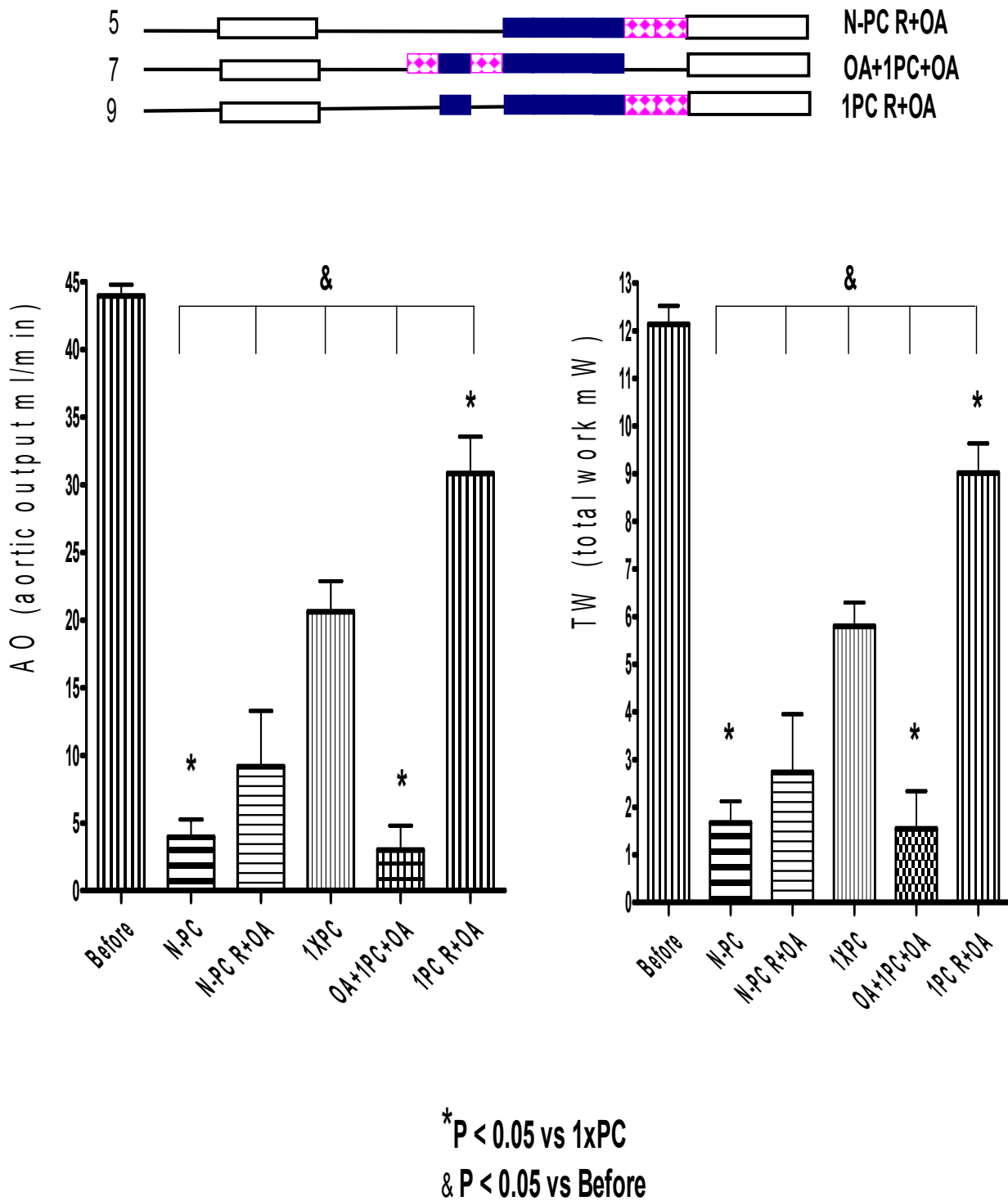
† p < 0.05 vs Before ischaemia

\* P < 0.05 vs 1xPC

& P < 0.05 vs N-PC(15'GI)

# P < 0.05 vs 1xPC

Abbreviations: as in Table 2. Data are means ± SE, n = 6 per group.



**Fig 11.** Effect of **okadaic acid** (7.5nM) treatment on cardiac function during 30 min reperfusion after 20 min global ischaemia. Abbreviations as in Table 2. Data are means  $\pm$  SE, n = 6 per group.

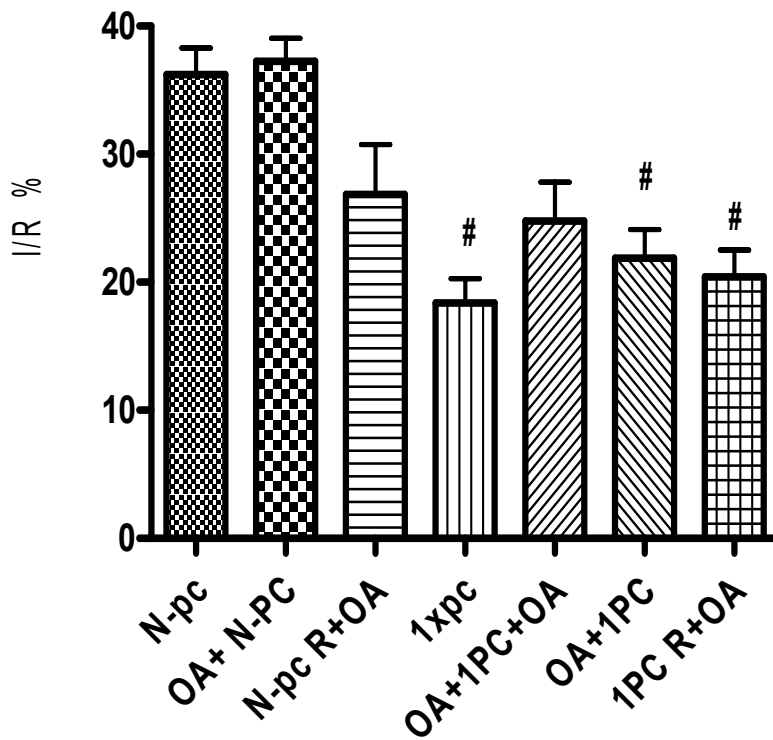
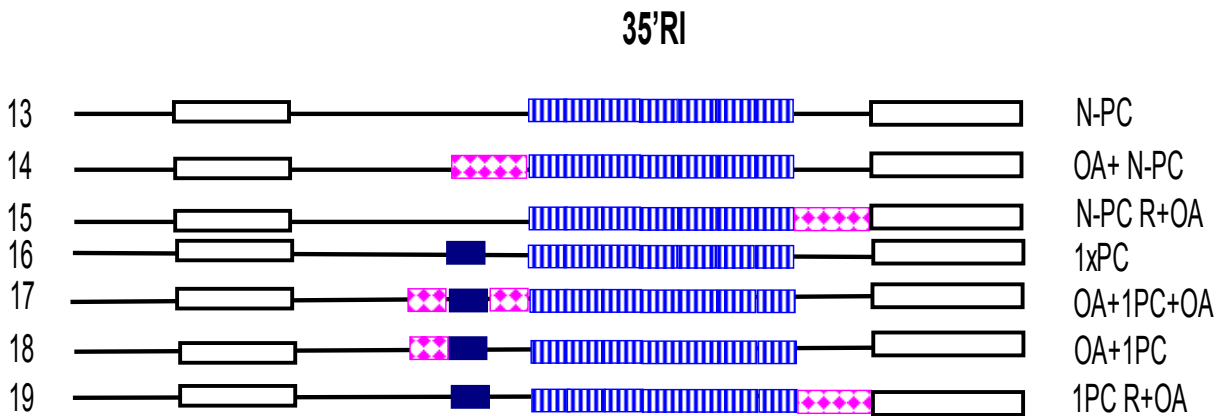
#### **4.3.4.2 Effects of okadaic acid on infarct size.**

Okadaic acid (7.5 nM) was administered for 10 min before 35 min sustained regional ischaemia in N-PC hearts (protocol II 14), there was no change in infarct size versus untreated N-PC hearts.

In 1xPC hearts, infarct size was significantly reduced to  $18.4 \pm 1.9\%$  from  $36.2 \pm 2.1\%$  in N-PC hearts ( $p < 0.05$ ). Okadaic acid (7.5 nM) added 5 min before and 5 min after one 5-min global ischaemic preconditioning protocol (OA+1PC+OA, protocol 17), showed a significant increase in infarct size ( $24.8 \pm 3.0\%$ ), which did not differ from N-PC hearts ( $36.2 \pm 2.1\%$ ,  $p > 0.05$ , Fig 12). Washout of okadaic acid during a 1xPC protocol (OA+1PC) retained the beneficial effect of 1xPC (1xPC:  $18.4 \pm 1.9\%$  vs. OA+1PC:  $21.9 \pm 2.2\%$ ,  $p > 0.05$ ).

Okadaic acid (7.5 nM) added during the first 10 min of reperfusion (N-PC R+OA or 1PC R+OA, protocols 14 and 19), had no influence on infarct size of N-PC and PC hearts (N-PC R+OA:  $26.9 \pm 3.9\%$  vs. N-PC:  $36.2 \pm 2.1$  and 1PC R+OA:  $20.4 \pm 2.1\%$  vs. 1xPC:  $18.4 \pm 1.9\%$ ,  $p > 0.05$ , Fig. 12). Thus the presence of okadaic acid (7.5 nM) for the first 10 min of reperfusion did not abrogate the PC-induced reduction in infarct size, and was also without effect on infarct size of N-PC hearts.

However, administration of okadaic acid during a preconditioning phase, abolished the beneficial effects of this intervention on infarct size.



# P < 0.05 vs N-PC

**Fig 12.** Effect of **okadaic acid** (7.5nM) on **infarct size** (IS) of hearts subjected to 35 min coronary ligation and 30 min reperfusion. Infarct size expressed as a percentage of the ischaemic (risk) zone. Data are means  $\pm$  SE,  $n \geq 6$  per group, see protocol II.

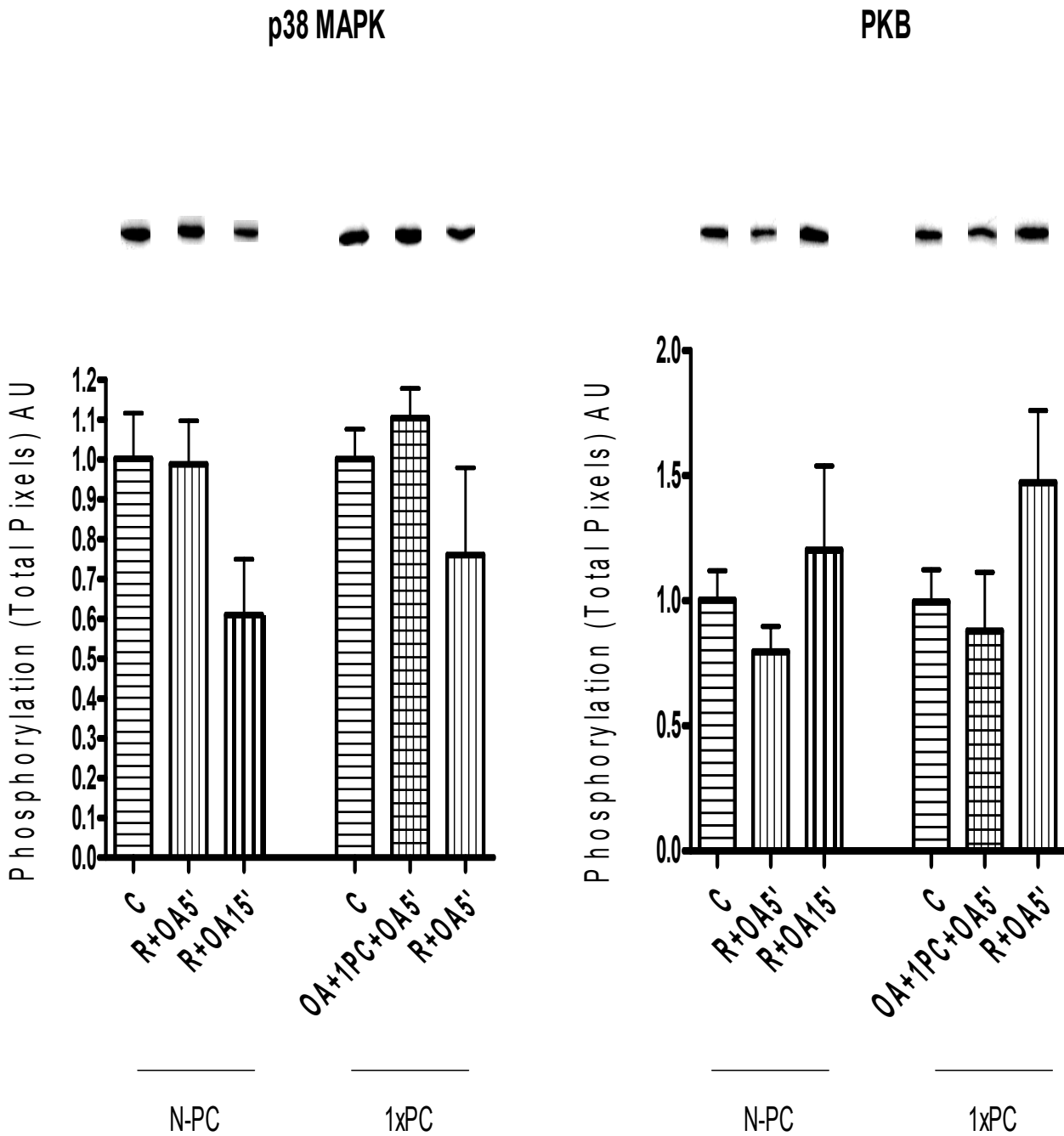


#### **4.3.4.3 Effect of okadaic acid on kinase activation during reperfusion.**

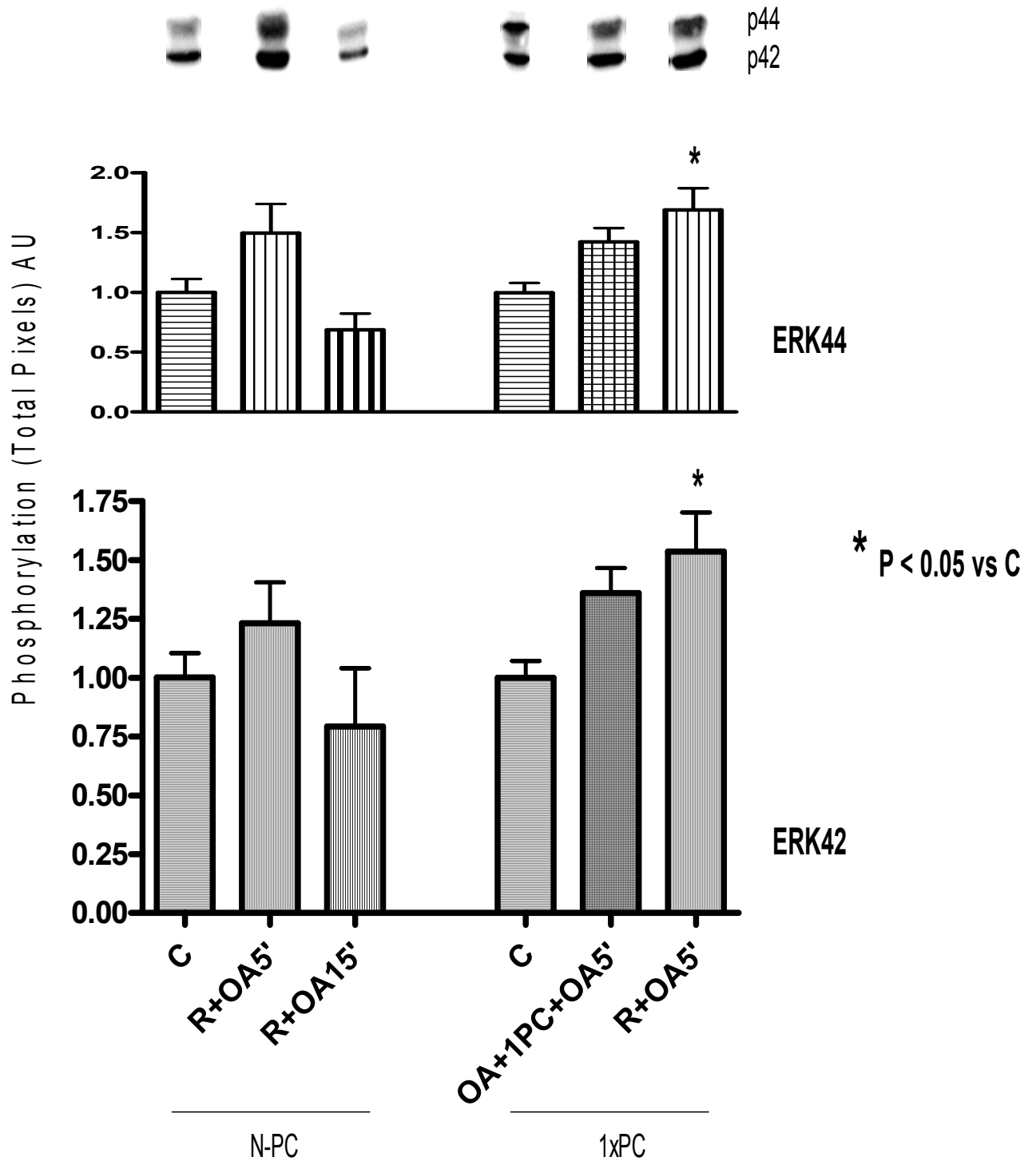
In these studies, the activation status of the kinases was evaluated after 5 min reperfusion in the preconditioned groups. In the case of N-PC, samples were taken at both 5 and 15 min reperfusion.

When okadaic acid (7.5 nM) was administered during preconditioning phase or during reperfusion in 1xPC hearts, no significant p38 MAPK and PKB phosphorylation occurred as measured after 5 min of reperfusion (Fig 13): p38 MAPK activity (a.u 5 min 1PC R+OA:  $0.87 \pm 0.21$ , OA+1PC+OA:  $1.10 \pm 0.07$ ), PKB activity (a.u 5 min 1PC R+OA:  $1.47 \pm 0.29$ , OA+1PC+OA:  $0.88 \pm 0.23$ ) compared with untreated 1xPC hearts. Also in N-PC hearts (N-PC R+OA), no significant p38 MAPK and PKB activation occurred after 5 min and 15 min reperfusion.

When okadaic acid (7.5 nM) was administered during the PC phase (OA+1PC+OA), no marked ERK42/44 activity was seen after 5 min reperfusion, but when added during reperfusion in 1xPC protocol (1PC R+OA), had significantly increased phosphorylation of ERK42/44 phosphorylation after 5 min reperfusion (a.u  $1.54 \pm 0.17/1.69 \pm 0.18$  vs. 1.00 in untreated 1xPC hearts,  $p < 0.05$ , respectively, Fig 14). When administered during reperfusion of N-PC hearts, okadaic acid had no significant effect on ERK42/44 phosphorylation after both 5 and 15 min reperfusion.



**Fig 13.** Effect of *okadaic acid* (7.5nM) treatment on phosphorylation of **p38 MAPK** and **PKB** during reperfusion. AU: arbitrary units. C= Untreated. Data are means  $\pm$  SE, n = 6 per group. See protocol II.



**Fig 14.** Effect of *okadaic acid* (7.5nM) treatment on phosphorylation of ERK42/44 during reperfusion. AU: arbitrary units. C= Untreated. Data are means  $\pm$  SE, n = 6 per group. See protocol II.

#### **4.4 Discussion**

As mentioned previously, it was decided to further investigate the possibility that the phosphatases PP1 and PP2A are involved in the cardioprotection of preconditioning.

As discussed in the literature survey, PP2A directly or indirectly regulate protein dephosphorylation including the MAPK pathway members ERK42/44 and the pro-survival Bcl-2 protein (45, 269). Proteins thought to be dephosphorylated by PP1 include the pro-apoptotic Bcl-2 family member, Bad (38). The multiple interactions between Bcl-2 and PP1/PP2A phosphatases, may explain how these phosphatases control cell survival.

The results obtained with the inhibitors, cantharidin and okadaic acid, suggested that these phosphatases may indeed play a role in the outcome of ischaemia/reperfusion. In our study, we find that pretreatment with the drugs before sustained ischaemia, all abolished the cardioprotection induced by preconditioning, but treatment after the onset of sustained ischaemia, during reperfusion, is still cardioprotective.

##### **Properties of inhibitors**

Both inhibitors have actions unrelated to their phosphatase inhibitory properties. Cantharidin has been demonstrated to act as a vasoconstrictor and positive inotrope in guinea pig (270) and human cardiac (271) tissue in vitro. These effects are mediated in part by cantharidin's action as a protein phosphatase inhibitor (272, 273). Cantharidin at 1  $\mu$ M reduces the activity of purified phosphatase catalytic subunits by 80-90% for PP2A and by 15-35% for PP1 (273, 274). In vivo studies suggest that the efficiency of cantharidin to inhibit phosphatases is slightly reduced compared with that in in vitro studies because of the lipophobic nature of cantharidin (275). It should also be noted that cantharidin is an economically feasible tool to study the functional effects of PP1 and PP2A in perfused hearts. Okadaic acid is a potent inhibitor of PP1 and PP2A at nanomolar concentrations but also has tumor promoting activity in vivo. However, okadaic acid is able to interact with a variety of molecular targets, resulting in an overall increase in phosphorylation of regulatory proteins (see reference 276). Both these phosphatase inhibitors can block apoptosis induced by staurosporine,

and etoposide in leukemic cell lines (277, 278).

PP1 and PP2A differ significantly in their sensitivity to phosphatase inhibitors in vitro (279, 280). The concentrations of phosphatase inhibitors needed to block PP1 and PP2A in cells are much higher than those shown to block PP1 or PP2A in vitro. The difference in concentration is presumably a consequence of cell permeability as well as the concentration of protein phosphatases in the cell. It should be kept in mind that the protein phosphatase activity such as in cell lysates in vitro does not reflect the true activity in cells due to partial or complete disassembly from regulatory components, and because of dephosphorylation and activation of phosphatase protein during analysis. The true activity of protein phosphatases in cells has, therefore, been difficult to measure.

### **Endpoints**

For the purpose of this study, three endpoints were used, namely, post-ischaemic mechanical recovery during reperfusion, infarct size and the activation patterns of p38 MAPK, ERK 42/44 and PKB/Akt during ischaemia/reperfusion of the working rat heart model. Infarct size determination has been used as the “gold standard” in studies on preconditioning (always showing a reduction). Use of post-ischaemic recovery as endpoint, has proved a much more difficult endpoint to attain. Furthermore, a reduction in infarct size is not always associated with improved functional recovery and probably depends on the experimental model (281). However, in the globally ischaemic heart, as used in this study, preconditioning (1xPC and 3xPC) always resulted in a significant improvement in functional performance, associated with a reduction of infarct size. In addition to functional recovery and infarct size, activation of the reperfusion injury salvage kinase (RISK) pathway (10) has increasingly been used as indicator of protection.

Ischaemia–reperfusion after ischaemic preconditioning has been shown to activate the pro-survival kinase signalling cascades, namely the phosphatidylinositol-3-OH kinase (PI3K)–Akt (7, 7A, 8) and MEK-1/2-ERK-1/2 (9, 9A) kinase cascades (RISK pathway), both of which have been implicated in cellular survival, through their recruitment of anti-apoptotic pathways of protection (282). The PI3K–Akt signalling cascade is activated in response to the activation of a wide range of receptors, including those for growth factors and G-protein-coupled receptors (282) and participates in numerous cellular processes by phosphorylating a diverse array of substrates, including glycogen synthase kinase-3 $\beta$  (glycogen and protein metabolism), apoptotic proteins (BAD, BAX, BIM, p53

and caspases), GLUT4 vesicles (glucose metabolism), transcription factors (IKK- $\alpha$  and Forkhead proteins), p70S6K, eNOS and PKC (282). These multiple signalling pathways can function as positive modulators of the PI3K/Akt pathway and PP2A serves as a major PKB/Akt phosphatase (for review see 283).

The MAPKs play important roles in cell survival and apoptosis and it is clear that their regulation involves a dynamic interplay between kinases and phosphatases. Three distinct MAPKs pathways have been characterized. The extracellular signal-regulated kinase ERK1/2 pathway (Raf $\rightarrow$ MEK1,2 $\rightarrow$ ERK1,2) is activated by mitogens via Ras and by phorbol esters via protein kinase C. The stress-activated MAPK pathways, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (MEK kinase 1,3 $\rightarrow$ MAPK kinase 4,7 [MKK4,7] $\rightarrow$ JNK1,2,3) and p38 MAPK (MAPK kinase kinase [MAPKKK] $\rightarrow$ MKK3,6 $\rightarrow$ p38 $\alpha,\beta,\gamma,\delta$ ) are activated by cellular stress, e.g., UV light, osmotic and oxidative stress, and inflammatory cytokines (284, 285). Despite some controversy, it is accepted by most that p38 MAPK and JNK signalling pathways play a proapoptotic role in cardiomyocytes subjected to ischaemia or oxidative stress (286-288). The dynamic balance between the opposing effects of ERK and JNK/p38 MAPK is therefore important in determining whether a cell will survive or undergo apoptosis (289). ERK protects cardiomyocytes from oxidative stress-induced apoptosis (9, 290, 291) and p38 MAPK promotes apoptosis (286-288). Phosphorylation of MAPKs results in their translocation to the nucleus, where they activate transcription factors by phosphorylation.

Extensive biochemical, pharmacological, and genetic evidence suggests that PP1 and PP2A negatively regulates several of the kinases involved in the MAPK cascade. Inhibition of these phosphatases would allow for the continual activation of both these kinases in cell cycling (42, 283, 292). PP1 and PP2A inhibit the activity of the ERK pathway by dephosphorylation of MEK1,2 and ERK1/2 (14, 283, 293). On the other hand, inhibition of PP1 and PP2A activity results in activation of ERK and in enhancement of AP-1-dependent gene expression (283, 293, 294). While PP2A regulates ERK phosphorylation, ERK has not been shown to be a substrate for PP2A, but is more likely a downstream target of PP2A activity (45).

### **Cantharidin and okadaic acid**

There were several major findings in our study in the in vitro rat hearts. Firstly, in isolated ischaemic-reperfused hearts, cantharidin added during the preconditioning phase abolished functional recovery during reperfusion induced by preconditioning; however, the effects of cantharidin can be attenuated by washing out the drug before the onset of sustained global ischaemia. When cantharidin was administered during reperfusion only, no deleterious effects were observed, but surprisingly, cantharidin, when added during first 10 min of reperfusion in 3xPC hearts, significantly increased postischaemic CF, AO, CO ( $p < 0.05$ ) and TW ( $0.05 < p < 0.1$ ). Secondly, these observations were also reflected by the changes in infarct size. Cantharidin administered during preconditioning phase increased the infarct size, while its presence during reperfusion had no effect on this endpoint. Thirdly, the effects of the inhibitor on infarct size and functional recovery during reperfusion were reflected in changes in the phosphorylation of p38 MAPK, ERK42/44 and PKB/Akt. For example, pretreatment of N-PC hearts caused a significantly increased phosphorylation of p38 MAPK after 15 min reperfusion, with no activation of ERK42/44. Similarly, cantharidin administered during the preconditioning phase, caused a significantly increased p38 MAPK phosphorylation at 5 min and 15 min reperfusion, and decreased phosphorylation of ERK42/44 during 5 min reperfusion. On the other hand, cantharidin administered during reperfusion did not upregulate p38 MAPK phosphorylation, but significantly increased ERK42/44 activation in 1xPC hearts after 10 min reperfusion, in 3xPC and N-PC hearts after 15 min reperfusion (Fig 9). When cantharidin was administered during the preconditioning phase, there were significant increases in PKB phosphorylation at 5 min reperfusion. When added during reperfusion, a marked increase in PKB activity was observed in preconditioned hearts.

It should be kept in mind that the significant increase in PKB phosphorylation during reperfusion may be partially due to the use of ethanol as solvent added during reperfusion. However, ethanol present before sustained ischaemia, did not have a confounding effect on phosphorylation of these kinases (p38 MAPK, ERK and PKB).

As in the case of cantharidin, okadaic acid added during preconditioning phase abolished preconditioning-induced protection and showed a significant increase in infarct size, while okadaic acid administered during reperfusion only, was without effect. When okadaic acid was administered

during the preconditioning phase, no marked ERK42/44 and PKB phosphorylation occurred after 5 min reperfusion, but okadaic acid, when added during reperfusion in 1xPC, caused significant phosphorylation of ERK42/44 after 5 min reperfusion. The differences between the effects of the two inhibitors on kinase phosphorylation may be due to the fact that cantharidin inhibits both PP1 and PP2A, while okadaic acid selectively inhibits PP2A.

To summarize, our data demonstrated that inhibition of PP1 and PP2A during the preconditioning phase abolished protective effect of preconditioning associated with activation of p38 MAPK and PKB, dephosphorylation of ERK42/44 and an increased infarct size, while inhibition of PP1 and PP2A during reperfusion improved protection in preconditioned hearts associated with activation of ERK42/44 (especially after 10 min and 15 min reperfusion) and PKB, but no upregulation of p38 MAPK.

To our knowledge, the effects of PP1 and PP2A inhibitors have not yet been studied on preconditioned hearts, using the working heart model. The cardioprotective effect of PP1 and PP2A inhibition in isolated buffer-perfused rat hearts during reperfusion may be in part mediated by its activation of the pro-survival kinase signalling cascades, the PI3K–Akt and MEK-1/2-ERK-1/2 both of which have been implicated in cellular survival, possibly through their recruitment of anti-apoptotic pathways of protection (282) or effects on the opening of the mitochondrial permeability transition pore (MPTP) (211, 212). Abolishment of ischaemic preconditioning-induced cardioprotection by administering the drugs during the preconditioning phase, may be attributed to upregulation of p38 MAPK phosphorylation during reperfusion. It is also possible that the sustained activation of p38 MAPK in particular, induced by administration of cantharidin, at the onset of global iachaemia, contributed to the mechanical failure (see Fig 5).

Although controversial, increased phosphorylation and activation of p38 MAPK during ischaemia/reperfusion has been associated with a reduction in functional recovery and an increase in infarct size (for review see 295) and increased apoptosis in the isolated working rat heart (242).

Our data are consistent with the view that both PP1 and PP2A exert regulatory actions at multiple sites of the ERK and p38 MAPK signalling pathways and the phosphorylation status of p38 MAPK, ERK and PKB may be mediated directly by these protein phosphatases.



The results obtained in this study also indicate that the timing of phosphatase inhibitor administration determines the outcome of the ischaemia/reperfusion injury. If added during the preconditioning phase, it abrogates protection, while having beneficial effect when added during reperfusion. Pretreatment with the inhibitor before the onset of sustained ischaemia (in the absence of preconditioning) also severely depressed functional recovery (see Table 3). These findings are in contrast with those obtained in cardiomyocytes: pretreatment with fostriecin (239, 240), calyculin A (245, 296) or okadaic acid was found to protect cardiomyocytes (296). An explanation for these contradictory findings is not readily available. However, Mackay and Mochley-Rosen (241) found that vanadate, a inhibitor of tyrosine phosphatase, enhanced tyrosine residue phosphorylation. As far as we know, the role of phosphatases in preconditioning has been studied by only one other group. Ladilov et.al ( see reference 250) found that both cantharidin and okadaic acid were able to abolish hypoxia-induced preconditioning, suggesting PP1 involvement in the triggering process. These findings are in agreement with our data.

### **Summary**

Finally, in this study we observed that phosphatase inhibition during preconditioning phase abolishes protection while inhibition during reperfusion after sustained ischaemia either has no effect or enhances the cardioprotective effects of preconditioning. The exact mechanism is not clear, perhaps maintenance of kinases in a phosphorylated state until the onset of reperfusion is undesirable while phosphatase inhibition during reperfusion may extend the period of RISK activation and thus protect the heart. This may be an explanation for the increased functional recovery observed in preconditioned hearts when the inhibitor was administered during early reperfusion (Figs 3 and 11). The ability to manipulate and up-regulate the RISK pathway by the use of appropriate phosphatase inhibitors, during the early reperfusion phase may thus provide a potential approach to limiting reperfusion-induced cell death.

## CHAPTER 5

### **Role of Mitogen-Activated Protein Kinase Phosphatases (MKP-1) on Response of Heart to Ischaemia/Reperfusion: Effect of Dexamethasone**

#### **5.1 Introduction**

The mitogen-activated protein kinase phosphatases (MKPs) constitute a family of 11 dual-specificity phosphatases that inactivate the MAPKs by dephosphorylation of specific Thr/Tyr residues. Some of these MKPs are often active only against specific MAPKs, although MKP-1 is able to dephosphorylate ERK, JNK and p38 MAPK in cell culture (14). The contribution of the MAPKs to cell death (78) and survival has been studied extensively (for review see 295), but it remains unclear whether the MKPs play a regulatory role in these processes. A recent study by Wu and Bennet (297) showed that in fibroblasts MKP-1 promotes cell survival by attenuating stress-responsive MAPK-mediated apoptosis. A previous study from our laboratory also showed that cardioprotection induced by long-chain fatty acids was associated with upregulation of MKP-1 (298).

It has been shown that MKP-1 preferentially inactivates p38 MAPK, then JNK and to a lesser extent ERK (78). Previous studies from our laboratory (242) suggested that activation of p38 MAPK during ischaemia and reperfusion is harmful and we hypothesized that inactivation of this kinase by upregulation of MKP-1 should be cardioprotective. The aim of this study was therefore to evaluate the effects of upregulation of this dual-specificity phosphatase on functional recovery and infarct size of the isolated rat heart subjected to ischaemia/reperfusion. Dexamethasone, which causes sustained expression of MKP-1 (299) has been used for this purpose.

## 5.2 **Methods**

Untreated control (C ): isolated hearts were stabilized for 60 min (15 min retrograde perfusion, 15 min working heart mode, 30 min retrograde perfusion) before 20 min sustained global ischaemia or 35 min regional ischaemia followed by 30 min reperfusion (10 min retrograde, 20 min working heart ) (protocol III: 1, 6)

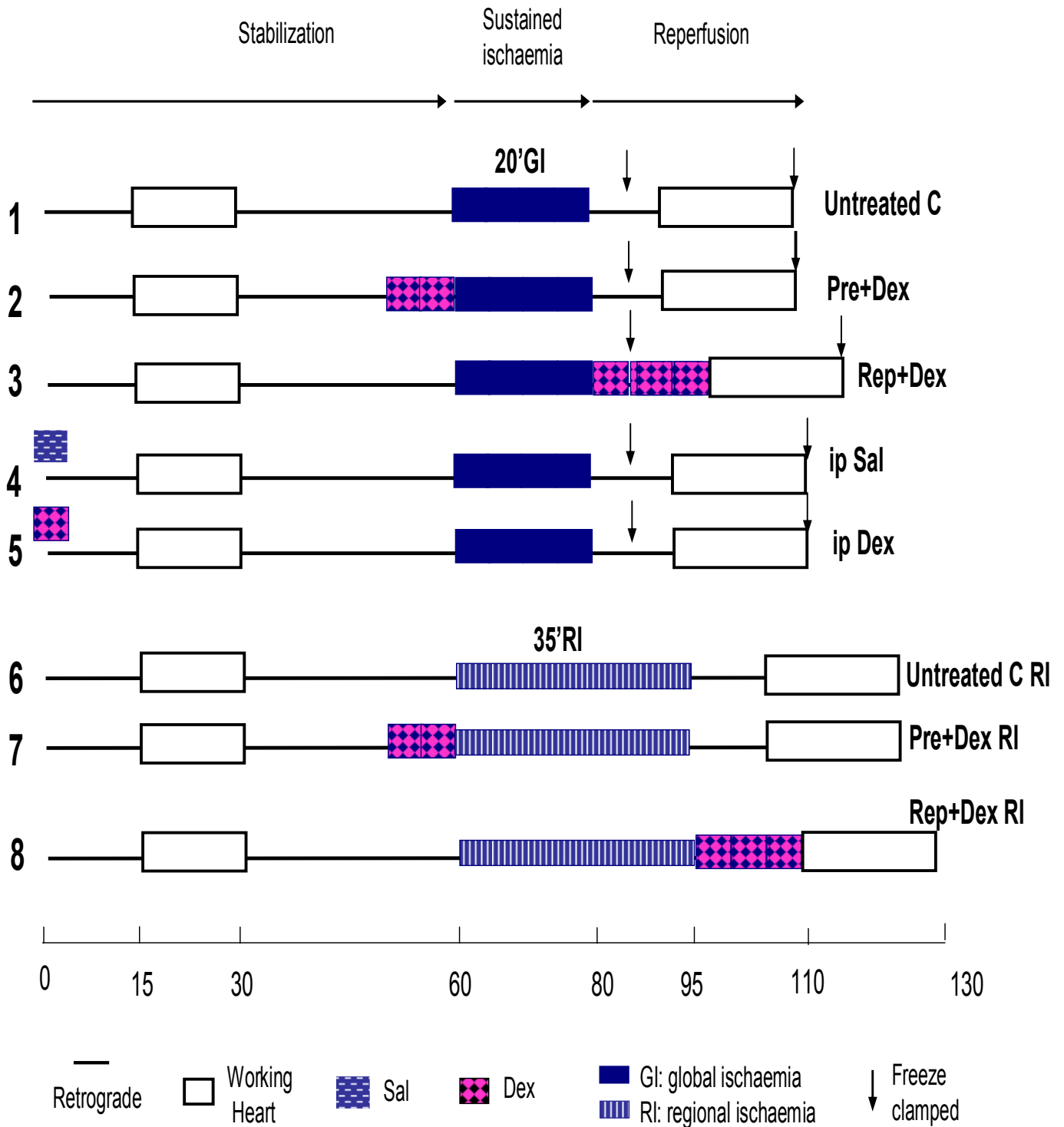
### **Drug treatment (protocol III)**

(1). After isolated hearts had been stabilized for 50 min (15 min retrograde, 15 min working heart mode, then perfused retrogradely 20 min), dexamethasone(1  $\mu$ M) was added for 10 min before 20 min sustained global ischaemia or 35 min regional ischaemia followed by 30 min reperfusion (Pre+Dex, protocols 2, 7).

(2). Isolated hearts were stabilized for 60 min (15 min retrograde, 15 min working heart mode, then perfused retrogradely 30 min), then subjected to 20 min sustained global ischaemia or 35 min regional ischaemia followed by 15 min retrograde reperfusion in the presence of dexamethasone(1  $\mu$ M) and 20 min reperfusion in the working model (Rep+Dex, protocols 3, 8).

(3). In one series of experiments, dexamethasone (3mg/kg/day) or an equivalent volume of saline was administered intraperitoneally to the rats for 10 days. After anaesthesia, hearts were removed from rats and stabilized on the perfusion rig for 60 min folowed by 20 min sustained global ischaemia or 35 min regional ischaemia and 30 min reperfusion as described above (ip Dex and ip Sal, protocols 4, 5).

# Protocol III



### **5.3 Results**

#### **5.3.1 Effect of dexamethasone on cardiac mechanical recovery.**

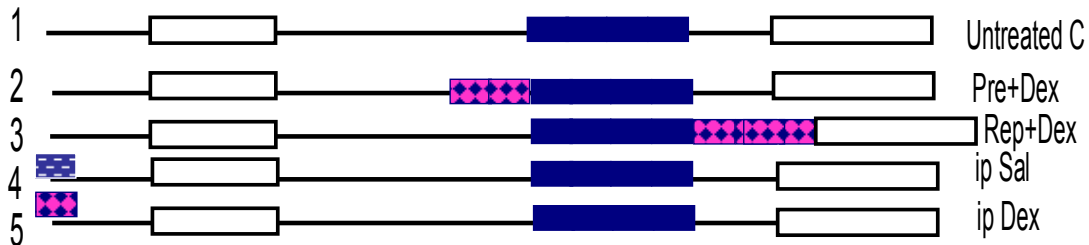
Table 5 shows that dexamethasone (3mg/kg daily) administered intraperitoneally to the rats for 10 days before experimentation (ip Dex) or dexamethasone (1  $\mu$ M) added for 10 min before (Pre+Dex) or 15 min after (Rep+Dex) 20 min sustained global ischaemia, resulted in a significant increase in all parameters of post-ischaemic functional recovery (CF, AO, CO, Psp, HR, TW) during 30 min reperfusion (Fig 15), when compared with values obtained from untreated control hearts ( $p < 0.05$ , for all parameters). More surprisingly, in ip Dex protocol, all parameters of functional recovery during 30 min reperfusion were increased so markedly that they were not only significantly different from untreated control values ( $p < 0.05$ ), but were similar to those obtained before subjecting hearts to sustained ischaemia ( $p > 0.05$ ). Please note that the reperfusion time of hearts treated with dexamethasone during reperfusion, averaged 35 min (rather than 30 min as in other groups).

#### **5.3.2 Effect of dexamethasone on infarct size**

Infarct size is expressed as a percentage of the area at risk in rat hearts. Dexamethasone (1  $\mu$ M), when added for 10 min before (Pre+Dex) or 15 min after (Rep+Dex) 35 min regional ischaemia, significantly reduced infarct size to  $20.5 \pm 1.3\%$  and  $25.4 \pm 1.52\%$ , respectively, from  $36.2 \pm 2.1\%$  in untreated control hearts ( $p < 0.05$ , Fig 16). In these studies, the area at risk did not differ between the groups. The averaged value was  $46.8 \pm 4.3\%$ .

**Table 5**

**Effect of dexamethasone (1µM) on functional recovery during reperfusion after 20 min sustained global ischaemia.**



	Protocols	CF	AO	CO	Psp	HR	TW
Before ischaemia	All	15.1±0.3	44.4±0.5	59.6±0.7	92±1	303±3	12.43±0.18
After ischaemia	Untreated Control	5.6±1.4 †	3.9±1.3 †	9.5±2.5 †	39±10 †	128±32 †	1.67±0.45 †
	Pre+Dex	12.2±0.58 *	28.5±1.9 *†	40.7±2.1*†	95±4 *	253±4 *	8.64±0.77 *†
	Rep+Dex	10.3±0.7 *†	16±5.6 *†	26.3±6.1 *†	85±10 *	244±13 *	5.38±1.42 *†
	ip Sal	6.8±2.3 †	7.8±5.0 †	14.7±6.7 †	51±17 †	156±64 †	2.82±1.45 †
	ip Dex	16.8±0.48*#	36.8±1.1*#	53.7±1.3*#	98±1*#	259±9*#	10.89±0.63*#

Abbreviations: as in Table 2. C=control

Pre+Dex: dexamethasone added before sustained ischaemia.

Rep+Dex: dexamethasone added during reperfusion

ip Sal: intraperitoneal saline before experiment

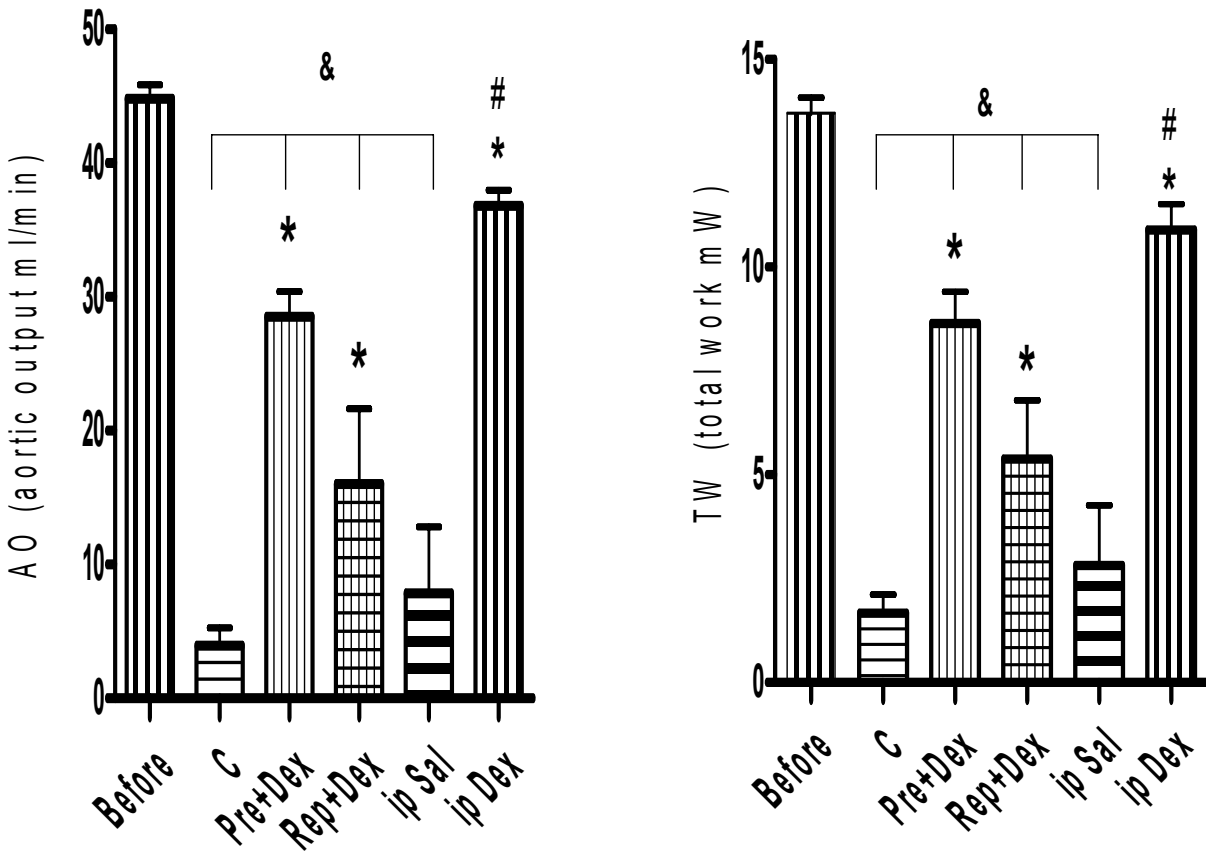
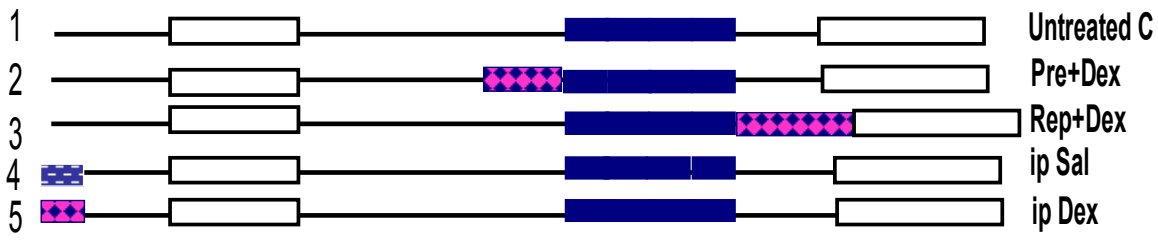
ip Dex: intraperitoneal dexamethasone before experiment

Data are means ± SE, n ≥6 per group. Protocol III.

**\* P < 0.05 vs Untreated C**

**# P < 0.05 vs ip Sal**

**† p < 0.05 vs Before ischaemia**

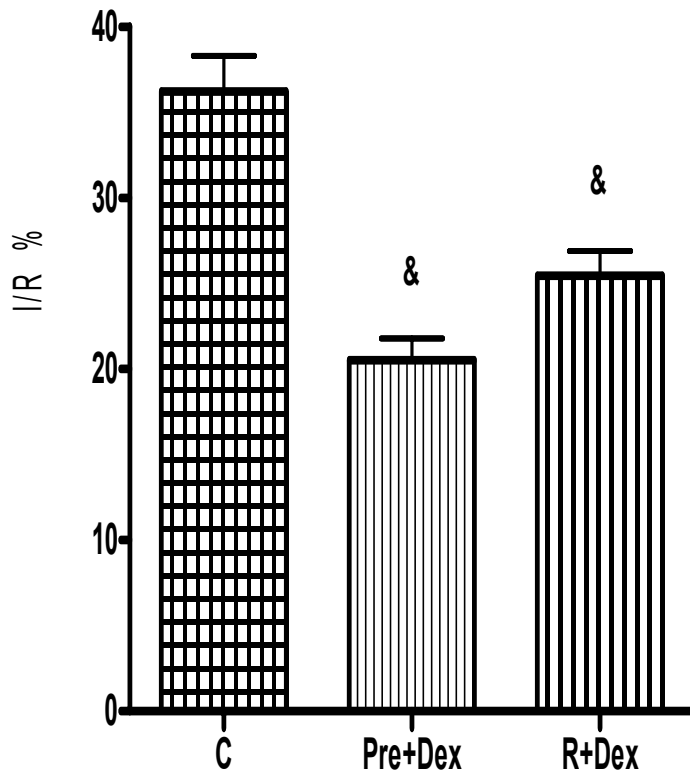
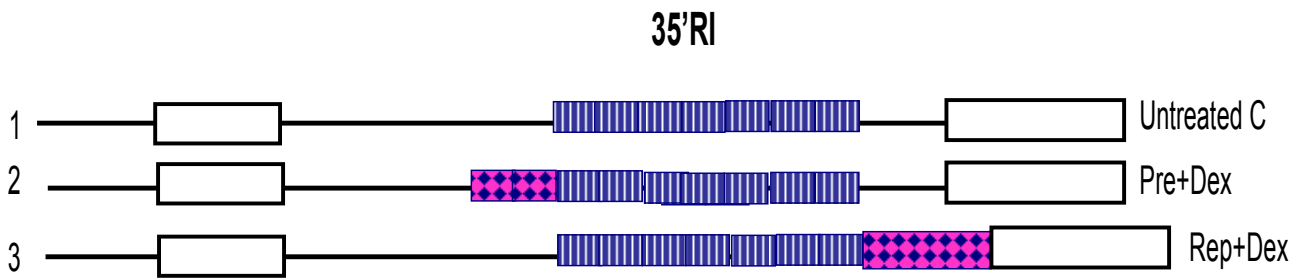


\*P < 0.05 vs C

# P < 0.05 vs ip Sal

&P < 0.05 vs Before

**Fig 15.** Effect of dexamethasone (1µM) treatment on aortic output (ml/min) and total work (mW) after 20 min global ischaemia and 30 min reperfusion. C= Untreated Control. Data are means ± SE, n ≥ 6 per group.



**& P < 0.05 vs untreated control**

**Fig 16.** Effect of dexamethasone (1 $\mu$ M) on infarct size (IS) of hearts subjected to 35 min coronary ligation and 30 min reperfusion. Infarct size expressed as a percentage of the ischaemic (risk) zone. C= Control. Data are means  $\pm$  SE,  $n \geq 6$  per group, see protocol III.

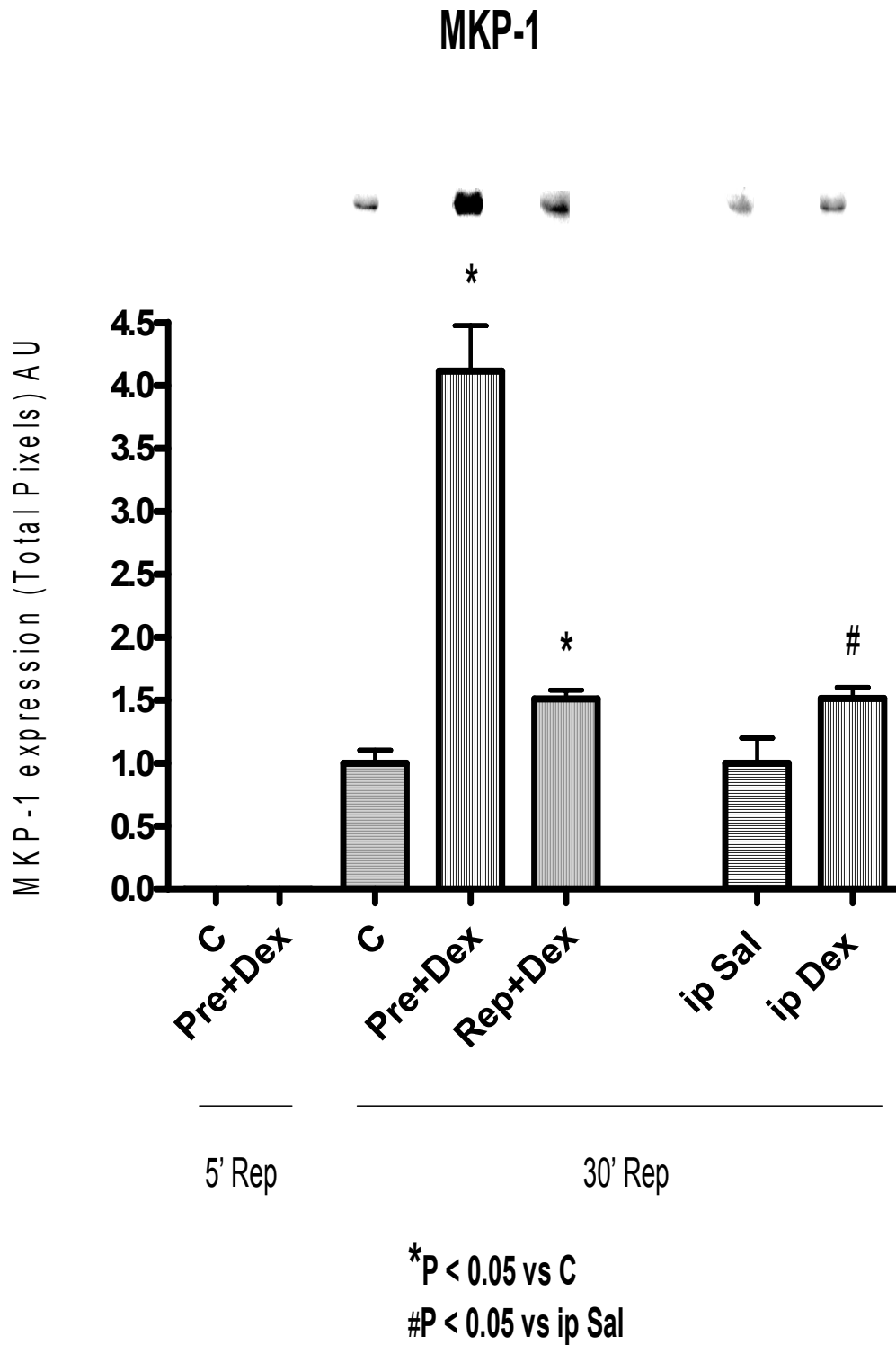


### **5.3.3 Effects of dexamethasone on expression of MKP-1 and kinase activation.**

Regardless of the mode of dexamethasone administration ( intraperitoneal 3 mg/kg daily for 10 days (ip Dex) or added (1 $\mu$ M) for 10 min before (Pre+Dex) or 15 min after (Rep+Dex) 20 min sustained global ischaemia), a marked increase in MKP-1 expression was observed after 30 min reperfusion when compared with untreated controls (a.u ip-Dex: 1.51 $\pm$ 0.09, Pre+Dex: 4.11 $\pm$ 0.36, Rep+Dex: 1.51 $\pm$ 0.07 vs. C: 1.00,  $p < 0.05$ , respectively. Fig. 17). However, after 5 min reperfusion, no MKP-1 expression could be seen in hearts pretreated for 10 min with dexamethasone (1 $\mu$ M).

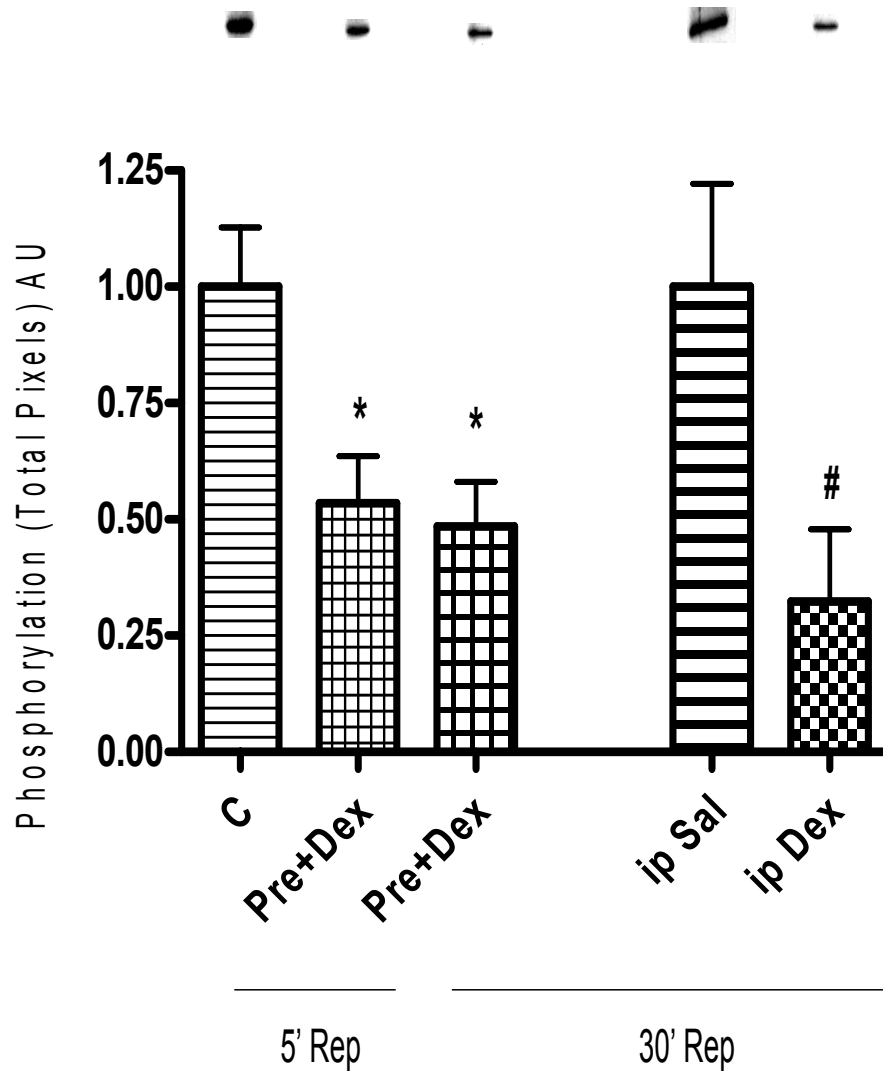
In ip-Dex and Pre+Dex protocols, a significant decrease in p38 MAPK phosphorylation during reperfusion was observed versus untreated controls (a.u Pre+Dex: 5 min 0.53 $\pm$ 0.10 and 30 min 0.41 $\pm$ 0.08; ip-Dex: 30 min 0.32 $\pm$ 0.16, respectively,  $p < 0.05$ , Fig. 18).

In Pre+Dex protocol, after 5 min reperfusion, an increased phosphorylation of ERK42/44 was seen (a.u 1.42 $\pm$ 0.19/1.91 $\pm$ 0.48), but only ERK44 activity was significantly elevated (1.91 $\pm$ 0.48 vs. 1.00 in untreated control,  $p < 0.05$ , Fig 19). However, after 30 min reperfusion, no marked change in ERK42/44 phosphorylation was observed. Also in this protocol, dexamethasone had no effect on PKB phosphorylation after 5 min reperfusion (a.u 1.19 $\pm$ 0.21, Fig 20). In ip-Dex protocol, there was also no significant difference in ERK42/44 activity after 30 min reperfusion.



**Fig 17.** Effect of **dexamethasone** (1 $\mu$ M) treatment on **MKP-1** expression during reperfusion. AU: arbitrary units. C= Untreated Control. 5' and 30' indicate time of reperfusion. Data are means  $\pm$  SE, n  $\geq$  6 per group. See protocol III.

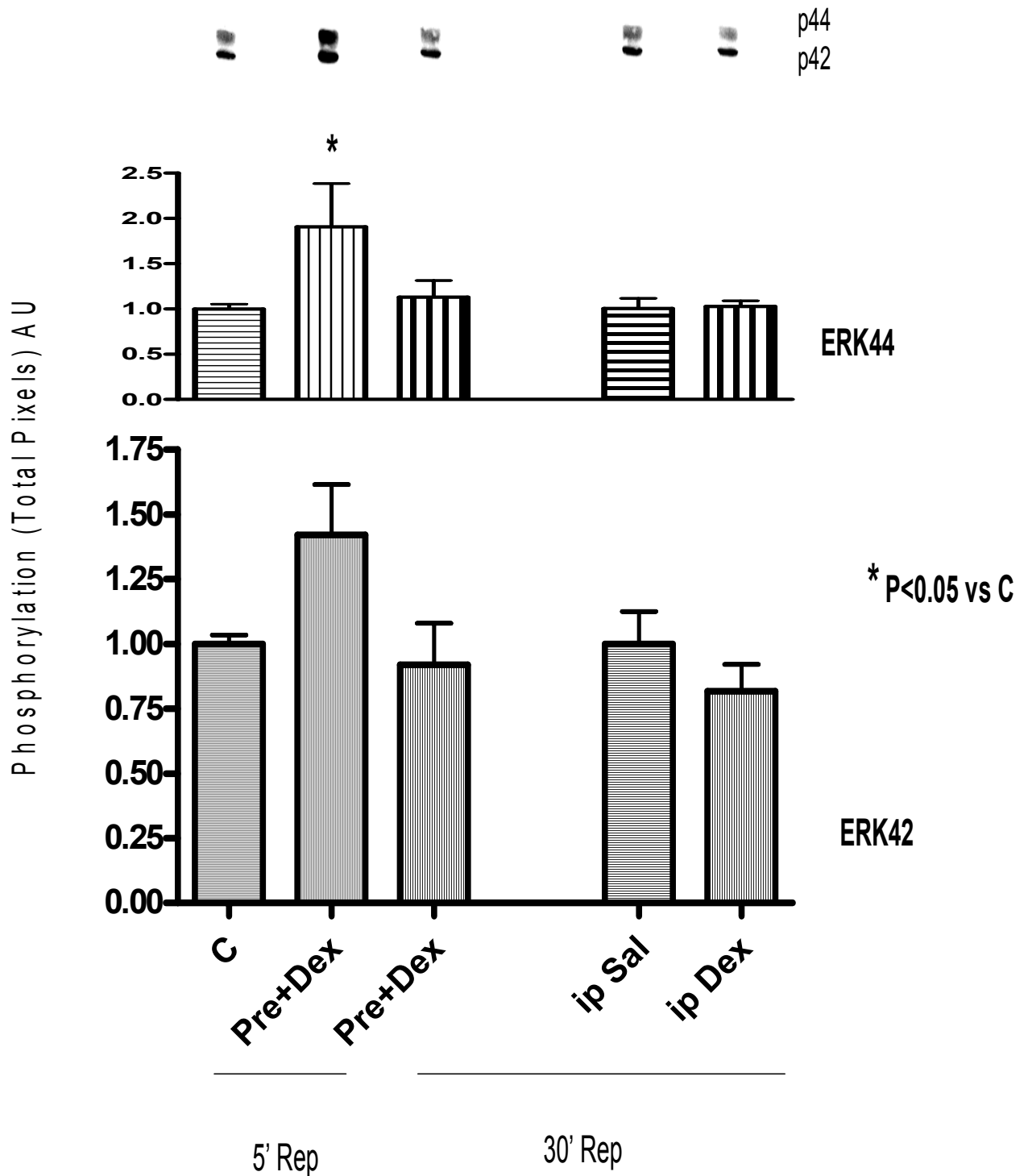
### p38 MAPK



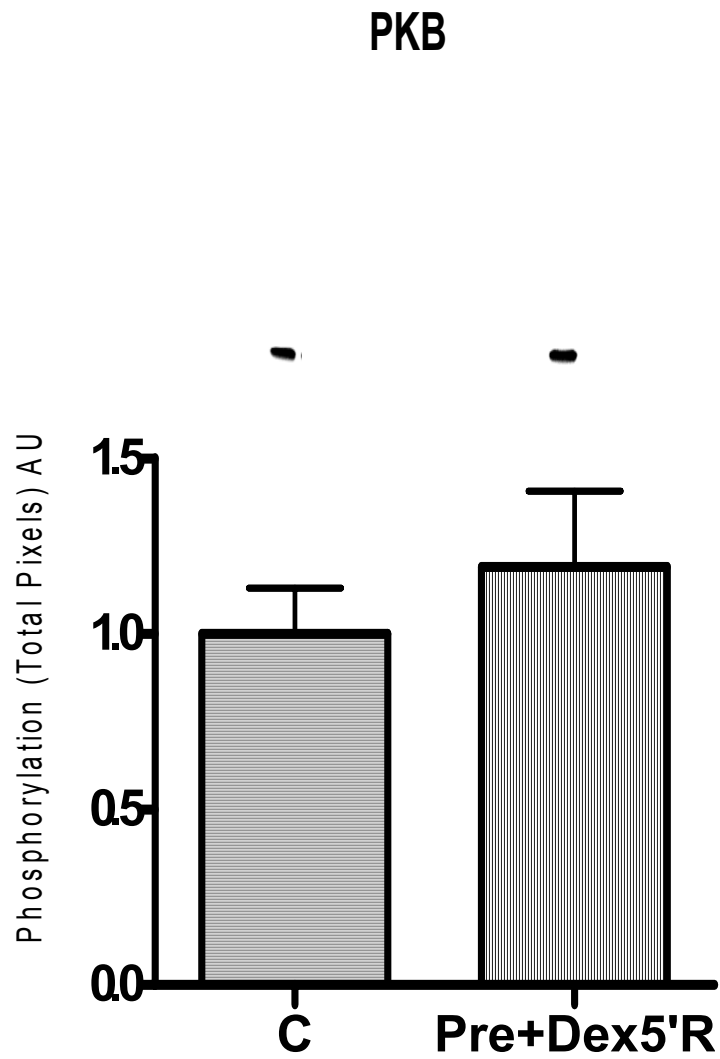
\* P < 0.05 vs C

# P < 0.05 vs ip Sal

**Fig 18.** Effect of dexamethasone (1µM) treatment on phosphorylation of p38 MAPK during reperfusion. AU: arbitrary units. C= Untreated Control. Data are means ± SE, n = 6 per group. See protocol III.



**Fig 19.** Effect of dexamethasone (1 $\mu$ M) treatment on phosphorylation of ERK42/44 during reperfusion. AU: arbitrary units. C= Untreated Control. Data are means  $\pm$  SE, n = 6 per group. See protocol III.



**Fig 20.** Effect of **dexamethasone** (1 $\mu$ M) treatment on phosphorylation of **PKB** during reperfusion. AU: arbitrary units. C= Untreated Control. Data are means  $\pm$  SE, n = 6 per group. See protocol III.

## 5.4 Discussion

The results obtained in this study showed clearly that dexamethasone, whether administered intraperitoneally for 10 days before experimentation or directly added to the perfusate of the isolated heart, afforded significant protection against ischaemia/reperfusion damage. The significant upregulation of MKP-1 occurring during reperfusion, suggests a role for this phosphatase in dexamethasone-mediated cardioprotection.

Induction of MPK-1 by dexamethasone in the working heart model appears to be time-dependent. Regardless of the mode of dexamethasone administration, after 5 min reperfusion, no MPK-1 expression could be seen, but after 30 min reperfusion, a marked increase in MPK-1 expression was observed. This led us to conclude that the ability of dexamethasone to induce MKP-1 expression is time-dependent. However, a reduction in p38 MAPK phosphorylation during reperfusion is evident before upregulation of MKP-1 is visible (Fig 17,18). A similar time-dependent effect was observed in a mast cell line, where dexamethasone induced MKP-1 expression after 5h (300).

### **Effects of glucocorticoids:**

It has long been known that dexamethasone protects against ischaemia/reperfusion damage. It was first shown in 1980 by Lefer *et al* (301) that dexamethasone normalized ST-segment elevation and reduced necrosis in cats subjected to coronary artery ligation. Similarly, Bernauer (302) showed that dexamethasone inhibited myocardial necrosis in rats, with and without reperfusion after ischaemia. At the time, this was suggested to be due to *de novo* synthesis of macrocortin, an antiphospholipase protein (303). The cardioprotective effects of the glucocorticoid, methylprednisolone, were investigated by Valen and coworkers. Her studies suggested roles for HSP72 (304) as well increased tissue antioxidant activity (305) during reperfusion as mediators for cardioprotection. Engelman and coworkers (306) showed that steroid-induced myocardial preservation was associated with decreased membrane microviscosity. The anti-inflammatory actions of dexamethasone have been shown to block sepsis-induced protection of the heart from ischaemia-reperfusion injury (307). The contribution of dexamethasone treatment to the recovery of postischaemic cardiac function was also studied by Varga and coworkers (308). In their study, rats

were treated with dexamethasone 24 h before being subjected to 30 min global ischaemia and 120 min reperfusion. Dexamethasone pretreatment significantly reduced the occurrence of ventricular fibrillation, and release of cytochrome C, while increasing postischaemic function. In their study actinomycin D inhibited the cardioprotective effects of dexamethasone, probably by inhibiting RNA synthesis. However, despite the above studies, the mechanism of the beneficial effects of dexamethasone pretreatment still remains unclear. It is possible that the changes observed by the above workers, are merely events associated with cardioprotection (or epiphenomena) and not the cause.

Glucocorticoids influence a great variety of cellular functions, for example, the repression (309) or activation of genes (310, 311), the induction of apoptosis (312) or protection from apoptosis (312). Another possible mechanism for glucocorticoid action, which may be particularly important in the setting of ischaemia/reperfusion, is the fact that it significantly inhibits MAPK signalling. This effect requires gene induction, possibly of a MAPK phosphatase (MKP) which could inhibit both transcriptional and posttranscriptional mechanisms controlled by the MAP kinases. For example, it has been shown that glucocorticoids inhibit ERK 1/2 activation via increased expression and decreased proteasomal degradation of MKP-1 (300).

#### **MKP-1 actions:**

It has been suggested that the dual-specificity phosphatase MKP-1 may function as a critical anti-apoptotic regulator. Overexpression of MKP-1 is associated with decreased apoptosis, suggesting that MKP-1 overexpression may be causally linked to the prevention of cardiomyocyte apoptosis (313, 314). MKP-1 can be induced by growth factors (305), oxidative stress (316), arachidonic acid (317), and 12-O-tetradecanoylphorbol-13-acetate (318) and is regulated at the transcriptional level. The expression of MKP-1 in response to dexamethasone is sustained, in contrast to the transient induction by growth factors and cellular stresses demonstrated elsewhere (71, 319, 320). However, the mechanism(s) of inhibition of MKP-1 degradation by glucocorticoids is still unknown. It is possible that MKP-1 exerts beneficial effects on the heart via inactivation of the MAP kinases, p38 MAPK in particular. The harmful effects of this kinase in ischaemia/reperfusion has been reviewed by Steenbergen et al (295). Originally isolated as a stress-responsive protein phosphatase, MKP-1 was initially shown to dephosphorylate ERK (319, 321). A combination of in

in vitro and overexpression studies in cultured cells subsequently demonstrated that MKP-1 dephosphorylated the MAPKs with a rank order of p38 MAPK > JNK > ERK (76-78). In turn, MKP-1 may be catalytically activated by p38 MAPK (70). These observations suggest the participation of MKP-1 in a complex feedback loop which modulates p38 MAPK activity.

Although these studies supported the interpretation that MKP-1 has a higher potency of dephosphorylation toward the stress-responsive MAPKs, it remained unclear whether MKP-1 was absolutely required for the physiological inactivation of these MAPKs. Studies using mice in which MKP-1 expression was disrupted (322), suggested that MKP-1 plays a redundant role in the regulation of ERK activity and cell growth.

In the present study, we demonstrate that under our experimental circumstances dexamethasone significantly improved post-ischaemic cardiac functional recovery and reduced infarct size associated with a marked increase in MKP-1 expression and decrease of p38 MAPK phosphorylation after 30 min reperfusion. Based on a report from Zhao et al. (323) who demonstrated that macrophages isolated from MKP-1-deficient mice exhibited hyperactivation of ERK, JNK, and p38 MAPK in response to lipopolysaccharide stimulation in the livers of MKP-1-deficient mice. We propose that dexamethasone-induced MKP-1 regulation is responsible for dephosphorylation of p38 MAPK in particular. Thus, these data suggest that MKP-1 plays a critical role in the negative regulation of p38 MAPK in response to ischaemia/reperfusion to promote cell survival.

However, in our system, the PKB/Akt phosphorylation level was not affected by dexamethasone treatment suggesting that the PI3K-Akt pathway is not involved in dexamethasone protection against ischaemia/reperfusion damage. As far as we know, MKP-1 does not dephosphorylate PKB/Akt. In contrast to previous reports, the upregulation of MKP-1 was not associated with a reduced phosphorylation of ERK42/44 (76-78).

Studies in other cell types have also indicated that p38 MAPK is the preferred substrate for MKP-1 (77). On the other hand, other MAPK, such as JNK has also been identified as targets for negative regulation by glucocorticoids (324-327). Glucocorticoid-mediated inhibition of JNK occurs after a



short time of hormone treatment (324, 326), does not require new protein synthesis and is independent of the transactivation function of the glucocorticoid receptor (324). JNK has not been studied in our model. The inhibition of p38, on the other hand, occurs after a short time of treatment with glucocorticoids but requires de novo protein expression (327).

In contrast to the above, other investigators have reported that dexamethasone either has no effect (325, 328) or causes a modest inhibition (329) or activates p38 MAPK (330). These contrasting observations presumably reflect cell-specific differences in the cross talk between the glucocorticoid and p38 signalling pathways.

However, according to our data, MKP-1 plays a critical role in the inactivation of p38 MAPK. Furthermore, the data presented here are in line with those recently published providing strong support for the conclusion that MKP-1 is a critical factor in the attenuation of p38 MAPK activity in neonatal cardiomyocytes exposed to hypoxia/reoxygenation (298).

### **Summary**

The results of this study demonstrated that the glucocorticoid, dexamethasone, improved post-ischaemic cardiac functional recovery, reduced infarct size associated with increased expression of MKP-1 and ERK44 phosphorylation and suppressed phosphorylation of p38 MAPK. We conclude that glucocorticoids play an important modulatory role in the regulation of reactive signalling pathways in cardiac myocytes in ischaemia/reperfusion. To our knowledge, this is the first demonstration that the well-known cardiac actions of dexamethasone can be attributed to upregulation of the phosphatase MKP-1.

The specific upregulation of endogenous dual-specificity phosphatases in the heart might offer an additional therapeutic strategy to benefit certain forms of heart disease. From a therapeutic point of view, our understanding of the molecular mechanisms regulating MAPK and MAPK phosphatase activities by glucocorticoids could lead to new strategies for the effective prevention and control of heart disease.

## Chapter 6

### Conclusion

Since the first report on the phenomenon of ischaemic preconditioning more than two decades ago, many articles have appeared, characterizing the phenomenon in different animal species, as well as attempting to elucidate its mechanism. Although unravelling the final steps in the mechanism of protection has not been successful thus far, our knowledge regarding signalling events in the ischaemic/reperfused heart has increased exponentially over the last years. As described in Chapter I, the focus of research has been mainly on the role of kinases in this scenario, while very little indeed is known about the contributions of the phosphatases. The chief objective of this study has therefore been to gain more information about the significance of the phosphatases in the setting of ischaemia/reperfusion and ischaemic preconditioning.

A number of interesting and possibly significant observations were made:

(i) PSP and PP1 activation, as measured by an in vitro assay, does not change during either a relatively long period of global ischaemia or during reperfusion; moreover, no difference could be discerned in the phosphatase activity of tissues obtained from non-preconditioned and preconditioned hearts. This could be due to the relatively short periods of reperfusion.

(ii) Despite the above negative results, indications are that phosphatases play a significant role in the preconditioning protocol: administration of the phosphatase inhibitors, cantharidin or okadaic acid during the preconditioning phase completely abolished cardioprotection, as evidenced by mechanical failure during reperfusion, increased infarct size and activation of p38 MAPK and PKB, and dephosphorylation of ERK42/44; but inhibition of PP1 and PP2A during reperfusion after sustained global ischaemia in preconditioned hearts increased cardioprotection, particularly in the case of hearts subjected to 3x5 min preconditioning, associated with increased phosphorylation of the RISK pathway, and dephosphorylation of p38 MAPK. Non-specificity of inhibitors also may be the limitation of this study.

(iii) Upregulation of the phosphatase MKP-1 is cardioprotective. The phosphatase MKP-1 selectively inactivates p38 MAPK by dephosphorylation of the regulatory Thr and Tyr residues. We demonstrate that under our experimental circumstances the glucocorticoid, dexamethasone, improved functional recovery, reduced infarct size associated with upregulation of the MKP-1 and dephosphorylation of the pro-apoptotic p38 MAPK during reperfusion. These data suggest that MKP-1 plays a critical role in the negative regulation of p38 MAPK in response to ischaemia/reperfusion to promote cell survival.

The finding that inhibition of PP1 and PP2A during the preconditioning phase completely abolished cardioprotection suggests that the transient activation of p38 MAPK which occurs during a multi-cycle preconditioning phase (242) may be due to dephosphorylation induced by these phosphatases. Administration of okadaic acid, which specifically inhibits PP2A, suggests a role for this particular phosphatase in the phenomenon of preconditioning.

As far as we know, this is the first demonstration that manipulation of the RISK pathway by phosphatase inhibition during reperfusion is associated with cardioprotection.

The cardioprotective effects of dexamethasone have been described before, but the present study is the first demonstration that this is associated with upregulation of the phosphatase MKP-1 and dephosphorylation of p38 MAPK. However, the significance of the RISK pathway as a target for phosphatase manipulation and the end-effectors involved, need further investigation.

Thus manipulation of the pro-survival PI3K–Akt and MEK1/2–ERK1/2 kinase cascades during the early reperfusion by administration of phosphatase inhibitors has great promise for new pharmacological approaches to reduce the morbidity and mortality of ischaemic heart disease.

However, the data presented in this study have merely scratched the surface of what potentially may be a promising new approach. Not only should a thorough study be made of the phosphatases involved, but also of the factors involved in their regulation. More sophisticated experimental approaches are required, for example, use of knockout or transgenic animals or the siRNA technique.

## REFERENCES

1. Plowman G.D, Sudarsanam S, Bingham J, Whyte D and Hunter T. The protein kinases of *Caenorhabditis elegans*: a model for signal transduction in multicellular organisms. *Proc Natl Acad Sci USA* 1999, 96: 13603–13610
2. Ceulemans H, Stalmans W and Bollen M. Regulator-driven functional diversification of protein phosphatase-1 in eukaryotic evolution. *Bioessays* 2002, 24: 371–381
3. Klumpp S, Krieglstein J. Serine/threonine protein phosphatases in apoptosis. *J Pharmacology Science* 2002, volue 2, 2: 458-462.
4. Hunter T. Signaling – 2000 and beyond. *Cell* 2000,100: 113–127.
5. Sim A.T.R and J.D Scott. Targeting of PKA, PKC and protein phosphatases to cellular microdomains. *Cell Calcium* 1999, 26: 209–217.
6. Viktor D, Krieglstein J and Klumpp S. Regulating the regulators Conference on protein phosphorylation and protein phosphatases. *EMBO Rep* 2002, 3(2): 120–124.
7. Matsui T, J Tao, F del Monte, K.H Lee, L Li, M Picard et al. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation* 2001,104(3): 330–335. 7A Mocanu M.M, Bell R.M and Yellon D.M. PI3 kinase and not p42/p44 appears to be implicated in the protection conferred by ischemic preconditioning. *J Mol Cell Cardiol* 2002, 34: 661–668.
8. Fujio Y, T Nguyen, D Wencker, R.N Kitsis and K Walsh. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 2000, 101(6): 660–667.
9. Yue T.L, Wang C, Gu J.L, Ma X.L, Kumar S, Lee J.C, Feuerstein G.Z, Thomas H, Maleeff B and Ohlstein E.H. Inhibition of extracellular signal-regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circ Res* 2000, 86: 692–699. 9A Fryer R.M, Pratt P.F, Hsu A.K and Gross G.J. Differential activation of extracellular signal-regulated kinase isoforms in preconditioning and opioid-induced cardioprotection. *J Pharmacol Exp Ther* 2001, 296: 642–649.
10. Hausenloy D.J and Yellon D.M. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the reperfusion injury salvage kinase (RISK) pathway. *Cardiovasc Res* 2004, 61: 448–460.
11. Wurgler-Murphy S.M, Saito H. Two-component signal transducers and MAPK cascades. *Trends Biochem Sci* 1997, 22: 172–176.
12. Saxena M, Williams S, Brockdorff J, Gilman J and Mustelin T. Inhibition of T cell signaling by mitogen-activated protein kinase-targeted hematopoietic tyrosine phosphatase (HePTP). *J Biol Chem* 1999, 274: 11693–11700.
13. Blanco-Aparicio C, Torres J and Pulido R. A novel regulatory mechanism of MAP kinases activation and nuclear translocation mediated by PKA and the PTP-SL tyrosine phosphatase. *J Cell Biol* 1999, 147: 1129–1136.
14. Keyse S.M. Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. *Curr Opin Cell Biol* 2000, 12: 186–192.
15. Kong M et al. The PP2A-associated protein alpha4 is an essential inhibitor of apoptosis. *Science* 2004, 306: 695–698.

16. Cohen P.T.W. Nomenclature and chromosomal localization of human protein serine/threonine phosphatase genes. *Adv Prot Phosphatases* 1994, 8: 371–376.
17. Aggen J.B, Nairn A.C and Chamberlin R. Regulation of protein phosphatase-1. *Chem Biol* 2000, 7: 13–23.
18. Barford D, Das A.K and Egloff M.P. The structure and mechanism of protein phosphatases: insights into catalysis and regulation. *Annu Rev Biophys Biomol Struct* 1998, 27: 133–164.
19. Cohen P.T. Protein phosphatase 1—targetted in many directions. *J Cell Sci* 2002, 115: 241–256.
20. Doonan J.H, MacKintosh C, Osmani S, Cohen P, Bai G, Lee E.Y and Morris N.R. A cDNA encoding rabbit muscle protein phosphatase 1 alpha complements the *Aspergillus* cell cycle mutation, bimG11. *J Biol Chem* 1991, 266: 18889–18894.
21. Sangrador A, Andres I, Eguiraun A, Lorenzo M.L and Ortiz J.M. Growth arrest of *Schizosaccharomyces pombe* following overexpression of mouse type 1 protein phosphatases. *Mol Gen Genet* 1998, 259: 449–456.
22. Bollen M and Stalmans W. The structure, role, and regulation of type 1 protein phosphatases. *Crit Rev Biochem Mol Biol* 1992, 27: 227–281.
23. Cohen P and Cohen P.T. Protein phosphatases come of age. *J Biol Chem* 1989, 264: 21435–21438.
24. Hubbard M.J and Cohen P. On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem Sci* 1993, 18: 172–177.
25. Aschenbach W.G, Suzuki Y, Breeden K, Prats C, Hirshman M.F, Dufresne S.D, Sakamoto K, Vilardo P.G, Steele M, Kim J.H, Jing S.L, Goodyear L.J and DePaoli-Roach A.A. The muscle-specific protein phosphatase PP1G/R(GL)(G(M)) is essential for activation of glycogen synthase by exercise. *J Biol Chem* 2001, 276: 39959–39967.
26. Gasa R, Jensen P.B, Berman H.K, Brady M.J, DePaoli-Roach A.A and Newgard C.B. Distinctive regulatory and metabolic properties of glycogen-targetting subunits of protein phosphatase-1 (PTG, GL, GM/RGI) expressed in hepatocytes. *J Biol Chem* 2000, 275: 26396–26403.
27. Alberts A.S, Montminy M, Shenolikar S and Feramisco J.R. Expression of a peptide inhibitor of protein phosphatase 1 increases phosphorylation and activity of CREB in NIH 3T3 fibroblasts. *Mol Cell Biol* 1994, 14: 4398–4407.
28. Bito H, Deisseroth K and Tsien R.W. CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. *Cell* 1996, 87: 1203–1214.
29. Hagiwara M, Alberts A, Brindle P, Meinkoth J, Feramisco J, Deng T, Karin M, Shenolikar S and Montminy M. Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. *Cell* 1992, 70: 105–113.
30. Canettieri G, Morantte I, Guzman E, Asahara H, Herzig S, Anderson S.D, Yates J.R and Montminy M. Attenuation of a phosphorylation-dependent activator by an HDAC-PP1 complex. *Nat Struct Biol* 2003, 10: 175–181.
31. Tamura Y, Simizu S, Osada H. The phosphorylation status and anti-apoptotic activity of Bcl-2 are regulated by ERK and protein phosphatase 2A on the mitochondria. *FEBS Lett* 2004, 569:249–255.
32. Dagda R.K, Zaucha J.A, Wadzinski B.E, Strack S. A developmentally regulated, neuron-specific splice variant of the variable subunit B $\beta$  targets protein phosphatase 2A to mitochondria and modulates apoptosis. *J Biol Chem* 2003, 278: 24976–24985.

33. Foley T.D, Armstrong J.J, Kupchak B.R. Identification and H<sub>2</sub>O<sub>2</sub> sensitivity of the major constitutive MAPK phosphatase from rat brain. *Biochem Biophys Res Commun*. 2004, 315: 568–574.
34. Zha J, H Harada, E Yang, J Jockel and S.J Korsmeyer. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-XL. *Cell* 1996, 87: 619–628.
35. Lizcano J.M, N Morrice and P Cohen. Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser155. *Biochem J* 2000, 349: 547–557.
36. Tan Y, M.R Demeter, H Ruan and M.J Comb. BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. *J Biol Chem* 2000, 275: 25865–25869.
37. Dramsi S, M.P Scheid, A Maiti, P Hojabrpour, X Chen, K Schubert, D.R Goodlett, R Aebersold and V Duronio. Identification of a novel phosphorylation site, ser-170, as a regulator of Bad pro-apoptotic activity. *J Biol Chem* 2002, 277: 6399–6405.
38. Ayllon V, A.C Martinez, A Garcia, X Cayla and A Rebollo. Protein phosphatase 1 $\alpha$  is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. *J EMBO* 2000, 19: 2237–2246.
39. Chiang C.W, G Harris, C Ellig, S.C Masters, R Subramanian, S Shenolikar, B.E Wadzinski and E Yang. Protein phosphatase 2A activates the proapoptotic function of BAD in interleukin-3-dependent lymphoid cells by a mechanism requiring 14-3-3 dissociation. *Blood* 2001, 97: 1289–1297.
40. Wang H.G, N Pathan, I.M Ethell, S Krajewski, Y Yamaguchi, F Shibasaki, F McKeon, T Bobo, T.F Franke and J.C Reed. Ca<sup>2+</sup>-induced apoptosis through calcineurin dephosphorylation of BAD. *Science* 1999, 284: 339–343.
41. Santoro M.F, R.R Annand, M.M Robertson, Y.W Peng, M.J Brady, J.A Mankovich, M.C Hackett, T Ghayur, G Walter, W.W Wong and D.A Giegel. Regulation of protein phosphatase 2A activity by caspase-3 during apoptosis. *J Biol Chem* 1998, 273: 13119–13128.
42. Shanley T.P, Vasi N, Denenberg A and Wong H.R. The serine/threonine phosphatase, PP2A: endogenous regulator of inflammatory cell signaling. *J Immunol* 2001, 166: 966–972.
43. Adams D.G, Coffee R.L Jr, Zhang H, Pelech S, Strack S, Wadzinski B.E. Positive regulation of Raf1-MEK1/2-ERK1/2 signaling by protein serine/threonine phosphatase 2A holoenzymes. *J Biol Chem* 2005, 280(52): 42644-54
44. Rossini G.P, C Pinna and C Malaguti. Different sensitivities of p42 mitogen-activated protein kinase to phorbol ester and okadaic acid tumor promoters among cell types. *Pharmacol Biochem* 1999, 58: 279–284.
45. Sontag E, S Federov, C Kamibayashi, D Robbins, M Cobb and M Mumby. The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the Map kinase pathway and induces cell proliferation. *Cell* 1993, 75: 887–897.
46. Wang P.Y, P Liu, J Weng, E Sontag and R.G Anderson. A cholesterol-regulated PP2A/HePTP complex with dual specificity ERK1/2 phosphatase activity. *J EMBO* 2003, 22(11): 2658-67.
47. Li S.P, M.R Junttila, J Han, V.M Kahari and J Westermarck. p38 Mitogen-activated protein kinase pathway suppresses cell survival by inducing dephosphorylation of mitogen-activated protein/extracellular signal-regulated kinase kinase1,2. *Cancer Res*

2003, 63913: 3473-7.

48. Gardai S.J, B.B Whitlock, Y.Q Xiao, D.B Bratton and P.M Henson. Oxidants inhibit ERK/MAPK and prevent its ability to delay neutrophil apoptosis downstream of mitochondrial changes and at the level of XIAP. *J Biol Chem* 2004, 279(43): 44695.
49. Liu Q and P.A Hofmann. Modulation of protein phosphatase 2a by adenosine A1 receptors in cardiomyocytes: role for p38 MAPK. *J Am Physiol Heart Circ Physiol* 2003, 285(1): 97-103.
50. Janssens V and J Goris. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *J Biochem* 2001, 353: 417.
51. Janssens V, J Goris and C Van Hoof. PP2A: the expected tumor suppressor. *Curr Opin Genet Dev* 2005, 15: 34-41.
52. Ivaska J, L Nissinen, N Immonen, J.E Eriksson, V.M Kahari and J Heino. Integrin alpha 2 beta 1 promotes activation of protein phosphatase 2A and dephosphorylation of Akt and glycogen synthase kinase 3 beta. *Mol Cell Biol* 2002, 22: 1352-9.
53. Guthridge M.A, Bellosta P, Tavoloni N and Basilico C. FIN13, a novel growth factor-inducible serine-threonine phosphatase which can inhibit cell cycle progression. *Mol Cell Biol* 1997, 17: 5485–5498.
54. Tong Y, Quirion R and Shen S.H. Cloning and characterization of a novel mammalian PP2C isozyme. *J Biol Chem* 1998, 273: 35282–35290.
55. Kitani T, Ishida A, Okuno S, Takeuchi M, Kameshita I and Fujisawa H. Molecular cloning of Ca<sup>2+</sup>/calmodulin-dependent protein kinase phosphatase. *J Biochem* 1999, 125: 1022–1028.
56. Takekawa M, Maeda T and Saito H. Protein phosphatase 2C $\alpha$  inhibits the human stress-responsive p38 and JNK MAPK pathways. *J EMBO* 1998,17: 4744–4752.
57. Hanada M, Kobayashi T, Ohnishi M, Ikeda S, Wang H, Katsura K, Yanagawa Y, Hiraga A, Kanamaru R and Tamura S. Selective suppression of stress-activated protein kinase pathway by protein phosphatase 2C in mammalian cells. *FEBS Lett* 1998, 437: 172–176.
58. Cheng A, Kaldis P and Solomon M.J. Dephosphorylation of human cyclin-dependent kinases by protein phosphatase type 2C $\alpha$  and  $\beta$ 2 isoforms. *J Biol Chem*. 2000, 275: 34744–34749.
59. Aramburu J, A Rao and C.B Klee. Calcineurin: from structure to function. *Curr Top Cell Regul* 2000, 36: 237–295.
60. Muda M, Theodosiou A, Rodrigues N, Boschert U, Camps M, Gillieron C, Davies K, Ashworth A and Arkinstall S. The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. *J Biol Chem* 1996, 271: 27205–27208.
61. Camps M, Nichols A and Arkinstall S. Dual specificity phosphatases: a gene family for control of MAP kinase function. *J FASEB* 2000, 14: 6-16.
62. Dickinson R.J and Keyse S.M. Diverse physiological functions for dual-specificity MAP kinase phosphatases. *J Cell Sci* 2006, 119: 4607–4615.
63. Guan K, Broyles S.S, Dixon J.E. A Tyr/Ser protein phosphatase encoded by vaccinia virus. *Nature* 1991, 350: 359–362. Cloning of VH1, the first member of the dual specificity phosphatase family. Demonstration that recombinant VH1 hydrolyzes substrates containing both phosphotyrosine and phosphoserine.
64. Guan K.L and Butch E. Isolation and characterization of a novel dual specific phosphatase,

- HVH2, which selectively dephosphorylates the mitogen-activated protein kinase. *J Biol Chem* 1995, 270: 7197–7203. Isolation and characterization of human HVH2 (DUSP4), a nuclear MKP that shows substrate selectivity for ERK1 and ERK2.
65. Kwak S.P and Dixon J.E. Multiple dual specificity protein tyrosine phosphatases are expressed and regulated differentially in liver cell lines. *J Biol Chem* 1995, 270: 1156–1160. Identification of human hVH-3 (DUSP5) and comparison of its levels of expression and tissue distribution to other known members of the family of MKPs.
  66. Rohan P.J, Davis P, Moskaluk C.A, Kearns M, Krutzsch H, Siebenlist U and Kelly K. PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. *Science* 1993, 259: 1763–1766.
  67. Muda M, Boschert U, Smith A, Antonsson B, Gillieron C, Chabert C, Camps M, Martinou I, Ashworth A, Arkinstall S. Molecular cloning and functional characterization of a novel mitogen activated protein kinase phosphatase, MKP-4. *J Biol Chem* 1997, 272: 5141–5151. Human MKP-4 (DUSP9), a phosphatase that is highly selective for ERK, maps to human Xq28 and displays distinct subcellular localization.
  68. Theodosiou A, Smith A, Gillieron C, Arkinstall S, Ashworth A. MKP5, a new member of the MAP kinase phosphatase family, which selectively dephosphorylates stress-activated kinases. *Oncogene* 1999, 18: 6981–6988. Cloning of human MKP5 (DUSP10) and mapping to human chromosome 1q32. Demonstration that MKP5 preferentially binds to and inactivates the p38 and JNK kinases.
  69. Tanoue T, Moriguchi T and Nishida E. Molecular cloning and characterization of a novel dual specificity phosphatase, MKP-5. *J Biol Chem* 1999, 274: 19949–19958.
  70. Hutter D, Chen P, Barnes J and Liu Y. Catalytic activation of mitogen-activated protein (MAP) kinase phosphatase-1 by binding to p38 MAP kinase: critical role of the p38 C-terminal domain in its negative regulation. *J Biol Chem* 2000, 275: 155–163.
  71. Keyse S.M and E.A Emslie. Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature* 1992, 359: 644–647.
  72. Li C, Hu Y, Mayr M and Xu Q. Cyclic strain stress-induced mitogen-activated protein kinase (MAPK) phosphatase 1 expression in vascular smooth muscle cells is regulated by Ras/Rac-MAPK pathways. *J Biol Chem* 1999, 274: 25273–25280.
  73. Bokemeyer D, Sorokin A and Dunn M.J. Differential regulation of the dual-specificity protein-tyrosine phosphatases CL100, B23 and PAC1 in mesangial cells. *J Am Soc Nephrol* 1997, 8: 40–50.
  74. Scimeca J.C, Servant M.J, Dyer J.O and Meloche S. Essential role of calcium in the regulation of MAP kinase phosphatase-1 expression. *Oncogene* 1997, 15: 717–725.
  75. Schliess F, Heinrich S and Haussinger D. Hyperosmotic induction of the mitogen-activated protein kinase phosphatase MKP-1 in H4IIE rat hepatoma cells. *Arch Biochem Biophys* 1998, 351: 35–40.
  76. Chu Y, Solski P.A, Khosravi-Far R.C.J and Kelly K. The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. *J Biol Chem* 1996, 271: 6497–6501.
  77. Franklin C.C and Kraft A.S. Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. *J Biol Chem* 1997, 272: 16917–16923.
  78. Franklin C.C, S Srikanth and A.S Kraft. Conditional expression of mitogen-activated protein kinase phosphatase-1, MKP-1, is cytoprotective against UV-induced apoptosis. *Proc Natl Acad Sci USA* 1998, 95: 3014–3019.



79. Brondello J.M, Pouyssegur J and McKenzie F.R. Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. *Science* 1999, 286: 2514–2517.
80. Bokemeyer D, Sorokin A, Yan M, Ahn N.G, Templeton D.J and Dunn M.J. Induction of mitogen-activated protein kinase phosphatase 1 by the stress-activated protein kinase signaling pathway but not by extracellular signal-regulated kinase in fibroblasts. *J Biol Chem* 1996, 271: 639–642.
81. Lim H.W, New L, Han J and Molkenin J.D. Essential role of calcium in the regulation of MAP kinase phosphatase-1 expression. *J Biol Chem* 2001, 276: 15913–15919.
82. Misra-Press A, Rim C.S, Yao H, Roberson M.S and Stork P.J. A novel mitogen-activated protein kinase phosphatase. Structure, expression, and regulation. *J Biol Chem* 1995, 270: 14587–14596.
83. Hirsch D.D and Stork P.J. Mitogen-activated protein kinase phosphatases inactivate stress-activated protein kinase pathways in vivo. *J Biol Chem* 1997, 272: 4568–4575.
84. Wiessner C. The dual specificity phosphatase PAC-1 is transcriptionally induced in the rat brain following transient forebrain ischemia. *Mol Brain Res*. 1995, 28: 353–356.
85. Boschert U, Muda M, Camps M, Dickinson R and Arkinstall S. Induction of the dual specificity phosphatase PAC1 in rat brain following seizure activity. *Neuroreport* 1997, 8: 3077–3080.
86. Martell K.J, Kwak S, Hakes D.J, Dixon J.E, Trent J.M. Chromosomal localization of four human VH1-like protein-tyrosine phosphatases. *Genomics*. 1994, 22: 462–464.
87. Grumont R.J, Rasko J.E, Strasser A and Gerondakis S. Activation of the mitogen-activated protein kinase pathway induces transcription of the PAC-1 phosphatase gene. *Mol Cell Biol* 1996, 16: 2913–2921.
88. Todd J.L, Tanner K.G, Denu J.M. Extracellular regulated kinases (ERK) 1 and ERK2 are authentic substrates for the dual-specificity protein-tyrosine phosphatase VHR. *J Biol Chem*. 1999, 274: 13271–13280. Demonstration that immunodepletion of endogenous VHR eliminates the dephosphorylation of cellular ERK, suggesting that VHR specifically inactivates ERK in vivo.
89. Groom L.A, Sneddon A.A, Alessi D.R, Dowd S and Keyse S.M. Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *J EMBO* 1996, 15: 3621–3632.
90. Muda M, A Theodosiou, C Gillieron, A Smith, C Chabert, M Camps, U Boschert, N Rodrigues, K Davies, A Ashworth and S Arkinstall. The mitogen-activated protein-kinase phosphatase-3 N-terminal noncatalytic region is responsible for tight substrate-binding and enzymatic specificity. *J Biol Chem* 1998, 273: 9323–9329. This paper demonstrates that the substrate specificity of dual specificity MKPs is determined by MAPK specific binding mediated by the noncatalytic domain of these proteins.
91. Muda M, Boschert U, Dickinson R, Martinou J.C, Martinou I, Camps M, Schlegel W, Arkinstall S. MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. *J Biol Chem* 1996, 271: 4319–4326. This paper describes the cloning and characterization of rat MKP-3 (DUSP6) and MKP-X (DUSP7), and shows that MKP-3 is a cytosolic MKP that specifically inactivates ERK. Expression studies of MKP-3 reveal that it is enriched within the CA1 layer of the hippocampus.

92. Ishibashi T, Bottaro D.P, Michieli P, Kelley C.A, Aaronson S.A. A novel dual specificity phosphatase induced by serum stimulation and heat shock. *J Biol Chem* 1994, 269: 29897–29902. Report of the isolation and characterization of human B23 (DUSP5). Demonstration that B23 displays substrate selectivity for ERK and that it is induced by serum stimulation and heat shock.
93. Camps M, Nichols A, Gillieron C, Antonsson B, Muda M, Chabert C, Boschert U and Arkinstall S. Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. *Science* 1998, 280: 1262–1265.
94. Dowd S, Sneddon A.A, Keyse S.M. Isolation of the human genes encoding the Pyst1 and Pyst2 phosphatases: characterization of Pyst2 as a cytosolic dual-specificity MAP kinase phosphatase and its catalytic activation by both MAP and SAP kinases. *J Cell Sci* 1998, 111: 3389–3399. Genomic organization of the human genes encoding the Pyst1 (DUSP6) and Pyst2 (DUSP7) phosphatases. The subcellular localization of Pyst2 protein is also determined.
95. Smith A, Price C, Cullen M, Muda M, King A, Ozanne B, Arkinstall S, Ashworth A. Chromosomal localization of three human dual specificity phosphatase genes (DUSP4, DUSP6, and DUSP7). *Genomics* 1997, 42: 524–527. Mapping the human phosphatases DUSP4, 6 and 7 to chromosomes 8p12-p11, 12q22-23 and 3p21, respectively.
96. Tanoue T, Yamamoto T, Maeda R, Nishida E. A novel MAPK phosphatase MKP-7 acts preferentially on JNK/SAPK and p38 $\alpha$  and  $\beta$  MAPKs. *J Biol Chem* 2001, 276: 26629–26639. Identification and characterization of human MKP-7 (DUSP16), which shows substrate preference for JNK and p38. The authors also propose a tentative classification of MKPs based on the sequence characteristics of their MAP kinase-docking sites.
97. Matsuguchi T, Musikacharoen T, Johnson T.R, Kraft A.S, Yoshikai Y. A novel mitogen-activated protein kinase phosphatase is an important negative regulator of lipopolysaccharide-mediated c-Jun N-terminal kinase activation in mouse macrophage cell lines. *Mol Cell Biol* 2001, 21: 6999–7009. Cloning of mouse MKP-M (mouse ortholog of DUSP16), a cytosolic MKP, which shows substrate specificity for JNK and p38. MKP-M is constitutively expressed in mouse macrophage cell lines with increased expression upon LPS stimulation.
98. Johnson T.R, Biggs J.R, Winbourn S.E and Kraft A.S. Regulation of dual-specificity phosphatases M3/6 and hVH5 by phorbol esters. Analysis of a delta-like domain. *J Biol Chem* 2000, 275: 31755–31762.
99. Martell K.J, Seasholtz A.F, Kwak S.P, Clemens K.K, Dixon J.E. hVH-5: a protein tyrosine phosphatase abundant in brain that inactivates mitogen-activated protein kinase. *J Neurochem* 1995, 65: 1823–1833. Isolation and characterization of human hVH-5 (DUSP8), which is abundant in brain. The expression pattern of hVH-5 in mouse embryos reveals abundant and wide distribution in the central and peripheral nervous system.
100. Marti F, Krause A, Post N.H, Lyddane C, Dupont B, Sadelain M and King P.D. Negative-feedback regulation of CD28 costimulation by a novel mitogen-activated protein kinase phosphatase, MKP6. *J Immunol* 2001, 166: 197–206.
101. Neel B. G and Tonks N. K. Protein tyrosine phosphatases in signal transduction. *Curr Opin Cell Biol* 1997, 9: 193-204.
102. Hooft van Huijsduijnen R. Protein tyrosine phosphatases: counting the trees in the forest. *Gene* 1998, 225: 1-8.

103. Denu J.M and Dixon J.E. Protein tyrosine phosphatases: mechanisms of catalysis and regulation. *Curr Opin J Chem Biol* 1998, 2: 633-641.
104. Alessi D.R, Gomez N, Moorhead G, Lewis T, Keyse S.M, Cohen P. Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. *Curr Biol* 1995, 5: 283-295 .
105. van der Wijk T, C Blanchetot and J den Hertog. Regulation of receptor protein-tyrosine phosphatase dimerization. *Methods* 2005, 35: 73.
106. López-Neblina F and Luis H. Toledo-Pereyra Phosphoregulation of Signal Transduction Pathways in Ischemia and Reperfusion. *J Surgical Research* 2006, Volume 134, 2: 292-299.
107. Andersen J.N, Jansen P.G, Echwald S.M, Mortensen O.H, Fukada T, Del Vecchio R, Tonks N.K, Moller N.P.H. A genomic perspective on protein tyrosine phosphatases: gene structure, pseudogenes, and genetic disease linkage. *J FASEB* 2004, 18: 8–30.
108. Andersen J.N, Mortensen O.H, Peters G.H, Drake P.G, Iversen L.F, Olsen O.H, Jansen P.G, Andersen H.S, Tonks N.K, Moller N.P. Structural and evolutionary relationships among protein tyrosine phosphatase domains. *Mol Cell Biol* 2001, 21: 7117–7136.
109. Zhang Z.Y. Mechanistic studies on protein tyrosine phosphatases. *Prog Nucleic Acid Res Mol Biol* 2003, 73: 171–220.
110. Wurgler-Murphy S.M, T Maeda, E.A Witten and H Saito. Regulation of the *Saccharomyces cerevisiae* HOG1 mitogen-activated protein-kinase by the PTP2 and PTP3 protein-tyrosine phosphatases. *Mol Cell Biol* 1997, 17: 1289–1297.
111. Jacoby T, H Flanagan, A Faykin, A.G Seto, C Mattison and I Ota. 2 protein-tyrosine phosphatases inactivate the osmotic-stress response pathway in yeast by targeting the mitogen-activated protein kinase, Hog1. *J Biol Chem* 1997, 272: 17749–17755.
112. Pulido R, A Zuniga and A Ullrich. PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. *J EMBO* 1998, 17: 7337–7350. This paper identifies a new class of tyrosine-specific phosphatases which regulate MAPK in mammalian cells. It also defines an interaction motif within the amino-terminal noncatalytic domain of these enzymes, which mediates ERK binding.
113. Saxena M, Williams S, Tasken K and Mustelin T. Crosstalk between cAMP-dependent kinase and MAP kinase through a protein tyrosine phosphatase. *Nat Cell Biol* 1999, 1: 305–311.
114. Millar J.B.A, V Buck and M.G Wilkinson. Pyp1 and pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell-size at division in fission yeast. *Genes Dev* 1995, 9: 2117–2130.
115. Shiozaki K and P Russell. Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. *Nature* 1995, 378: 739–743.
116. West A.H and Stock A.M. Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* 2001, 26: 369–376.
117. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang S.I et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997, 275: 1943–1947.
118. Steck P.A, Pershouse M.A, Jasser S.A, Yung W.K, Lin H, Ligon A.H et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997, 15: 356–362.

119. Leslie N.R, Downes C.P. PTEN function: how normal cells control it and tumour cells lose it. *J Biol Chem* 2004, 382: 1–11.
120. Hlobilkova A, Knillova J, Bartek J, Lukas J, Kolar Z. The mechanism of action of the tumour suppressor gene PTEN. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2003, 147 (1): 19–25.
121. Vazquez F, Ramaswamy S, Nakamura N, Sellers W.R. Phosphorylation of the PTEN tail regulates protein stability and function. *Mol Cell Biol* 2000, 20: 5010–5018.
122. Vazquez F, Grossman S.R, Takahashi Y, Rokas M.V, Nakamura N, Sellers W.R. Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *J Biol Chem* 2001, 276: 48627–48630.
123. Leslie N.R, Bennett D, Lindsay Y.E, Stewart H, Gray A, Downes C.P. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *J EMBO* 2003, 22: 5501–5510.
124. Mocanu M.M and D.M Yellon. PTEN, the Achilles' heel of myocardial ischaemia/reperfusion injury? *British J Pharmacology* 2007, 150: 833–838.
125. Ghebranious N, Donehower L.A. Mouse models in tumor suppression. *Oncogene* 1998, 17: 3385–3400.
126. Schmid A.C, Byrne R.D, Vilar R, Woscholski R. Bisperoxovanadium compounds are potent PTEN inhibitors. *FEBS Lett* 2004, 566: 35–38.
127. Wu D.N, Pei D.S, Wang Q, Zhang G.Y. Down-regulation of PTEN by sodium orthovanadate inhibits ASK1 activation via PI3-K/Akt during cerebral ischemia in rat hippocampus. *Neurosci Lett*. 2006, 287: 28258–28263.
128. Wu W, Wang X, Zhang W, Reed W, Samet J.M, Whang Y.E et al. Zinc-induced PTEN protein degradation through the proteasome pathway in human airway epithelial cells. *J Biol Chem* 2003, 278: 28258–28263.
129. Sasaoka T, Wada T, Tsuneki H. Lipid phosphatases as a possible therapeutic target in cases of type 2 diabetes and obesity. *Pharmacol Ther* 2006, 112: 799–809.
130. Cai Z, Semenza G.L. PTEN activity is modulated during ischemia and reperfusion: involvement in the induction and decay of preconditioning. *Circ Res* 2005, 97: 1351–1359.
131. Murry C.E, R.B Jennings and K.A Reimer. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986, 74 5: 1124–1136.
132. Das D.K and N Maulik. Cardiac genomic response following preconditioning stimulus. *Cardiovasc Res* 2006, 70: 254–263.
133. Shintani-Ishida K, M Nakajima, K Uemura and K Yoshida. Ischemic preconditioning protects cardiomyocytes against ischemic injury by inducing GRP78. *Biochem Biophys Res Commun* 2006, 345: 1600–1605.
134. Halestrap A.P. Mitochondria and preconditioning: a connexin connection? *Circ Res* 2006, 99: 10–12.
135. Eisena Alon, Enrique Z. Fisman, Melvyn Rubenfirec, Dov Freimarka, Ronald McKechniec, Alexander Tenenbauma, Michael Motroa and Yehuda Adler. Ischemic preconditioning: nearly two decades of research. A comprehensive review *Atherosclerosis* 2004, Volume 172, 2: 201-210.
136. Yellon D.M and Downey J.M. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev*. 2003, 83 4: 1113-51.
137. Downey J.M, Davis A.M, Cohen M.V. Signaling pathways in ischemic preconditioning *Heart Fail Rev* 2007, 12 3-4:181-8.
138. Liu G.S, J Thornton, D.M Van Winkle et al. Protection against infarction afforded by

- preconditioning is mediated by A1 adenosine receptors in rabbit hearts. *Circulation* 1991, 84: 350–356.
139. Miura T, T Adachi, T Ogawa et al. Myocardial infarct size-limiting effect of ischemic preconditioning: its natural decay and the effect of repetitive preconditioning. *Cardiovasc Pathol* 1992, 1: 147–154.
140. Nakano A, C.P Baines, S.O Kim, S.L Pelech, J.M Downey, M.V Cohen and S.D Critz. Ischemic preconditioning activates MAPKAPK2 in the isolated rabbit heart: Evidence for involvement of p38 MAPK. *Circulation Research* 2000, 86: 144–151.
141. Li G.C, J.A Vasquez, K.P Gallagher and B.R Lucchesi. Myocardial protection with preconditioning. *Circulation* 1990, 82: 609–619.
142. Liu Y and J.M Downey. Ischemic preconditioning protects against infarction in rat hearts. *Am J Physiol* 1992, 263: 1107–1112.
143. Beguin P.C, Joyeux-Faure M, Godin-Ribuot D, Levy P, Ribouot C. Acute intermittent hypoxia improves rat myocardium tolerance to ischemia. *J Appl Physiol* 2005, 99(3): 1064–9.
144. Fenton R.A, E.W Dickson, T.E Meyer and J.G Dobson Jr. Aging reduces the cardioprotective effect of ischemic preconditioning in rat heart. *J Molecular and Cellular Cardiology* 2000, 321: 1371–1375.
145. Schott R.J, S Rohmann, E.R Braun et al. Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res* 1990, 66: 1133–1142.
146. Vahlhaus C, R Schulz, H Post, J Rose and G Heusch. Prevention of ischemic preconditioning only by combined inhibition of protein kinase C and protein tyrosine kinase in pigs. *J Mol Cell Cardiol* 1998, 30: 197–209.
147. Ikonomidis J.S, L.C Tumati, R.D Weisel et al. Preconditioning human ventricular cardiomyocytes with brief periods of simulated ischemia. *Cardiovasc Res* 1994, 28: 1285–1291.
148. Walker D.M, J.M Walker, W.B Pugsley et al. Preconditioning in isolated superfused human muscle. *J Mol Cell Cardiol* 1995, 27: 1349–1357.
149. Deutsch E, M Berger, W.G Kussmaul et al. Adaptation to ischemia during percutaneous transluminal coronary angioplasty. Clinical, hemodynamic and metabolic features. *Circulation* 1990, 82: 2044–2051.
150. Cribier A, L Korsatz, R Koning et al. Improved myocardial ischemic response and enhanced collateral circulation with long repetitive coronary occlusion during angioplasty: a prospective study. *J Am Coll Cardiol* 1992, 20: 578–586.
151. Yellon D.M, A.M Alkhulaif and W.B Pugsley. Preconditioning the human myocardium. *Lancet* 1993, 342: 276–277.
152. Abete P, N Ferrara, A Cioppa, P Ferrara, S Bianco, C Calabrese, F Cacciatore, G Longobardi and F Rengo. Preconditioning does not prevent postischemic dysfunction in aging heart. *J Am Coll Cardiology* 1996, 27: 1777–1786.
153. Van Winkle D.M, J.D Thornton, D.M Downey and J.M Downey. The natural history of preconditioning: cardioprotection depends on duration of transient ischemia and time to subsequent ischemia. *Coron Artery Dis* 1991, 2: 613–619.
154. Schulz R, H Post, C Vahlhaus and G Heusch. Ischemic preconditioning in pigs: a graded phenomenon. Its relation to adenosine and bradykinin. *Circulation* 1998, 98: 1022–1029.
155. Matsubara S, S Minatoguchi, H Matsuo et al. Three minute, but not one minute. Ischemia and nicorandil have a preconditioning effect in patients with coronary artery disease. *J Am Coll Cardiol* 2000, 35: 345–351.

156. Jennings R.B, L Sebbag, L.M Schwartz et al. Metabolism of preconditioned myocardium: effect of loss and reinstatement of cardioprotection. *J Mol Cell Cardiol* 2001, 33: 1571–1588.
157. Kuzuya T, S Hoshida, N Yamashita et al. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993, 72: 1293–1299.
158. Sandhu R, R.J Diaz, G.D Mao and G.J Wilson. Ischemic preconditioning-difference in protection and susceptibility to blockade with single cycle versus multicycle transient ischemia. *Circulation* 1997, 96: 984–995.
159. Murry C.E, V.J Richard, R.B Jennings and K.A Reimer. Myocardial protection is lost before contractile function recovers from ischemic preconditioning. *J Am Physiol Heart Circ Physiol* 1991, 260: H796–H804.
160. Marber M.S, D.S Latchman, J.M Walker et al. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993, 88: 1264–1272.
161. Baxter G, F Goma and D Yellon. Characterisation of the infarct-limiting effect of delayed preconditioning: time-course and dose-dependency studies in rabbit myocardium. *Basic Res Cardiol* 1997, 92: 159–167.
162. Rizvi A, X.L Tang, Y Qiu, Y.T Xuan, H Takano and A.K Jadoon et al. Increased protein synthesis is necessary for the development of late preconditioning against myocardial stunning. *J Am Physiol* 1999, 277: H874–H884.
163. Murry C.E, V.J Richard, K.A Reimer and R.B Jennings. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* 1990, 66 (4): 913–931.
164. Murry C.E, Jennings R.B, Reimer K.A. New insights into potential mechanisms of ischemic preconditioning. *Circulation* 1991, 84: 442–445.
165. DeFily D.V, Chilian W.M. Preconditioning protects coronary arteriolar endothelium from ischemia-reperfusion injury. *J Am Physiol* 1993, 265: 700–706.
166. Richard V, N Karffer, C Tron and C Thuillez. Ischemic preconditioning protects against coronary endothelial dysfunction induced by ischemia and reperfusion. *Circulation* 1994, 89: 1254–1261.
167. Miura T, Ishimoto R, Sakamoto J et al. Suppression of reperfusion arrhythmia by ischemic preconditioning in the rat: Is it mediated by the adenosine receptor, prostaglandin, or bradykinin receptor? *Basic Res Cardiol* 1995, 90: 240–246.
168. Cave A.C and D.J Hearse. Ischemic preconditioning and contractile function: studies with normothermic and hypothermic global ischemia. *J Mol Cell Cardiol* 1992, 24: 1113–1123.
169. Armstrong S and C.E Ganote. Adenosine receptor specificity in preconditioning of isolated rabbit cardiomyocytes: evidence of A<sub>3</sub> receptor involvement. *Cardiovasc Res* 1994, 28: 1049–1056.
170. Tong H, Chen W, Steenbergen C and Murphy E. Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. *Circ Res* 2000, 87: 309–315.
171. Datta S.R, H Dudek, X Tao, S Masters, H Fu, Y Gotoh et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997, 91(2): pp. 231–241.
172. Tsuruta F, N Masuyama and Y Gotoh. The phosphatidylinositol 3-kinase (PI3K)–Akt pathway suppresses Bax translocation to mitochondria. *J Biol Chem* 2002, 277(16): 14040–14047.

173. Weston C.R, K Balmanno, C Chalmers, K Hadfield, S.A Molton, R Ley et al. Activation of ERK1/2 by deltaRaf-1:ER represses Bim expression independently of the JNK or PI3K pathways. *Oncogene* 2003, 22(90): 1281–1293.
174. Erhardt P, E.J Schremser and G.M Cooper. B-Raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/Erk pathway. *Mol Cell Biol* 1999, 19(8): 5308–5315.
175. Cardone M.H, N Roy, H.R Stennicke, G.S Salvesen, T.F Franke, E Stanbridge et al. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 1998, 282: 1318–1321.
176. Dimmeler S, I Fleming, B Fisslthaler, C Hermann, R Busse and A.M Zeiher. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999, 399: 601–605.
177. Chung J, T.C Grammer, K.P Lemon, A Kazlauskas and J Blenis. PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase. *Nature* 1994, 370: 71–75.
178. Lehman J.A and J Gomez-Cambronero. Molecular crosstalk between p70S6k and MAPK cell signaling pathways. *Biochem Biophys Res Commun* 2002, 293(1): 463–469.
179. Le Good J.A, W.H Ziegler, D.B Parekh, D.R Alessi, P Cohen and P.J Parker. Protein kinase C isoforms controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* 1998, 281: 2042–2045.
180. Freude B, Masters T.N, Kostin S, Robicsek F, Schaper J. Cardiomyocyte apoptosis in acute and chronic conditions. *Basic Res Cardiol* 1998, 93: 85–89.
181. Maulik N, Engelman R.M, Rousou J.A, Flack J.E, Deaton D, Das D.K. Ischemic preconditioning reduces apoptosis by upregulating anti-death gene Bcl-2. *Circulation* 1999, 100(19): 369–375.
182. Wang N.P, Bufkin B.L, Nakamura M, et al. Ischemic preconditioning reduces neutrophil accumulation and myocardial apoptosis. *Ann Thorac Surg* 1999, 67: 1689–95.
183. Nakamura M, Wang N.P, Zhao Z.Q et al. Preconditioning decreases Bax expression, PMN accumulation and apoptosis in reperfused rat heart. *Cardiovasc Res* 2000, 45: 661–70.
184. McCully J.D, Y Toyoda, M Uematsu, R.D Stewart and S Levitsky. Adenosine enhanced ischemic preconditioning: adenosine receptor involvement during ischemia and reperfusion. *J Am Physiol Heart Circ Physiol* 2001, 280: 591–602.
185. Lasley R.D, J.W Rhee, D.G.L.V Wylen and J.R.M Mentzer. Adenosine A1 receptor mediated protection of the globally ischemic isolated rat heart. *J Mol Cell Cardiol* 1990, 22: 39.
186. Schultz J.E.J, E Rose, Z Yao et al. Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. *J Am Physiol* 1995, 268: 2157–2161.
187. Schwartz L.M, R.B Jennings and K.A Reimer. Premedication with the opioid analgesic butorphanol raises the threshold for ischemic preconditioning in dogs. *Basic Res Cardiol* 1997, 92: 106–114.
188. Wang G.Y, S Wu, J.M Pei, X.C Yu and T.M Wong.  $\kappa$  but not  $\delta$ -opioid receptors mediate effects of ischemic preconditioning on both infarct and arrhythmia in rats. *J Am Physiol Heart Circ Physiol* 2001, 280: 384–391.
189. Wall T.M, R Sheehy and J.C Hartman. Role of bradykinin in myocardial preconditioning. *J Pharmacol Exp Ther* 1994, 2: 681–689.
190. Goto M, Y Liu, X.M Yang et al. Role of bradykinin in protection of ischemic preconditioning

- in rabbit hearts. *Circ Res* 1995, 77: 611–621.
191. Li Y and R.A Kloner. Cardioprotective effects of ischemic preconditioning are not mediated by prostanoids. *Cardiovasc Res* 1992, 26: 226–231.
192. Moolman J.A, S Genade, E Tromp and A Lochner. No evidence for mediation of ischemic preconditioning by alpha1-adrenergic signal transduction pathway or protein kinase C in isolated rat heart. *Cardiovasc Drugs Ther* 1996, 10: 125–136.
193. Lochner A, E Marais, S Genade and J.A Moolman. Nitric oxide: a trigger for classic preconditioning. *J Am Physiol Heart Circ Physiol* 2000, 279: 752–765.
194. Nakano A, G.S Liu, G Heusch, J.M Downey and M.V Cohen. Exogenous nitric oxide can trigger a preconditioned state through a free radical mechanism, but endogenous nitric oxide is not a trigger of classical ischemic preconditioning. *J Mol Cell Cardiol* 2000, 32: 11159–11167.
195. Post H, R Schulz, M Behrends et al. No involvement of endogenous nitric oxide in classical ischemic preconditioning in swine. *J Mol Cell Cardiol* 2000, 32: 725–733.
196. Baines C.P, M Goto and J.M Downey. Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J Mol Cell Cardiol* 1997, 29: 207–216.
197. Tokube K, T Kiyosue and M Arita. Openings of cardiac KATP channel by oxygen free radicals produced by xanthine oxidase reaction. *J Am Physiol Heart Circ Physiol* 1996, 271: 478–489.
198. Cain B.S, D.R Meldrum, J.C Cleveland Jr et al. Clinical  $\alpha_1$ -type calcium channel blockade prevents ischemic preconditioning of human myocardium. *J Mol Cell Cardiol*. 2000, 31: 2191–2197.
199. Jennings R.B, Reimer K.A, Steenbergen C. Effect of inhibition of the mitochondrial ATPase on net myocardial ATP in total ischemia. *J Mol Cell Cardiol* 1991, 23: 1383–1395.
200. Kobara M, Tatsumi T, Matoba S, Yamahara Y, Nakagawa C, Ohta B, Matsumoto T, Inoue D, Asayama J, Nakagawa M. Effect of ischemic preconditioning on mitochondrial oxidative phosphorylation and high energy phosphates in rat hearts. *J Mol Cell Cardiol* 1996, 28: 417–428.
201. Xu W, Liu Y, Wang S, McDonald T, van Eyk J. E, Sidor A, O'Rourke B. Cytoprotective role of  $Ca^{2+}$ -activated channels of the inner mitochondrial membrane. *Science* 2002, 298: 1029–1033.
202. Cohen M.V and J.M Downey. Myocardial preconditioning promises to be a novel approach to the treatment of ischemic heart disease. *Annual Review of Medicine* 1996, 47: 21–29.
203. Krieg T and Qin Q. ACh and adenosine activate PI3-kinase in rabbit hearts through transactivation of receptor tyrosine kinases. *J Am Physiol Heart Circ Physiol* 2002, 283(6): 2322–30.
204. Oldenburg O, Qin Q, Krieg T, Yang X.M, Philipp S, Critz S.D, Cohen M.V, Downey J.M. Bradykinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mitoKATP channel opening and leads to cardioprotection. *J Am Physiol Heart Circ Physiol* 2004, 286(1): 468–76.
205. Costa A.D, Garlid K.D, West I.C, Lincoln T.M, Downey J.M, Cohen M.V, Critz S.D. Protein kinase G transmits the cardioprotective signal from cytosol to mitochondria *Circ Res* 2005, 97: 329–336.
206. Halestrap A.P, Clarke S.J, Khaliulin I. The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta* 2007, 1767(8): 1007–31



207. Hausenloy D.J, M.M Mocanu and D.M Yellon. Cross-talk between the survival kinases during early reperfusion: its contribution to ischemic preconditioning. *Cardiovascular Research* 2004, 63: 305-312.
208. Solenkova N.V, Solodushko V, Cohen M.V, Downey J.M. Endogenous adenosine protects preconditioned heart during early minutes of reperfusion by activating Akt. *J Am Heart Circ Physiol* 2006, 290(1): 441-9.
209. Bijur G.N, Jope R.S. Rapid accumulation of Akt in mitochondria following phosphatidylinositol 3-kinase activation. *J Neurochem* 2003, 87: 1427–1435.
210. Tong H, Imahashi K, Steenbergen C, Murphy E. Phosphorylation of glycogen synthase kinase-3 $\beta$  during preconditioning through a phosphatidylinositol-3-kinase--dependent pathway is cardioprotective. *Circ Res* 2002, 90: 377-379.
211. Hausenloy D.J, Yellon D.M, Mani-Babu S, Duchon M.R. Preconditioning protects by inhibiting the mitochondrial permeability transition. *J Am Physiol Heart Circ Physiol*. 2004, 287(2): 841-9.
212. Crompton M, Virji S, Ward J.M. Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. *J Eur Biochem* 1998, 258: 729–735.
213. Gombosova I, Boknik P, Kirchhefer U, Knapp J, Luss H, Muller F.U, Muller T, Vahlensieck U, Schmitz W, Bodor G.S, Neumann J. Postnatal changes in contractile time parameters, calcium regulatory proteins, and phosphatases. *J Am Physiol* 1998, 274: 2123-32.
214. Lu C, Kumar R, Akita T, Joyner R.W. Developmental changes in the actions of phosphatase inhibitors on calcium current of rabbit heart cells. *Pflugers Arch* 1994, 427(5-6): 389-98.
215. Huang B, Wang S, Qin D, Boutjdir M, El-Sherif N. Diminished basal phosphorylation level of phospholamban in the postinfarction remodeled rat ventricle: role of beta-adrenergic pathway, G(i) protein, phosphodiesterase, and phosphatases. *Circ Res* 1999, 85(9): 848-55.
216. Neumann J, Eschenhagen T. Increased expression of cardiac phosphatases in patients with end-stage heart failure. *J Mol Cell Cardiol* 1997, 29: 265-272 .
217. Boknik P, Fockenbrock M, Herzig S, Knapp J, Linck B, Lüss H, Müller F.U, Müller T, Schmitz W, Schröder F, Neumann J. Protein phosphatase activity is increased in a rat model of long-term beta-adrenergic stimulation. *Naunyn Schmiedebergs Arch pharmacol* 2000, 262: 222-231.
218. MacDougall L.K, Jones L.R, and Cohen P. Identification of the major protein phosphatases in mammalian cardiac muscle which dephosphorylate phospholamban. *J Eur Biochem* 1991, 196: 725–734.
219. Carr A.N, Schmidt A.G, Suzuki Y, del Monte F, Sato Y, Lanner C, Breeden K, Jing S.L, Allen P.B, Greengard P, Yatani A, Hoit B.D, Grupp I.L, Hajjar R.J, DePaoli-Roach A.A, and Kranias E.G. Type 1 phosphatase, a negative regulator of cardiac function. *Mol Cell Biol* 2002, 22: 4124–4135.
220. Mishra S, Gupta R.C, Tiwari N, Sharov V.G, and Sabbah H.N. Molecular mechanisms of reduced sarcoplasmic reticulum Ca(2+) uptake in human failing left ventricular myocardium. *J Heart Lung Transplant* 2002, 21: 366–373.
221. Neumann J. Altered phosphatase activity in heart failure, influence on Ca<sup>2+</sup> movement. *Basic Res Cardiol* 2002, 97: 191-5.
222. Sorrentino V, Barone V and Rossi D. Intracellular Ca(2+) release channels in evolution. *Curr Opin Genet Dev* 2000, 10: 662–667.

223. Marx S.O, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosembliit N and Marks A.R. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* 2000, 101: 365–376.
224. McAvoy T, Allen P.B, Obaishi H, Nakanishi H, Takai Y, Greengard P, Nairn A.C and Hemmings H.C Jr. Regulation of neurabin I interaction with protein phosphatase 1 by phosphorylation. *Biochemistry* 1999, 38: 12943–12949.
225. Oliver C.J, Terry-Lorenzo R.T, Elliott E, Bloomer W.A, Li S, Brautigan D.L, Colbran R.J and Shenolikar S. Targetting protein phosphatase 1 (PP1) to the actin cytoskeleton: the neurabin I/PP1 complex regulates cell morphology. *Mol Cell Biol* 2002, 22: 4690–4701.
226. Dickson E.W, D.J Blehar, M Lorbar et al. Cardioprotective factor released from rabbit heart during brief preconditioning ischemia is captured by a hydrophobic matrix. *Circulation* 2000, 102: 27.
227. Ceulemans H, Bollen M. Functional Diversity of Protein Phosphatase-1, a Cellular Economizer and Reset Button. *Physiol Rev* 2004, 84: 1-39.
228. Fernandez A, Brautigan D.L, Mumby M and Lamb N.J. Protein phosphatase type-1, not type-2A, modulates actin microfilament integrity and myosin light chain phosphorylation in living non-muscle cells. *J Cell Biol* 1990, 111: 103–112.
229. Alessi D, MacDougall L.K, Sola M.M, Ikebe M and Cohen P. The control of protein phosphatase-1 by targetting subunits. The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. *J Eur Biochem* 1992, 210: 1023–1035.
230. Moorhead G, Johnson D, Morrice N and Cohen P. The major myosin phosphatase in skeletal muscle is a complex between the beta-isoform of protein phosphatase 1 and the MYPT2 gene product. *FEBS Lett* 1998, 438: 141–144.
231. Tan I, Ng C.H, Lim L and Leung T. Phosphorylation of a novel myosin binding subunit of protein phosphatase 1 reveals a conserved mechanism in the regulation of actin cytoskeleton. *J Biol Chem* 2001, 276: 21209–21216.
232. Arimura T, Suematsu N, Zhou Y.B, Nishimura J, Satoh S, Takeshita A, Kanaide H and Kimura A. Identification, characterization, and functional analysis of heart-specific myosin light chain phosphatase small subunit. *J Biol Chem* 2001, 276: 6073–6082
233. Shirazi A, Iizuka K, Fadden P, Mosse C, Somlyo A.P, Somlyo A.V and Haystead T.A. Purification and characterization of the mammalian myosin light chain phosphatase holoenzyme. The differential effects of the holoenzyme and its subunits on smooth muscle. *J Biol Chem* 1994, 269: 31598–31606.
234. Johnson D, Cohen P, Chen M.X, Chen Y.H, and Cohen P.T. Identification of the regions on the M110 subunit of protein phosphatase 1M that interact with the M21 subunit and with myosin. *J Eur Biochem* 1997, 244: 931–939.
235. Johnson D.F, Moorhead G, Caudwell F.B, Cohen P, Chen Y.H, Chen M.X and Cohen P.T. Identification of protein-phosphatase-1-binding domains on the glycogen and myofibrillar targetting subunits. *J Eur Biochem* 1996, 239: 317–325.
236. Ingebritsen T.S, Stewart A.A, Cohen P. The protein phosphatases involved in cellular regulation 6. Measurement of type-1 and type-2 protein phosphatases in extracts of mammalian tissues; an assessment of their physiological roles. *J Eur Biochem* 1983, 132(2): 297-307.
237. Braconi Quintaje S.B, Church D.J, Rebsamen M, Valloton M.B, Hemmings B.A, Lang U. Role of protein phosphatase 2A in the regulation of mitogen-activated protein kinase activity in ventricular cardiomyocytes. *Biochem Biophys Res Commun* 1996, 221(3):

238. Armstrong S.C, Gao W, Lane J.R, and Ganote C.E. Protein phosphatase inhibitors calyculin A and fostriecin protect rabbit cardiomyocytes in late ischemia. *J Mol Cell Cardiol* 1998, 30: 61-73.
239. Weinbrenner C, C.P Baines, G.S Liu, S.C Armstrong, C.E Ganote, A.H Walsh, R.E Honkanen, M.V Cohen and J.M Downey. Fostriecin, an inhibitor of protein phosphatase 2A, limits myocardial infarct size even when administered after onset of ischemia. *Circulation* 1998, 98: 899–905. 239A Weinbrenner C, Liu G.S, Cohen M.V, Downey J.M. Phosphorylation of tyrosine 182 of p38 mitogen-activated protein kinase correlates with the protection of preconditioning in the rabbit heart. *J Mol Cell Cardiol* 1997, 29(9): 2383-91.
240. Armstrong S.C, Kao R, Gao W, Shivell L.C, Downey J.M, Honkanen R.E and Ganote C.E. Comparison of in vitro preconditioning responses of isolated pig and rabbit cardiomyocytes: effects of a protein phosphatase inhibitor, fostriecin. *J Mol Cell Cardiol* 1997, 29: 3009-3024.
241. Mackay K, Mochly-Rosen D. Involvement of a p38 mitogen-activated protein kinase phosphatase in protecting neonatal rat cardiac myocytes from ischemia. *J Mol Cell Cardiol*. 2000, 32(8): 1585-8.
242. Marais E, Genade S, Huisamen B, Strijdom J.G, Moolman J.A, Lochner A. Activation of p38 MAPK induced by a multi-cycle ischaemic preconditioning protocol is associated with attenuated p38 MAPK activity during sustained ischaemia and reperfusion. *J Mol Cell Cardiol* 2001, 33(4): 769-78.
243. Marais E, Genade S, Strijdom H, Moolman J.A, Lochner A. p38 MAPK activation triggers pharmacologically-induced beta-adrenergic preconditioning, but not ischaemic preconditioning. *J Mol Cell Cardiol* 2001, 33(12): 2157-77.
244. Barancik M, P Htun and W Shaper. Okadaic acid and anisomycin are protective and stimulate the SAPK/JNK pathway. *J Cardiovascular Pharmacology* 1999, 34: 182–190.
245. Armstrong S.C, D.B Hoover, M.H Delacey and C.E Ganote. Translocation of PKC, protein phosphatase inhibition and preconditioning of rabbit cardiomyocytes. *J Molecular and Cellular Cardiology* 1996, 28: 1479–1492.
246. Lochner A, Marais E, Genade S et al. Myocardial ischaemia and preconditioning. Edit N S Dhalla et al. Kluwer Academic publishers USA 2002, 249-273.
247. Cano E, Doza Y.N, Ben-Levy R, Cohen P, Mahadevan L.C. Identification of anisomycin-activated kinases p45 and p55 in murine cells as MAPKAP kinase-2. *Oncogene* 1996,12(4): 805-12.
248. Sato M, Cordis G.A, Maulik N, Das D.K. SAPKs regulation of ischemic preconditioning. *J Am Physiol Heart Circ Physiol* 2000, 279(3): 901-7.
249. Baines C.P, Wang L, Cohen M.V, Downey J.M. Protein tyrosine kinase is downstream of protein kinase C for ischemic preconditioning's anti-infarct effect in the rabbit heart. *J Mol Cell Cardiol* 1998, 30(2): 383-92.
250. Ladilov Y.V, H Maxeiner, C Wolf, C Schäfer, K Meuter and H.M Piper. Role of protein phosphatases in hypoxic preconditioning. *J Am Physiol Heart Circ Physiol* 2002, 283(3): 1092-1098.
251. Opie L.H. *The Heart*. Lippincott-Raven Publishers 1998, Third edition: 522.
252. Ladilov YV, Balsler-Schafer C, Haffner S, Maxeiner H and Piper HM. Pretreatment with PKC activator protects cardiomyocytes against reoxygenation-induced hypercontracture independently of Ca<sup>2+</sup> overload. *Cardiovasc Res* 1999, 43: 408-416.

253. Piper HM, Probst I, Schwartz P, Hütter F.J and Spieckermann P.G. Culturing of calcium stable adult cardiac myocytes. *J Mol Cell Cardiol* 1982, 14: 397-412.
254. Vahlhaus, C, Schulz R, Post H, Onallah R and Heusch G. No prevention of ischemic preconditioning by the protein kinase C inhibitor staurosporine in swine. *Circ Res* 1996,79: 407-414.
255. Przyklenk K and Kloner R.A Ischemic preconditioning: exploring the paradox. *Prog Cardiovasc Dis* 1998, 40: 517-547.
256. Fryer R.M, Schultz J.E, Hsu A.K and Gross G.J. Importance of PKC and tyrosine kinase in single or multiple cycles of preconditioning in rat hearts. *J Am Physiol* 1999, 276: 1229-1235.
257. Simons T.J. Vanadate: a new tool for biologists. *Nature* 1979, 281: 337-338.
258. Palmer G, Bonjour J.P and Caverzasio J. Stimulation of inorganic phosphate transport by insulin-like growth factor I and vanadate in opossum kidney cells is mediated by distinct protein tyrosine phosphorylation processes. *Endocrinology* 1996, 137: 4699-4705.
259. Liem D.A, C.C Gho, B.C Gho, S Kazim, O.C Manintveld, P.D Verdouw and D.J Duncker. The Tyrosine Phosphatase Inhibitor Bis(Maltolato)Oxovanadium Attenuates Myocardial Reperfusion Injury by Opening ATP-Sensitive Potassium Channels. *J Pharmacology And Experimental Therapeutics Fast Forward* 2004, JPETb309: 1256-1262.
260. Kannengieser G.J, L.H Opie and T.J van der Werff. Impaired cardiac work and oxygen uptake after reperfusion of regionally ischaemic myocardium. *J Mol Cell Cardiol* 1979, 11: 197-207. 260A Marais E, Genade S, Salie R, Huisamen B, Maritz S, Moolman J.A, Lochner A. The temporal relationship between p38 MAPK and HSP27 activation in ischaemic and pharmacological preconditioning. *Basic Res Cardiol* 2005,100(1): 35-47.
261. Bradford M.M. A rapid sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, 71: 248-254.
262. Jonassen A.K, M.N Sack and O.D Mjos et al. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 2001, 89: 1191.
263. Eefting F, B Rensing and J Wigman et al. Role of apoptosis in reperfusion injury, *Cardiovasc Res* 2004, 61: 414.
264. Hausenloy D.J, M.M Mocanu and D.M Yellon. Activation of the pro-survival kinase cascades (PI3 kinase-Akt-p70S6K kinase and ERK 1/2-p70S6K kinase) at reperfusion are essential for preconditioning-induced protection. *Circulation* 2003,108: I-288.
265. Phillis J.W, D Song and M.H O'Regan. Inhibition of tyrosine phosphorylation attenuates amino acid neurotransmitter release from the ischemic/reperfused rat cerebral cortex. *Neurosci Lett* 1996, 207: 151.
266. Clerk K, S.J Fuller and A Michael et al. Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. *J Biol Chem* 1998, 273: 7228.
267. Li Y and T Sato. Dual signaling via protein kinase C and phosphatidylinositol 3'-kinase/Akt contributes to bradykinin B2 receptor-induced cardioprotection in guinea pig hearts. *J Mol Cell Cardiol* 2001, 33: 2047.
268. Cohen P and Cohen P.T. Protein phosphatases come of age. *J Biol Chem* 1989, 264: 21435-21438.
269. Ruvolo P.P, X Deng, T Ito, B.K Carr and S May. Ceramide induces Bcl2 dephosphorylation

- via a mechanism involving mitochondrial PP2. *J Biol Chem* 1999, 274: 20296–20300.
270. Neumann J, Herzig S, Boknik P et al. On the cardiac contractile, biochemical and electrophysiological effects of cantharidin, a phosphatase inhibitor. *J Pharmacol Exp Ther* 1995, 274: 530-539.
271. Linck B, Boknik P, Knapp J et al. Effects of cantharidin on force of contraction and phosphatase activity in nonfailing and failing human hearts. *Br J Pharmacol* 1996, 119: 545-550.
272. Li Y.M and Casida J.E. Cantharidin-binding protein: identification as protein phosphatase 2A. *Proc Natl Acad Sci U S A* 1992, 89: 11867-11870.
273. Honkanen R.E. Cantharidin, another natural toxin that inhibits the activity of serine/threonine protein phosphatases types 1 and 2A. *FEBS Lett* 1993, 330: 283-286.
274. Neumann J, Herzig S, Boknik P, Apel M, Kaspereit G, Schmitz W, Scholz H, Tepel M and Zimmermann N. On the cardiac contractile, biochemical and electrophysiological effects of cantharidin, a phosphatase inhibitor. *J Pharmacol Exp Ther* 1995, 274: 530-539.
275. Erdodi F, Toth B, Hirano K, Hirano M, Hartshorne D.J and Gergely P. Endothal thioanhydride inhibits protein phosphatases-1 and -2A in vivo. *J Am Physiol Cell Physiol* 1995, 269: 1176-1184.
276. Michelle M. Gehringer Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response 2004. *FEBS letters* Volume 557, 1-3: 1-8
277. Morana S, C.M Wolf, J. Li, J.E Reynolds, M.K Brown and A Eastman. The involvement of protein phosphatases in the activation of ICE/CED-3 protease, intracellular acidification, DNA digestion, and apoptosis. *J Biol Chem* 1996, 271: 18263–18271.
278. Wolf C.M and A Eastman. The temporal relationship between protein phosphatase, mitochondrial cytochrome c release and caspase activation in apoptosis. *Exp Cell Res* 1999, 247: 505–513.
279. Kennedy N.J and R.C Budd. Phosphorylation of FADD/MORT1 and Fas by kinases that associate with the membrane–proximal cytoplasmic domain of Fas. *J Immunol* 1998, 160: 4881–4888.
280. Hori M, J Magae, Y.G Han, D.J Hartshorne and H Karaki. A novel protein phosphatase inhibitor, tautomycin. Effect on smooth muscle. *FEBS Lett* 1991, 285: 145–148.
281. Lochner A, Genade S, Moolman J.A. Ischemic preconditioning: infarct size is a more reliable endpoint than functional recovery. *Basic Res Cardiol.* 2003, 98(5): 337-46
282. Cross T.G, D Scheel-Toellner, N.V Henriquez, E Deacon, M Salmon and J.M Lord. Serine/threonine protein kinases and apoptosis. *Exp Cell Res* 2000, 256(1): 34–41.
283. Millward T.A, Zolnierowicz S, Hemmings B.A. Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem Sci* 1999, 24: 186-191.
284. Garrington T P, Johnson G L. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol* 1999, 11: 211–218.
285. Lewis T S, Shapiro P S, Ahn N G. Signal transduction through MAP kinase cascades. *Adv Cancer Res.* 1998, 74: 49–139.
286. Cicconi S, Ventura N, Pastore D, Bonini P, Di Nardo P, Lauro R and Marlier LN. Characterization of apoptosis signal transduction pathways in HL-5 cardiomyocytes exposed to ischemia/reperfusion oxidative stress. *J Cell Physiol* 2003, 195: 27–37.
287. Iwakura A, Fujita M, Hasegawa K, Toyokuni S, Sawamura T, Nohara R, Sasayama S and Komeda M. Pericardial fluid from patients with ischemic heart disease induces myocardial

- cell apoptosis via an oxidant stress-sensitive p38 mitogen-activated protein kinase pathway. *J Mol Cell Cardiol* 2001, 33: 419–430.
288. Ma X.L, Kumar S, Gao F, Loudon C.S, Lopez B.L, Christopher T.A, Wang C, Lee J.C, Feuerstein G.Z and Yue T.L. Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. *Circulation* 1999, 99: 1685–1691.
289. Xia Z, Dickens M, Raingeaud J, Davis R.J, and Greenberg M.E. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995, 270: 1326–1331.
290. Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Tanaka M, Shiojima I, Hiroi Y and Yazaki Y. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. *J Clin Invest* 1997, 100: 1813–1821.
291. Bueno O.F, De Windt L.J, Tymitz K.M, Witt S.A, Kimball T.R, Klevitsky R, Hewett T.E, Jones S.P, Lefer D.J, Peng C.F, Kitsis R.N and Molkentin J.D. The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *J EMBO* 2000, 19: 6341–6350.
292. Silverstein A.M, Barrow C.A, Davis A.J, Mumby M.C. Actions of PP2A on the MAP kinase pathway and apoptosis are mediated by distinct regulatory subunits. *Proc Natl Acad Sci USA* 2002, 99: 4221–4226.
293. Virshup D.M. Protein phosphatase 2A: a panoply of enzymes. *Curr Opin Cell Biol* 2000, 12: 180–185.
294. Frost J.A, Alberts A.S, Sontag E, Guan K, Mumby M.C, Feramisco J.R. Simian virus 40 small t antigen cooperates with mitogen-activated kinases to stimulate AP-1 activity. *Mol Cell Biol* 1994, 14: 6244–6252.
295. Steenbergen C. The role of p38 mitogen-activated protein kinase in myocardial ischemia/reperfusion injury; relationship to ischemic preconditioning. *Basic Res Cardiol* 2002, 97(4): 276–85
296. Armstrong S.C, Ganote C.E. Effects of the protein phosphatase inhibitors okadaic acid and calyculin A on metabolically inhibited and ischaemic isolated myocytes. *J Mol Cell Cardiol.* 1992, 24(8): 869–84.
297. Wu J.J, Bennett A.M. Essential role for mitogen-activated protein (MAP) kinase phosphatase-1 in stress responsive MAP kinase and cell survival signalling. *J Biol Chem* 2005, 280: 16461–16466.
298. Engelbrecht A.M, Engelbrecht P, Genade S, Niesler C, Page C, Smuts M, Lochner A. Long-chain polyunsaturated fatty acids protect the heart against ischemia/reperfusion-induced injury via a MAPK dependent pathway. *J Mol Cell Cardiol.* 2005, 39(6): 940–54.
299. Lasa M, Abraham S.M, Boucheron C, Saklatvala J, Clark A.R. Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MPAK p38. *Mol Cell Biol* 2002, 22: 7802–7811.
300. Kassel O, A Sancono, J Kratzschmar, B Kreft, M Stassen and A.C Cato. Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *J EMBO* 2001, 20: 7108–7116.
301. Lefer A.M, Crossley K, Grigoris G, Lefer D.J. Mechanism of the beneficial effect of dexamethasone on myocardial cell integrity in acute myocardial ischemia. *Basic Res Cardiol* 1980, 75(2): 328–39.
302. Bernauer W. Inhibiting effect of dexamethasone on evolving myocardial necrosis in

- coronary-ligated rats, with and without reperfusion. *Pharmacology* 1985, 31(6): 328-36.
303. Koltai M, Leprán I, Nemezc G, Szekeres L. The possible mechanism of protection induced by dexamethasone against sudden death due to coronary ligation in conscious rats. *J Br Pharmacol* 1983, 79(2): 327-9.
304. Valen G, T Kawakami, P Tahepold, A Dumitrescu, C Lowbeer and J Vaage. Glucocorticoid pretreatment protects cardiac function and induces cardiac heat shock protein 72. *J Am Physiology Heart and Circulatory Physiology* 2000, 279 (2): 836–843.
305. Juhasz B, P Der, T Turoczi, I Bacskey, E Varga and A Tosaki. Preconditioning in intact and previously diseased myocardium: laboratory or clinical dilemma? *Antioxidants and Redox Signaling* 2004, 6 (2): 325–333.
306. Engelman R.M, M.R Prasad, J.A Rousou, R.H Breyer, M Bagchi and D.K Das. Steroid-induced myocardial preservation is associated with decreased cell membrane microviscosity. *Circulation* 1989, 80 (5): III36–III43.
307. Spanier A.J and K.H McDonough. Dexamethasone blocks sepsis-induced protection of the heart from ischemia reperfusion injury. *Proceedings of the Society for Experimental Biology and Medicine* 2000, 223 (1): 82–87.
308. Varga E, Nagy N, Lazar J, Czifra G, Bak I, Biro T, Tosaki A. Inhibition of ischemia/reperfusion-induced damage by dexamethasone in isolated working rat hearts: the role of cytochrome c release. *Life Sci.* 2004, 75(20): 2411-23.
309. Dumont A, Hehner S.P, Schmitz M.L, Gustafsson J.A, Lidén J, Okret S, van der Saag P.T, Wissink S, van der Burg B, Herrlich P, Haegeman G, De Bosscher K, Fiers W. Cross-talk between steroids and NF-kappa B: what language?. *Trends in Biochemical Sciences* 1998, 23 (7): 233–235.
310. Valen G. The basic biology of apoptosis and its implications for cardiac function and viability. *Annals of Thoracic Surgery* 2003, 75 (2): 656–660.
311. Hofmann T.G, S.P Hehner, S Bacher, W Droge and M.L Schmitz. Various glucocorticoids differ in their ability to induce gene expression, apoptosis and to repress NF-κB-dependent transcription. *FEBS Letters* 1998, 441 (3): 441–446.
312. Mikosz C.A, D.R Brickley, M.S Sharkey, T.W Moran and S.D Conzen. Glucocorticoid receptor-mediated protection from apoptosis is associated with induction of the serine/threonine survival kinase gene, *sgk-1*. *J Biological Chemistry* 2001, 276 (20): 16649–16654.
313. Srikanth S, Franklin C.C, Duke R.C, Kraft R.S. Human DU145 prostate cancer cells overexpressing mitogen-activated protein kinase phosphatase-1 are resistant to Fas ligand-induced mitochondrial perturbations and cellular apoptosis. *Mol Cell Biochem* 1999, 199 (1-2): 169-78.
314. Magi-Galluzzi C, Mishra R, Fiorentino M, Montironi R, Yao H, Capodiceci P, Wishnow K, Kaplan I, Stork P.J, Loda M. Mitogen-activated protein kinase phosphatase 1 is overexpressed in prostate cancers and is inversely related to apoptosis. *Lab Invest.* 1997, 76 (1): 37-51.
315. Duff J.L, Marrero M.B, Paxton W.G, Charles C.H, Lau L.F, Bernstein K.E, Berk B.C. Angiotensin II induces 3CH134, a protein-tyrosine phosphatase, in vascular smooth muscle cells. *J Biol Chem* 1993, 268 (35): 26037-40.
316. Guyton K.Z, Liu Y, Gorospe M, Xu Q, Holbrook N.J. Activation of mitogen-activated protein kinase by H<sub>2</sub>O<sub>2</sub>. Role in cell survival following oxidant injury. *J Biol Chem* 1996, 271(8): 4138-42.

317. Metzler B, Hu Y, Sturm G, Wick G, Xu Q. Induction of mitogen-activated protein kinase phosphatase-1 by arachidonic acid in vascular smooth muscle cells. *J Biol Chem* 1998, 273 (50): 33320-6.
318. Bokemeyer D, Lindemann M, Kramer H.J. Regulation of mitogen-activated protein kinase phosphatase-1 in vascular smooth muscle cells. *Hypertension* 1998, 32 (4): 661-7.
319. Charles C.H, H Sun, L.F Lau and N.K Tonks. The growth factor-inducible immediate-early gene 3CH134 encodes a protein-tyrosine-phosphatase. *Proc Natl Acad Sci USA* 1993, 90: 5292-5296.
320. Laderoute K. R, H.L Mendonca, J.M Calaoagan, A.M Knapp, A.J Giaccia and P.J Stork. Mitogen-activated protein kinase phosphatase-1 (MKP-1) expression is induced by low oxygen conditions found in solid tumor microenvironments. A candidate MKP for the inactivation of hypoxia-inducible stress-activated protein kinase/c-Jun N-terminal protein kinase activity. *J Biol Chem* 1999, 274: 12890-12897.
321. Sun H, C.H Charles, L.F Lau and N.K Tonks. MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. *Cell* 1993, 75: 487-493.
322. Dorfman K, D Carrasco, M Gruda, C Ryan, S.A Lira and R Bravo. Disruption of the *erp/mkp-1* gene does not affect mouse development: normal MAP kinase activity in ERP/MKP-1-deficient fibroblasts. *Oncogene* 1996, 13: 925-931.
323. Zhao Q, Shepherd E.G, Manson M.E, Nelin L.D, Sorokin A, Liu Y. The role of mitogen-activated protein kinase phosphatase-1 in the response of alveolar macrophages to lipopolysaccharide: attenuation of proinflammatory cytokine biosynthesis via feedback control of p38. *J Biol Chem* 2005, 280 (9): 8101-8.
324. Caelles C, González-Sancho J.M and Muñoz A. Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes Dev* 1997, 11: 3351-3364.
325. Swantek J.L, Cobb M.H and Geppert T.D. Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) translation: glucocorticoids inhibit TNF- $\alpha$  translation by blocking JNK/SAPK. *Mol Cell Biol* 1997, 17: 6274-6282.
326. Hirasawa N, Sato Y, Fujita Y, Mue S and Ohuchi K. Inhibition by dexamethasone of antigen-induced c-Jun N-terminal kinase activation in rat basophilic leukemia cells. *J Immunol* 1998, 161: 4939-4943.
327. Lasa M, Brook M, Saklatvala J and Clark A.R. Dexamethasone destabilizes cyclooxygenase 2 mRNA by inhibiting mitogen-activated protein kinase p38. *Mol Cell Biol* 2001, 21: 771-780.
328. Gonzalez M V, Gonzalez-Sancho J M, Caelles C, Munoz A, Jimenez B. Hormone-activated nuclear receptors inhibit the stimulation of the JNK and ERK signalling pathways in endothelial cells. *FEBS Lett* 1999, 459: 272-276.
329. Shalom-Barak T, Quach J, Lotz M. Interleukin-17-induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-kappaB. *J Biol Chem* 1998, 273: 27467-27473.
330. Zhang J P, Wong C K, Lam C W. Role of caspases in dexamethasone-induced apoptosis and activation of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase in human eosinophils. *Clin Exp Immunol* 2000, 122: 20-27.