The mechanism of pharmacological preconditioning of rat myocardium with beta-adrenergic agonists

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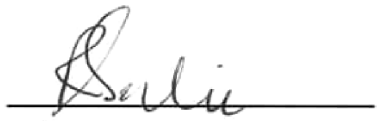
Declaration

I, the undersigned, hereby declare that this study project is my own original work and that all sources have been accurately reported and acknowledge, and that this document has not been previously in its entirety or in part submitted at any university in order to obtain an academic qualification.



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Abstract

The Mechanism of β-adrenergic preconditioning (β-PC)

Ischaemic preconditioning (IPC), a potent endogenous protective intervention against myocardial ischaemia, is induced by exposure of the heart to repetitive short episodes of ischaemia and reperfusion. The protective effects of this phenomenon have been demonstrated to be mediated by release of autocoids such as adenosine, opioids and bradykinin. Release of endogenous catecholamines and activation of the beta-adrenergic receptors (β-AR) have also been shown to be involved in ischaemic preconditioning. However, the exact mechanism whereby activation of the β-adrenergic signal transduction pathway leads to cardioprotection, is still unknown.

In view of the above, the aims of the present study were to evaluate:

(i) the respective roles of the β1-, β2- and β3-AR receptors as well as the contribution of Gi protein and PKA to β-adrenergic preconditioning,

(ii) the role of the prosurvival kinases, PKB/Akt and ERK 44/p42 MAPKinase in β-drenergic preconditioning,

(iii) whether β-AR stimulation protect via ischaemia and the formation of adenosine; the respective roles of the A_1-, A_2-, A_3-adenosine receptors as well as the involvement of the PI3-K/PKB/Akt and ERKp44/p42 signal transduction pathways, in the cardioprotective phenomemon of β-adrenergic preconditioning and

(iv) the contribution of the mitochondrial K_{ATP} channels (mK_{ATP}), reactive oxygen species and NO to the mechanism of β-AR-induced cardioprotection.

Methods: Isolated perfused rat hearts were subjected to 35 min regional ischaemia (RI) and reperfusion. Infarct size (IS) was determined using tetrazolium staining (TTC) and data were analyzed with ANOVA. Hearts were preconditioned with 5 min isoproterenol 0.1 μM (β1/β2-AR agonist), or formoterol 1 nM (β2-AR agonist) or BRL 37344 1 μM (β3-AR agonist) followed by 5 min reperfusion. The roles of the β1-, β2- and β3-ARs as well as NO were explored by using the selective antagonists CGP-20712A (300 nM), ICI -18551 (50 nM), SR59230A (100 nM) and NOS inhibitors L-NAME (50 μM) or LNNA (50 μM) respectively. Involvement of ROS and the mK^+_{ATP} channels was studied by administration of N-acetyl cysteine (NAC, 300 μM) and the $mitK^+_{ATP}$

channel blocker 5-HD (100 μM) during the triggering phase. The role of PKA and PI3-K/Akt was investigated by the administration of the blockers Rp-8-CPT-cAMPs (16 μM) and wortmannin (100 nM) respectively, prior to RI or at the onset of reperfusion. Pertussis toxin (PTX), 30 μg kg^{-1} was administered i.p., 48 h prior to experimentation.

The role of adenosine and the adenosine A_1, A_3, A_{2A} and A_{2B} receptors was studied by using adenosine deaminase and the selective antagonists DPCPX (1 μM), MRS 1191(1 μM), ZM241385 (1 μM) and MRS1754 (1 μM). Activation of PKB/Akt and ERKp44/p42 was determined by Western blot.

Results: Infarct sizes of hearts preconditioned with isoproterenol of formoterol were significantly smaller compared to those of non-preconditioned hearts. This was associated with an improvement in postischaemic mechanical performance. However the β_3-AR agonist BRL37344 could not reduce infarct size. The β_1- and β_2-AR blockers CGP-20712A and ICI-118551 completely abolished the isoproterenol-induced reduction in infarct size and improvement in mechanical recovery, while the β3-AR blocker was without effect.

Both Rp-8-CPT-cAMPs and wortmannin significantly increased infarct size when administered before β1/β2-AR preconditioning or at the onset of reperfusion while it reduced mechanical recovery during reperfusion. PTX pretreatment had no significant effect on the reduction in infarct size induced by β1/β2-AR or β2-AR preconditioning, however it reduced mechanical recovery in the latter. The NOS inhibitors had no effect on the reduction in infarct size induced by β1/β2-AR preconditioning, but depressed mechanical function during reperfusion.

The significant reduction in infarct size by β1/β2-PC, was associated with activation of ERKp44/p42 and PKB/Akt during the triggering phase, as well as during reperfusion. DPCPX (A_1-AdoR antagonist) had no effect on the β1/β2-PC-induced reduced infarct size or ERK p44/p42 and PKB activation.

A_{2A}-AdoR, but not A_{2b}-AdoR, blockade during the trigger phase abolished the reduction in infarct size of β1/β2-PC. Both antagonists significantly reduced ERK and PKB activation in the trigger phase. In addition, when applied at the onset of reperfusion they significantly reduced ERK p44 /

p42 MAPK and PKB/Akt activation to an even greater extent. MRS-1191 (A_3-AdoR antagonist) blocked β1/β2-PC when applied prior to index ischaemia or when added during early reperfusion, significantly inhibiting both ERK p44 and PKB activation.

Cardioprotection of β1/β2-PC was abolished by inhibition of ROS generation with NAC in the triggering phase as well as at the start of reperfusion. However, the mitoK^+_{ATP} channel blocker 5-HD was without effect.

Conclusions: Protection afforded by an acute transient stimulation of the β-ARs, depends on the activation of both β_1-AR and β_2-ARs but not the β_3-AR. PKA as well as PI3-K activation prior to sustained ischemia and at the onset of reperfusion were essential for cardioprotection. With functional recovery as endpoint, it appears that NO is involved in β1/β2-AR preconditioning, while the Gi protein may play a role in β_2-AR preconditioning.

The production of endogenous adenosine induced by transient β1/β2 stimulation of the isolated rat heart is involved in β−AR preconditioning. Cardioprotection was shown not to be dependent on the A_1AdoR while activation of the A_3-AdoR occurs during both the triggering and mediation phases. Both the adenosine A_{2A} and, to a lesser extent, the adenosine A_{2B} receptors participate in the triggering phase of β1/β2-PC. Generation of ROS during the triggering and reperfusion phases is involved in eliciting protection, but no role for the mK_{ATP} channels could be demonstrated. Finally, activation of the RISK pathway (PKB/Akt and ERKp44/p42) during the triggering phase is a prerequisite for protection. In addition, cardioprotection by β-AR is characterized by activation of the RISK pathway during reperfusion.

Uittreksel

Die Meganisme van β-adrenerge prekondisionering (β-PC)

Iskemiese prekondisionering (IPC) is 'n kragtige endogene beskerming teen miokardiale iskemie, wat deur blootstelling van die hart aan kort opeenvolgende episodes van iskemie en herperfusie, ontlok word. Hierdie beskerming word medieer deur vrystelling van outakoïede soos adenosine, opioïede en bradikinien. Vrystelling van endogene katekolamiene en aktivering van die beta-adrenerge reseptore (β-AR) is bewys om ook by hierdie proses betrokke te wees. Die presiese meganismes waardeur aktivering van die β-adrenerge seintransduksiepad tot miokardiale beskerming lei, is nog onbekend.

In die lig van bogenoemde, was die doel van die huidige studie om die volgende te evalueer: (i) die onderskeie rolle van die β1-, β2- en β3-AR sowel as die bydrae van die Gi proteïen en PKA in β-adrenerge prekondisionering, (ii) of β-AR stimulasie beskerming ontlok via iskemie en vorming van adenosien, die onderskeie rolle van die A_1-, A_2-, A_3-adenosien reseptore (AdoRs) sowel as die PI3-K/PKB/Akt en ERKp44/p42 seintransduksie paaie, (iv) die mitochondriale K_{ATP} (mK_{ATP}) kanale, vry suurstof radikale en NO in β−AR prekondisionering.

Metodes: Geïsoleerde, geperfuseerde rotharte is aan 35 minute streeksiskemie en herperfusie onderwerp. Infarktgrootte (IS) is deur die tetrazolium (TTC)-kleuringsmetode bepaal. Data is met behulp van ANOVA analiseer. Harte is geprekondisioneer vir 5 min met isoproterenol 0.1 μM (β1/β2-AR agonist), of formoterol 1 nM (β2-AR agonist) of BRL 37344 1 μM (β3-AR agonist), gevolg deur 5 min herperfusie, voor streeksiskemie. Die belang van die β1-, β2- en β3-ARs sowel as NO is ondersoek, deur onderskeidelik gebruik te maak van selektiewe antagoniste nl CGP-20712A (300 nM), ICI -18551 (50 nM), SR59230A (100 nM) en NOS inhibitore L-NAME (50μM) of LNNA (50μM). Die rol van die mK^+_{ATP} kanale en ROS is bepaal deur die toediening van die mK^+_{ATP} kanaal blokker 5-HD (100 μM) en die vrye-radikaal opruimer, N-asetiel cysteine (NAC, 300 μM). Die belang van PKA en PI3-K/Akt is bepaal deur toediening van die PKA blokker Rp-8-CPT-cAMPs (16μM) en wortmannin (100nM) respektiewelik. Pertussis toxin (PTX), 30 μg kg^{-1} is i.p toegedien, 48 uur voor eksperimentasie.

Die rol van adenosien en die adenosien A_1, A_{2A}, A_{2B} en A_3 reseptore is bestudeer, deur gebruik te maak van adenosien deaminase en die selektiewe antagoniste DPCPX (1 μM), MRS 1191(1 μM), ZM241385 (1 μM) and MRS1754 (1 μM),repektiewelik. Die middels is deurgaans toegedien tydens die prekondisioneringsprotokol ("snellerfase") of tydens vroeë herperfusie. Aktivering van PKB/Akt en ERK p44/p42 is deur Western blot analise bepaal.

Resultate: Infarktgrootte van harte wat geprekondisioneer is met of isoproterenol (β1/β2-PC) of formoterol (β2-PC), was beduidend kleiner as díe van ongeprekondisioneerde harte. Dit is geassosieer met 'n toename in postiskemiese meganiese herstel. Die β3-AR agonis BRL37344 (β3-PC) het egter geen effek op infarktgrootte gehad nie. Die selektiewe β1- en β2-AR blokkers CGP-20712A en ICI-118551 het die afname in infarktgrootte heeltemal opgehef, asook die verbetering in meganiese herstel tydens herperfusie terwyl die β3-AR blokker geen effek getoon het nie. Beide Rp-8-CPT-cAMPs en wortmannin het infarktgrootte beduidend vergroot en meganiese herstel beduidend verlaag, wanneer dit net voor β1/β2-prekondisionering of tydens die begin van herperfusie toegedien is. PTX voorafbehandeling het geen beduidende effek op die vermindering van infarktgrootte (geïnduseer deur β1/β2-PC of β2-PC) gehad nie. Meganiese herstel is egter verminder in die geval van β2-PC. Die NOS inhibitore het geen effek op die vermindering in infarktgrootte geïnduseer deur β1/β2 gehad nie, maar het ook meganiese herstel onderdruk.

Die beduidende afname in infarktgrootte deur β1/β2 prekondisionering is gekenmerk deur aktivering van ERKp42/p44 en PKB/Akt tydens die snellerfase. Soortgelyke aktivering van hierdie kinases is ook tydens herperfusie van β-AR geprekondisioneerde harte waargeneem.

DPCPX (A_1-AdoR antagonis) het geen effek op die infarkt-verminderde effek van β1/β2-prekondisionering of op ERK p44/p42 en PKB aktivering gehad nie. A_{2A}-AdoR, maar nie A_{2b} – AdoR, blokkade tydens die snellerfase, het die effek van β-AR prekondisionering op infarktgroottee opgehef. Beide antagoniste het die aktivering van ERKp42/p44 en PKB/Akt tydens die snellerfase onderdruk. Wanneer toegedien tydens herperfusie, het dit die aktivering van hierdie kinases tot 'n groter mate onderdruk. MRS-1191 (A_3-AdoR antagonis) het infarktgrootte beduidend verhoog en β1/β2-prekondisionering geblokkeer, beide wanneer dit voor indeks-iskemie toegedien is of tydens vroeë herperfusie, tesame met inhibisie van PKB en ERK p44/p44 aktivering.

Die kardiobeskerming van β1/β2-prekondisionering is opgehef deur middel van opruiming van vry suurstof radikale deur NAC in die snellerfase sowel as aan die begin van herperfusie. Die mK^+_{ATP} kanaal blokker 5-HD het geen effek op β-AR prekondisionering gehad nie.

Gevolgtrekking: Kardiobeskerming teweeggebring deur 'n kort periode van stimulasie van die β-ARs, is afhanklik van die aktivering van beide β1-AR en β2-ARs, maar nie β3-AR nie. PKA sowel as PI3-K aktivering, net voor volgehoue iskemie en tydens vroeë herperfusie, is aangedui om noodsaaklik vir β1/β2-AR prekondisionering te wees. Waar funksionele herstel as eindpunt gebruik is, blyk dit dat NO wel betrokke is by β1/β2-AR prekondisionering, terwyl die Gi protein 'n rol mag speel in β2-AR prekondisionering.

Vorming van endogene adenosien tydens β-adrenerge stimulasie is betrokke by β-AR prekondisionering. Hierdie beskerming is nie van die A_1-AdoR afhanklik nie, maar aktivering van die A_3-AdoR is nodig tydens beide die sneller en herperfusie fases. Beide die A_{2A}-AdoR, en tot 'n mindere mate die A_{2B}–AdoR, is ook betrokke by die snellerfase. Vorming van vry suurstof radikale is nodig vir β-AR prekondisionering, nterwyl die mK_{ATP} kanale nie betrokke is nie. Ten slotte, aktivering van die RISK seintransduksiepad (ERKp42/p44 en PKB/Akt) tydens die snellerfase is 'n voorvereiste vir die ontlokking van beskerming. Daarbenewens word β-AR prekondisionering gekarakteriseer deur aktivering van hierdie pad tydens herperfusie.

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Index

Chapter 5: The function of adenosine, its receptors (A_1, A_{2A}, A_{2B} and A_3) and downstream targets in the cardioprotective phenomemon of β-adrenergic preconditioning

Chapter 6: Investigation of the roles of the mitoK_{ATP} channel, reactive oxygen species (ROS) and nitric oxide in β-adrenergic preconditioning

List of Tables

List of Figures

Fig. 6.5: Cartoon showing the sequence of signaling events involved in triggering the preconditioned state as well as the cardioprotective strategy of β-PC prior to the ischemic insult and those that mediate protection in the first minutes of reperfusion

Chemicals, drugs and reagents

The following chemicals were purchased from Sigma-Aldrich, St Louis, MO, USA:

Isoproterenol (ISO);

β_1-AR antagonist (CGP-20712A) ((±)-2-Hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamidine methanesulfonate salt);

β_2- AR antagonist (ICI 118,551) ((±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride);

β_3-AR antagonist (SR 59230A) (3-(2-Ethylphenoxy)-1-[[(IS)-1,2,3,4-tetrahydronapth-1-yl]amino]-(2S)-2-propanol oxalate salt);

β_3-AR receptor agonist (BRL 37344) ((±)-(R,R)-[4-[2-[2-(3-Chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid sodium);

Pertussis toxin (PTX);

Carbamycholine chloride (Carbachol);

L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride);

L-NNA (Nω-Nitro-L-arginine); 5-HD (5-hydroxy decanoate);

NAC (N-acetyl-cysteine);

Adenosine deaminase (ADA);

A_1-AdoR antagonist (DPCPX) (1,3-Dipropyl-8-cyclopentylxanthine);

A_{2B} -AdoR antagonist (MRS1754) (8-[4- [((4-Cyanophenyl) Carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)xanthine hydrate);

A_3 -AdoR antagonist (MRS 1191) (3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate);

Wortmannin and PD 98,059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one);

8-(4-Chlorophenylthio)adenosine-3',5'-cyclic Monophosphphorothioate, Rp-isomer sodium salt (Rp-8-CPT-cAMPS)

The following chemicals were purchased from Tocris Bioscience, Bristol, UK:

The A_{2A} –AdoR antagonist (ZM241385) (4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol) and the β_2-ARs agonist Formoterol Hemifumarate (formoternol)(N-[2-Hydroxy-5-[1-hydroxy-2-[[2-(4-methoxyyphenyl)-1-methylethyl]amino]ethyl]phenyl formamide hemifumarate

Antibodies were purchased from **Cell Signalling Technology (Boston, MA, USA)** and all other routine chemicals were **MERCK** (analar grade).

Abbreviation List

Units of measurement:

%	percentage
µl	microlitre
µg	microgram
ml	milliliter
g	gram
M	molar
Min	minute
H	hour
mM	millimole
µM	micromole

Chemical compounds:

Ca^{2+}	Calcium
CO_2	Calcium chloride
H_2O	water
K^+	Potassium
KCL	Potassium chloride
$MgSO_4$	Magnesium sulphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
O_2	Oxygen
Tris	tris(hydroxymethyl) aminomethane hydrochloride

Other abbreviations:

NPC	non precconditioning
IPC	ischaemic preconditioning
SWOP	second window of protection
HF	heart failure
β-PC	β-adrenergic preconditioning
Ado	adenosine
ADA	adenosine deaminase
ACs	adenylyl cyclases
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
PKA	protein kinase A
AKAPs	A kinase anchoring proteins
PKG	protein kinase G
PKC	protein kinase C
PI3-K	phosphoinositide 3-kinase
PKB/Akt	protein kinase B
MAPK	Mitogen-activated protein kinases
ERK	extracellular signal-regulated kinases
JNK	c-Jun amino-terminal kinases
p38MAPK	p38Mitogen-activated protein kinase
NOS	nitric oxide synthase
iNOS	inducible nitric oxide synthase
eNOS	endothelial nitric oxide synthase

NO	nitric oxide
COX-2	cyclooxygenase-2
ROS	Reactive Oxygen Species
HSP	heat shock protein
RISK	Reperfusion Induced Salvage Kinases
mitoK_{ATP} channels	mitochondrial K_{ATP} channels

Chapter 1

Introduction

Myocardial cell death due to ischaemia-reperfusion is a major cause of morbidity and mortality. It has been debated whether cardiomyocytes suffer irreversible injury primarily during ischaemia, which may be revealed at the start of reperfusion, or whether additional injury occurs during reperfusion (reperfusion injury). This point has important clinical implications, because if additional injury occurs on reperfusion, this would allow an opportunity to intervene with cardioprotective strategies at this time. It has become clear that the myocardial response to ischaemia-reperfusion can be manipulated to delay injury, which in turn has motivated intense study of the mechanisms of cardioprotection. It follows then that cardioprotection should be aimed at the prevention of perioperative infarction, fewer myocardial infarct-associated ventricular arrhythmias and less mortality.

A large number of studies have investigated the capability of cardioprotective drugs or strategies administered at the onset of reperfusion to reduce infarct size. Postconditioning, characterized by short cycles of reperfusion/ischaemia applied at the onset of reperfusion [Zhao et al., 2003], Na^+-H^+ exchange inhibitors [Karmazyn, 1988], activation of kinases [Hausenloy, Mocanu and Yellon, 2004], perfusion with erythropoietin [Hanlon et al., 2005], inhibitors of protein kinase C (PKC-δ) [Inagaki et al., 2003], inhibitors of the mitochondrial permeability transition pore (MPT) [Hausenloy, Duchen and Yellon, 2003], inhibition of glycogen synthase kinase (GSK)-3β [Gross, Hsu and Gross, 2004] and other interventions have been reported to protect the myocardium when administered at the time of reperfusion. However, from failed clinical trails [Bolli et al., 1988; Flaherty et al., 1994] it appears that the window of opportunity during reperfusion is very limited. Although protection can be initiated at reperfusion, injury also occurs during ischaemia, and the relative proportion of each event likely depends on the duration of ischaemia [Stephanou et al., 2001]. Thus, if cardioprotective strategies can be initiated before or during ischaemia, it is likely that they will enhance protection, especially with longer durations of ischaemia [Murphy, and Steenbergen, 2007].

Early attempts to salvage myocardium exposed to ischaemia-reperfusion have been intensely explored but results obtained in these studies have mostly been unsatisfactory and controversial. However, in 1986 it was discovered that the heart has an endogenous protective mechanism, the so-called phenomenon of ischaemic preconditioning (IPC) [Murry et al., 1986]. This can be defined as a phenomenon whereby exposure of the myocardium to one or more brief episodes of ischaemia and reperfusion markedly reduces tissue necrosis induced by a subsequent prolonged period of ischaemia. IPC was shown to exert a very powerful anti-infarct effect, reduce reperfusion arrhythmias [Shiki and Hearse, 1987], reduce energy metabolism during the early stages of ischaemia [Murry et al., 1990] and improve post-ischaemic developed tension [Cave and Hearse, 1992]. This discovery led to intensive research into the mechanism(s) and signaling pathways involved since it is believed that this could lead to the development of new cardioprotective strategies and drugs aimed at salvage of ischaemic tissues. Several comprehensive reviews on ischaemic preconditioning have appeared in recent years, thus only some of the major findings in this regard are summarized below.

IPC has been shown to reduce infarct size in all species tested including rats [Liu and Downey, 1992], rabbits [Liu et al., 1991], pigs [Vahlhaus et al., 1996], dogs [Przyklenk et al., 1995] and mice [Miller and Winkle, 1999]. It was also illustrated that recovery of function in isolated human atrial trabeculae after an extended period of hypoxia was greatly enhanced by earlier hypoxic preconditioning [Speechly-Dick et al., 1995].

Furthermore, a standard ischaemic preconditioning stimulus of one or more brief episodes of non-lethal ischaemia and reperfusion elicits a bi-phasic pattern of cardioprotection. The first phase manifests almost immediately following the IPC stimulus and lasts for 1-2 hours after which its effects disappears (termed classic or early preconditioning) [Murray, Jennings and Reimer, 1986; Lawson and Downey, 1993]. The second phase of cardioprotection appears 12-24 hours later and lasts for 48-72 hours and is termed the Second Window of Protection [SWOP], delayed or late ischaemic preconditioning [Marber et al., 1993; Kuzuya et al., 1993]. The cardioprotection conferred by delayed IPC is robust and ubiquitous but not as powerful as early IPC. Although there are some similarities in the mechanisms underlying early and delayed IPC, one of the major distinctions between the two is the latter's requirement for de novo protein synthesis of distal

mediators such as iNOS, HSP and COX-2 which mediate the cardioprotection 24 hours after the IPC stimulus [for review see Hausenloy and Yellon, 2010].

The signal transduction cascades of IPC can be divided into a trigger and a mediator phase and in recent years it has become a major objective to identify the various triggers, mediators and end effectors that are activated in this phenomenon. **Triggers** are activated during the preconditioning ischaemia and reperfusion cycle(s), and blockade of a trigger during this time will attenuate or abolish the cardioprotection of IPC. **Mediators** are important during prolonged index ischaemia and the first few minutes of reperfusion after sustained ischaemia. Similarly, blockade of mediators during this time will abolish the cardioprotection of IPC. Elucidation of the signaling mechanisms involved in the cardioprotective effects of identified triggers and / or mediators in IPC could lead to the development of pharmacological applications to be used in clinical settings.

Although the protection of ischaemic or pharmacological preconditioning is powerful, it could not be effectively employed in patients with acute myocardial infarction since preconditioning has to be introduced before the onset of ischaemia. But if IPC exerts its protection at reperfusion, then therapeutic salvage could still be possible even after ischaemia had begun by intervening at reperfusion.

1.1 Receptor dependent triggers of early preconditioning

In the heart, adenosine (Ado) has been proposed to act as a regulatory "metabolite" in ischaemia [Berne et al., 1963] in view of its ability to limit oxygen demand by causing negative inotropy and chronotropy and increase oxygen delivery by vasodilation. As an antiarrhythmic agent, the effects of adenosine on the mammalian heart were first reported in 1929 by Drury and Szent-Gyorgyi. In 1991, Liu et al. discovered that stimulation of the Gi-coupled adenosine A_1 receptor was necessary to trigger IPC. An increase in interstitial adenosine concentration during preconditioning was shown to occur in rats [Kuzmin et al 2000], rabbits [Lasley et al., 1995], dogs [Mei et al., 1998], and pigs [Schulz et al., 1998]. Attenuation of the increase in interstitial adenosine concentration in pigs [Schulz et al., 1995] or blocking the adenosine A_1- and A_3-, but not the A_2- receptors [Liu et al., 1991; Thornton et al., 1992] almost completely abolished the infarct size reduction achieved by IPC. The role of the opioid receptors in the preconditioning stimulus has been widely studied, and evidence indicated the involvement of the δ opioid receptor type [Schultz, 1995; Genade et al., 2001; Lochner et al, 2001].

Several studies suggest that bradykinin contributes to infarct size reduction in IPC [Wall et al., 1994; Jalowy et al., 1998] and it was also illustrated that bradykinin and adenosine act synergistically as triggers of preconditioning [Goto et al., 1995].

The hypothesis that the cardioprotective effects of IPC are due to release of an endogenous substance derived from the cyclo-oxygenase pathway of arachidonic acid metabolism such as prostacyclin (PGI), was substantiated when cyclo-oxygenase inhibition in the dog heart prevented the anti-arrhythmic effect of preconditioning [Vegh et al., 1990]. However, the cardioprotective effects of IPC could not be prevented by aspirin, suggesting that this was not mediated by prostanoids in a rat model [Li and Kloner, 1992] or in an in situ and a blood perfused isolated heart model, respectively [Liu, Stanley and Downey, 1992].

However the prostanoids prostaglandin I_2 (PGI_2) and prostaglandin E_2 (PGE_2) were shown to mediate the protective effects of ischaemia-induced late preconditioning in rabbits and mice [Gao et al., 2000; Shinmura et al., 2000 and 2002]. In the rat heart it was later confirmed that the cardioprotective effects of the late phase of δ-opioid receptor-induced preconditioning appear to be linked to the functional coupling between COX-2 and upregulation of PGI_2 [Shinmura et al., 2002].

More recently, it was reported that certain arachidonic acid metabolites of the cytochrome P-450 epoxygenase (CYP) pathway, the epoxyeicosatrienoic acids (11, 12-EET and 14, 15-EET) produced similar cardioprotection as IPC and postconditioning (POC) when applied prior to sustained ischaemia or at the start of reperfusion, respectively [Nithipatikom et al., 2006]. It was later established in dog hearts that endogenous EETs had an essential role in both these cardioprotective strategies [Gross et al., 2008]. Interestingly, it was recently shown in a rat model, that the major cardioprotective effects of the EETS are dependent on activation of a Gi protein coupled δ- and / or κ-opioid receptor [Gross et al., 2010].

There are other neurohormonal agonists which can precondition the heart when administered exogenously which may not be released in sufficient quantities by the ischaemic myocardium to trigger protection endogenously, such as norepinephrine, endothelin and angiotensin. Therefore, administration of antagonists to α-adrenergic receptors [Moolman et al., 1996; Bugge and Ytrehus,

1995], angiotensin [Tanno et al., 2000; Liu et al., 1995], or endothelin [Wang et al., 1996] has no effect on the process of IPC.

1.2 Receptor independent triggers

Reports regarding the participation of NO in the signaling of classic (early) preconditioning have been quite controversial. Wolfson and coworkers (1995) were the first to test for the involvement of NO in IPC. Isolated rabbit hearts were treated with L-NAME, a NOS inhibitor, which had no effect on cardioprotection. However, they noted that L-NAME reduced infarct size of non-preconditioned hearts. On the other hand, using NO donors, NO was shown to be an important trigger of cardioprotection in the isolated rat heart [Lochner et al., 2000]. Loss of protection was also observed in a pacing model of preconditioning when NOS inhibitors were administered [Ferdinandy et al., 1997]. Furthermore, a study by Qin et al., (2004) illustrated that exogenous NO triggers the preconditioning effect in the isolated rabbit heart. Conversely, it was shown that exogenous NO could not trigger the preconditioning state [Cohen, Yang and Downey, 2006]. In addition, Nakano and co-workers (2000) could not demonstrate a role for endogenous NO in the cardioprotection of classic preconditioning [Nakano et al., 2000). Despite these initial controversies, it is generally accepted that endogenous NO plays an important role in the downstream signaling during the triggering phase of IPC [for review see Downey et al., 2008].

However, NO is very important role player in SWOP and the first indication that NO triggered this process was provided by a study in which a nonselective blocker of NOS (L-NA) blocked the development of delayed protection against myocardial stunning [Bolli et al., 1997]. Also, pre-treatment with NO donors in the absence of ischaemia induced a delayed protective effect against both myocardial stunning and infarction that was indistinguishable from that observed during the late phase of ischaemic preconditioning [Takano et al., 1998; Bolli, 2001].

Redox signaling in preconditioning is still not completely understood, but it is widely accepted that transient, low concentrations of ROS (Reactive Oxygen Species: O_2^- and H_2O_2) and / or RNS (Reactive Nitrogen Species: $NO^{\cdot}$, HNO and $ONOO^-$) may trigger protective mechanisms. Some of these may be included among the triggers of preconditioning and it is likely that they collaborate in inducing cardioprotection [for review see Penna et al., 2009; Baines et al., 1997; Cleveland et al., 1997; Vanden Hoek et al., 1998; Das et al., 1999].

The role of calcium in preconditioning is unclear. Calcium L-type channel blockade prevents IPC in the human myocardium [Cain et al., 2000], whereas no attenuation of ischaemic preconditioning could be illustrated in the anesthetized pig model using calcium antagonists [Wallbridge et al., 1996].

1.3 The signaling pathway of IPC (Fig. 1.1)

It is generally accepted that simultaneous activation of the adenosine, bradykinin and opioid receptors as well as the release of oxygen free radicals during the brief ischaemia / reperfusion episodes, all contribute to the triggering of IPC. It was hypothesized that this scheme would require the convergence of all stimuli on a common distal pathway, which appears to be protein kinase C (PKC), since inhibition of PKC effectively inhibit the cardioprotection associated with adenosine [Sakamoto et al., 1995], bradykinin [Goto et al., 1995], opioid receptor [Miki et al., 1998] as well as oxygen free radicals [Baines et al., 1997]. In addition, studies in the rabbit [Ytrehus et al 1994] and in the rat [Mitchell et al., 1995] concluded that PKC activation is central to the protection by IPC.

Adenosine, bradykinin and opioids act via Gi-proteins to activate very divergent pathways despite the fact that their signaling converges on a single target. Adenosine receptors are thought to activate PKC via the phospholipases synthesizing diacylglycerol from membrane phospholipid [Cohen, Yang and Liu et al., 2001]. Opioid receptors are proposed to depend on metalloproteinase-mediated transactivation of the epidermal growth factor receptor (EGFR) which activates PI3-K [Cohen, Philipp and Krieg, 2007]. The receptor tyrosine kinase auto-phosphorylates its tyrosine residues when bound to its triggering growth factor. Bradykinin also triggers through PI3-K activation but is independent of EGFR [Cohen et al., 2007]. The steps downstream of PI3-K for both opioids and bradykinin appear to be similar. PI3-K causes phosphorylation of Akt through the phospholipid-dependent kinases. Phosphorylated Akt subsequently activates eNOS to produce NO, which then stimulates guanylyl cyclase to produce cGMP which in turn stimulates PKG [Cohen, Yang and Liu et al., 2001; Oldenburg et al., 2004].

Ligands to several other Gi-coupled receptors in the heart were also found to have the ability to mimic preconditioning through PKC activation including catecholamines [Banerjee et al., 1993], angiotensin II [Liu et al., 1995] and endothelin [Wang et al., 1996]. However, inhibition of the

receptors for any of these additional ligands does not raise the threshold for IPC, indicating that these substances are not released by ischaemia in large enough quantities to participate in IPC.

Reactive oxygen species, previously categorized as a receptor independent trigger, can simulate the protection of IPC by transient exposure of the heart to an oxygen radical generating system [for review see Yang, Cohen and Downey 2010], and conversely a ROS scavenger can abolish the cardioprotection of IPC [Baines, Goto and Downey, 1997; Tritto, D'Andrea and Eramo, 1997]. The cardioprotection from ROS could be blocked by a PKC inhibitor indicating that the ROS signal occurred upstream of PKC [Kuno et al., 2008].

The source of ROS appears to be the **mitochondria where the mitoK_{ATP} channels** play an essential role. It is proposed that activation of PKG opens the mitoK_{ATP} channels on the inner mitochondrial membrane permitting K^+ to enter the matrix along its electrochemical gradient [Costa et al., 2005]. However, the mitoK_{ATP} channels are localized on the inner mitochondrial membrane which is not accessible to cytosolic PKG and the connection between PKG and PKC-ε [Costa et al., 2005] dependent opening of the mitoK_{ATP} channel is not known [for review see Yang, Cohen and Downey, 2010]. Opening of the channels and the resulting K^+ influx is balanced by electrogenic H^+ efflux driven by the respiratory chain which consequently results in increased amounts of ROS generation [Costa and Garlid, 2008]. Generation of free radicals leads to activation of PKC. According to Downey and co-workers (2010) PKC activation signifies the end of the trigger phase and kinase activity is the first step in the mediatory phase. Interestingly, although the adenosine receptors activate PI3-K, they can also directly couple to PKC and thus circumvent the mitochondrial pathway and the mitoK_{ATP} channel.

However, it is still controversial which PKC isozyme mediates this protection but it seems that both PKC-ε and PKC-δ are involved [for review see Yang 2010; Dorn et al., 1999; Ping et al., 2001]. Also, peptide inhibitors of PKC-ε abolished ischaemic / hypoxic or pharmacological preconditioning in mice, rats, rabbits and pigs [Dorn et al., 1999; Gray et al., 1997; Inagaki K et al., 2005].

Another unresolved issue is the target of PKC. Because protection from a PKC activator could be aborted by adenosine A_{2B} receptor blocker [Philipp et al., 2006], and since PKC inhibition does not affect A_{2B} receptor mediated protection [Kuno et al., 2007], it is believed that the adenosine A_{2B}

receptor resides downstream of PKC and that PKC sensitizes the adenosine A_{2B} receptor to the heart's endogenous adenosine. Consequently, the adenosine A_{2B} receptor was shown to an essential element in the cardioprotection of IPC [Solenkova et al., 2006], as well as in postconditioning [Philipp et al., 2006]. It should not be surprising that an important kinase like PKC has many targets and it is known that PKC can directly or indirectly modulate components, associated with mitochondrial membranes such as the mitoK_{ATP} channel, mPTP, BAX / BAD and Bcl-2 [Costa et al., 2005 and 2006; Murphy, 2004] which are important molecules in the determination of cell survival or death.

1.3.1 IPC exerts its protection at reperfusion

It was proposed that IPC protects the heart by inducing activation of PI3-K /Akt and MEK1/2 / ERK 1/2 cascades at reperfusion [Hausenloy et al., 2005], the so-called "Reperfusion Injury Salvage Kinases" or RISK pathway. Pharmacological inhibition of either these cascades early in reperfusion abolishes IPC-induced cardioprotection. It was then concluded that IPC actually exerts its protection early in reperfusion following lethal ischaemia. This provided enormous hope for the clinical translation of IPC, especially when blood supply to the affected area is restored after clinical procedures. Indeed, in the past several years it was found that many pharmacological agents can protect the myocardium when given at the time of reperfusion, e.g. insulin [Baines et al., 1999], the adenosine A1 / A2 agonist Bay 60-6583 [Xu et al., 2000], transforming growth factor-β1 [Baxter et al., 2001], urocortin [Schulman, Latchman and Yellon, 2002], the adenosine agonist 5'-(N-ethylcarboxyamido) adenosine (NECA) [Yang et al., 2004], bradykinin [Yang et al., 2004], erythropoietin [Cai and Semenza, 2004], natriuretic peptide [Yang et al., 2006], cyclosporine A [Hausenloy, Ong and Yellon, 2009]. Like IPC, all of these reagents except cyclosporine A depend on the activation of PI3-K /Akt and MEK1/2 / ERK 1/2 cascades for protection to occur.

1.3.2 GSK-3β and the mPTP

As described above the end effector of IPC may be PKC-ε 2 which acts to inhibit the opening of of the mitochondrial permeability transition pore (mPTP) and it is currently thought to be a major role player in determining cell death or survival [Hunter et al. (1976)], despite the fact that its molecular structure is still unknown.

The immunosuppressant drug cyclosporine A can inhibit mPTP opening induced by calcium, phosphatase and oxidative stress [Crompton, Ellinger and Costi, 1988], providing an important pharmacological tool for investigating the function of mPTP in cardioprotection. It was found that the mPTP remained closed during ischaemia and open only in the first few minutes of ischaemia, a convenient time-point for clinical therapeutic intevention [Griffiths and Halestrap, 1995]. However, it is not yet kown how IPC actually inhibits opening of the pore at reperfusion. Although phosphorylation and thus inhibition of GSK-3β mimics IPC by reducing infarct size [Tong, Imahashi, Steenbergen and Murphy, 2002; Juhaszova et al., 2004], the role of this kinase in IPC is still not clear. It has been shown that the survival kinases PKB/Akt and ERK form tight couplings with the mPTP [Juhaszova et al., 2004] to prevent mPTP formation in the reperfused heart model [Solenkova et al., 2006].

In summary, after more than 20 years since the discovery of IPC, and despite the vast amounts of knowledge that have evolved from studies of intracellular events, the exact mechanism of this endogenous protective phenomenon still remains to be fully elucidated. Most of the studies aimed at elucidating the mechanisms of ischaemic preconditioning have used a pharmacological approach. This has led to an array of suggested receptors and signaling pathways and an increased focus on events during reperfusion. However, it is also believed that meticulous elucidation of events during an IPC protocol will yield more insight in the mechanis(s) of cardioprotection. In this regard, it was observed that cyclic increases in tissue cAMP characterizes a multi-cycle IPC protocol, suggesting a role also for activation of the β-adrenergic signaling pathway. The significance of these changes, was underscored by the fact that β-adrenergic receptor blockade abolishes IPC [Lochner et al., 1999].

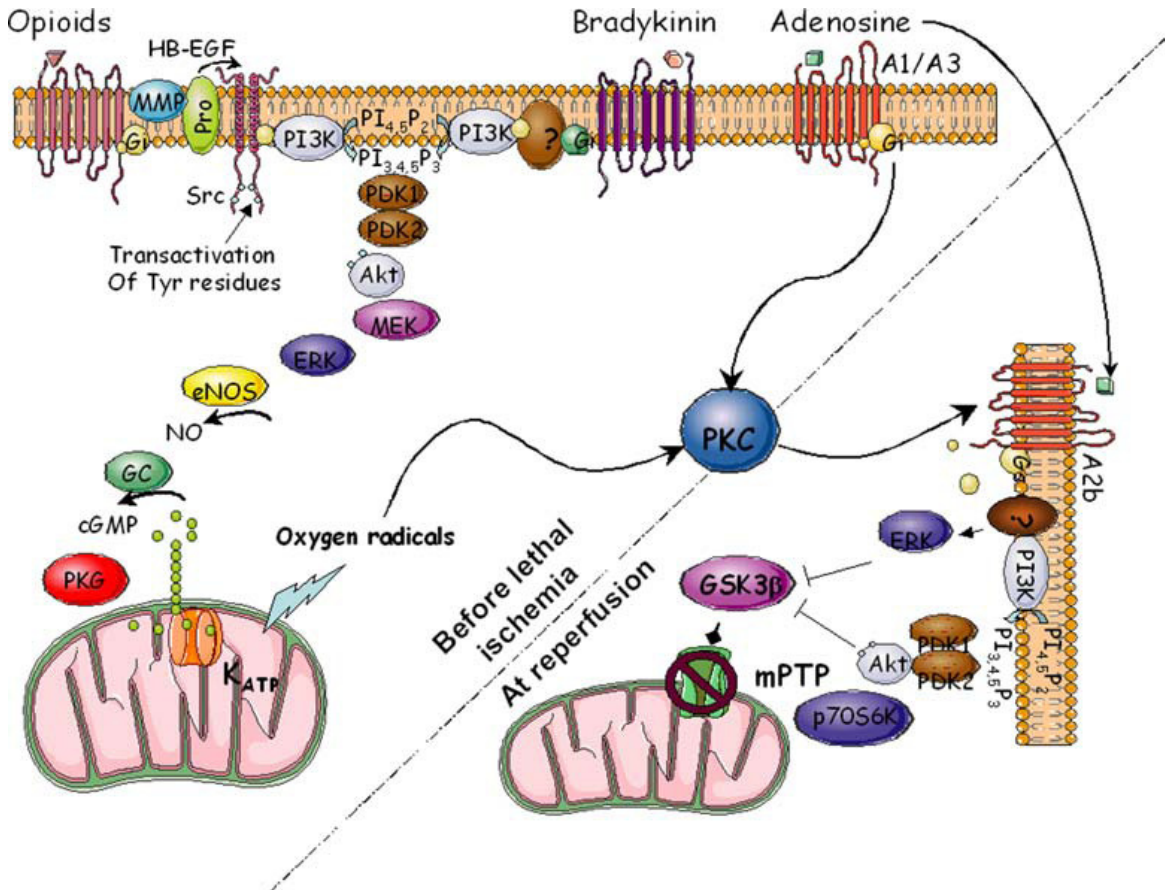


Fig. 1.1: A cartoon showing the sequence of signaling events involved in triggering the preconditioned state prior to the ischemic insult (events above the dividing line) and those that mediate protection in the first minutes of reperfusion (events below the dividing line). See text for details [Tissier, Cohen, and Downey, 2007; Downey, Krieg, Cohen, 2008].

1.4 β-adrenergic preconditioning (β-PC)

As referred to in the previous section, cardiovascular disease remains a leading cause of morbidity and mortality in the Western world. Thus there is continued interest in developing new drugs and interventions that will limit the extent of infarction and prevent cell death and explains the enormous effort investigated in elucidating the mechanism of IPC.

It is now well established that three endogenous triggers are released during exposure of the heart to short episodes of ischaemia/reperfusion, namely, adenosine, opioids and bradykinin [Downey, Davis, Cohen, 2005, 2007]. However, the role of the release of endogenous catecholamines in eliciting preconditioning has received surprisingly little attention. Ischaemia-mediated release of catecholamines and a concomitant increase in tissue cAMP have been known for many years [Schömig et al, 1984]. Even though the α1-adrenergic receptor was advocated to play a role in this regard, our laboratory could not find evidence for this receptor or PKC activation in the mechanism of IPC [Moolman et al., 1996]. In retrospect, this could be due to the perfusion model (working heart), and endpoint (functional recovery) used in these early studies.

The approach employed in our laboratory was that thorough investigation of events during an IPC protocol should serve as a guide for further studies. Thus the observation that the cyclic nucleotide cAMP increased in a cyclic fashion at the end of each preconditioning episode suggested a role for the β-AR signal transduction system [Lochner et al., 1998, 2000) as trigger in the preconditioning process. Should this be the case, then pharmacological activation of this pathway should be able to elicit protection against ischaemia. This was first demonstrated by Asimakis et al. (1994) who reported that pharmacological preconditioning with isoproterenol protected against ischaemia. It was subsequently reported that transient β-AR stimulation with ligands such as isoproterenol and dobutamine mimicked IPC and elicited protection against a subsequent period of ischaemia-the so-called phenomenon of β-preconditioning (β-PC) [Lochner et al., 1999; Miyawaki and Ashraf, 1997; Nasa, Yabe, Takeo, 1997).

The role of β_1-AR activation as trigger in β-PC was indicated by the use of blockers: (i) propanolol (a non-selective β-blocker) and atenolol (a more selective β_1-blocker) abolished isoproterenol-induced protection, while the selective β_2-blocker ICI-118551 was without effect [Francis et al., 2003]; (ii) the specific β_1-adrenergic agonist xamoterol could elicit protection against ischaemia, which could be attenuated atenolol and PKA inhibition [Robinet, Hoizy and Millart, 2005]; (iii) hypoxic preconditioning was attenuated by a β_1-selective blocker metoprolol [Mallet et al., 2006]; (iv) desflurane and sevoflurane preconditioning was shown to be dependent on β_1-AR activation, since it could be blocked by esmolol and H89, a β_1-AR blocker and PKA inhibitor respectively [Lange et al., 2006]. These findings suggest that ischaemic and anaesthetic preconditioning share a common pathway, namely the β_1-AR signal transduction pathway.

The fact that activation of most membrane receptors coupled to the Gi protein are able to elicit cardioprotection, suggests that the β_2-AR may also be a strong candidate for triggering β-PC. Indeed, Tong and coworkers (2005) found that preconditioning could not be triggered by isoproterenol in transgenic β_2-AR knockout mice. Furthermore, it was found that the Gi inhibitor pertussis toxin blocked isoproterenol-induced improvement in postischaemic function and reduction in infarct size [Tong et al., 2005]. As far as we know, no information is available regarding a role for the β_3-AR in β-AR preconditioning and this possibility should still be investigated

1.4.1 Downstream events

As stated above, elevation in tissue cAMP levels during a preconditioning protocol by ischaemia or isoproterenol, appear to be pivotal in eliciting cardioprotection. However, the significance of downstream events such as PKA and p38MAPK activation has not yet been established without a doubt. Using isolated perfused hearts, 55% and 87% increases in PKA activation after ischaemia (1x10 min) or β-AR preconditioning with forskolin ($0.3x10^{-6}$ M; 1x5 min), respectively, have been observed (Makaula et al., 2006). Cyclic elevations in PKA during a preconditioning protocol were also observed by others [Lan, Wang and Zhang, 2005; Inserte et al., 2004].

As in the case of cAMP, activation of PKA during ischaemia is potentially harmful, for example, causing phosphorylation and activation of the L-type Ca^{2+} channels, thereby promoting the harmful effects of Ca^{2+} into the cardiomyocyte [Tsien et al., 1983; Buneman et al., 1999] and hyperphosphorylation of the ryanodine receptor to liberate excess amounts of Ca^{2+}, as may occur in heart failure [Marks, 2003]. The harmful effects of cAMP accumulation during ischaemia may therefore be due to, at least in part, to activation of PKA. However, activation of PKA has also been linked to cardioprotection. Brief exposure to β-agonists [Sanada et al., 2004; Lochner et al., 1999] or an adenylyl cylase activator [Lochner et al., 1999; Makaula et al., 2005] or phosphodiesterase type III inhibitors [Sanada et al., 2001; Nomura et al., 2003], all which cause rapid activation of PKA, protect the heart against subsequent ischaemia, independently from PKC.

The necessity of PKA activation for successful preconditioning is demonstrated by the fact that PKA inhibitors such as H89 [Sanada et al., 2004; Inserte et al., 2004] and Rp-cAMPs [Sanada et al., 2004] blunted ischaemic and dibutyryl-cAMP-induced preconditioning.

The putative role of PKA activation after β1-AR stimulation with xamoterol was further conformed by the finding that both atenolol and H89 completely abolished protection [Robinet, Hoizy and Millart, 2005]. These workers also showed that, besides PKA, transduction mechanisms following β1-AR stimulation, also involved PI3-K and PKC, with PKA activation occurring prior to PKC.

It should be kept in mind that PKA-independent cAMP pathways may be activated by the preconditioning trigger to promote contractile recovery and to decrease infarct size following ischaemia / reperfusion. Many of the cAMP functions previously attributed to PKA, may be dependent on the cAMP receptor protein, the guanine nucleotide exchange factor Epac [Kawasaki et al.,1998; Mei et al., 2002]. In HEK cells activation of Epac leads to a prosurvival response via phosphatidylinositol-3-kinase dependent kinase PKB (Akt) activation while stimulation of PKA inhibits Akt [Mei et al., 2002]. Recent studies from our laboratory showed that Epac is rapidly activated by ischaemia as well as by β-AR preconditioning and is enhanced by simultaneous PKA inhibition. The latter confirms the evidence of an alternative β-AR signaling pathway in the myocardium [Marais and Lochner, unpublished observations].

1.4.2 p38MAPK and HSP27

Each subfamily of the MAPK family, ERK, JNK and p38MAPK, has been suggested to play a role in cardioprotection elicited by prior IPC [Schulz et al., 2002]. In our laboratory the involvement of p38MAPK activation was demonstrated in the triggering phase of β-PC, but not IPC, in the isolated rat heart model, using functional recovery as endpoint [Marais et al., 2001]. Downstream of p38MAPK, activation of the 27kDa small-heat shock protein (HSP27) may protect against ischaemic stress [Martin et al., 1997]. Interestingly, the marked but transient activation of p38MAPK during a multi-cycle IPC protocol was associated with sustained activation of HSP27 [Marais et al., 2005]. As in the case of HSP27, CREB was activated by exposure of the heart to 5 min of ischaemia, followed by reperfusion, and it remained activated throughout a multi-cycle IPC protocol [Marais et al., 2008]. CREB activation by events downstream of receptor activation included activation of PKA, PKC, ERK, MSK-1 as well as p38MAPK and these observations indicate that CREB may be a convergence point for several signaling pathways during the triggering process of preconditioning [Marais et al., 2008].

1.4.3 Possible mechanisms of β–PC: a decrease of cAMP during sustained ischaemia

The role of cAMP accumulation during a long period of ischaemia in mediating necrosis and arrhythmias is well recognized [Marks, 2003]. Previous studies have shown that accumulation of intracellular cAMP levels during sustained ischaemia was less in ischaemic preconditioned hearts than in controls, both in rats [Moolman et al.,1996] and rabbits [Sandhu et al. 1996,1997], and interventions such as β-AR blockade and depletion of endogenous catecholamines by prior reserpine treatment mimicked the effects of preconditioning, causing less cAMP accumulation during ischaemia and resulting in functional protection [Moolman et al., 1996]. These observations suggested a decrease in β-AR signal transduction during sustained ischaemia.

The above observations raised the question as to the mechanism of the decrease in cAMP, and whether the decrease in cAMP during sustained ischaemia was responsible for the protective effect of preconditioning. A study of the state of the β-AR signal transduction system in terms of β-adrenergic receptor density and affinity, forskolin stimulated adenylyl cyclase activity and PKA activity immediately following the preconditioning protocol, i.e. immediately prior to sustained ischaemia showed an increase in density and affinity of the β-receptor following an IPC protocol of 3x5 min ischaemia/ reperfusion (Bmax increased by 39% and Kd decreased by 35%, with a significant increase in adenylyl cyclase activity and PKA activity with each cycle of preconditioning) (Lochner et al., 1999). The effect of these changes was assessed by investigating cAMP generation in response to isoproterenol of hearts preconditioned with a 3x5 min cycle of ischaemia/reperfusion. cAMP increased significantly in non-preconditioned hearts but remained unchanged in preconditioned hearts, indicating desensitization. These findings were supported by Simonis, Weinberger and Strasser [2003] who found that β-AR density increased with repeated cycles, but sensitization of adenylyl cyclase was lost after more than one cycle of 5 min ischaemia/reperfusion. These findings suggest reduced responsiveness of the β-AR signal transduction pathway in IPC hearts as the mechanism of reduced cAMP accumulation during sustained ischaemia. However, this seems to differ from rabbits, as Sandhu et al. [1996] found no evidence for reduced responsiveness to isoproterenol in preconditioned rabbit hearts, and based on results obtained with propranolol concluded that reduced cAMP accumulation in preconditioned rabbit hearts was mediated by attenuated norepinephrine release.

The question remained how reduced cAMP accumulation during sustained ischaemia related to the mechanism of IPC. Administration of forskolin to preconditioned hearts resulted in an increase in cAMP during sustained ischaemia, but did not abolish protection in an isolated working rat heart model [Moolman et al., 1996]. Sandhu and coworkers [1996] used NKH477 to activate adenylyl cyclase and increase cAMP in preconditioned hearts during ischaemia, and likewise found no loss of protection as measured with infarct size in rabbits. These data strongly suggests that the preconditioned-induced reduction in cAMP accumulation seen during sustained ischaemia is a reflection of protection and not a causal factor.

Previous studies from our laboratory suggested a dual role for p38 MAPK in both ischaemic and β-adrenergic preconditioning [Marais et al., 2005]: activation of the kinase during the preconditioning protocol had a triggering action, while attenuation of its phosphorylation during sustained ischaemia may act as mediator of protection. Significant phosphorylation of cytosolic and myofibrillar HSP27 also occurred during both protocols; this was maintained throughout the sustained ischaemic period. It was subsequently hypothesized that attenuation of p38 MAPK activation and elevation of HSP27 phosphorylation during sustained ischaemia are prerequisites for cardioprotection. However, contradictory results regarding the exact role of p38 MAPK have been published (Mocanu et al., 2000, Steenbergen, 2002) and the matter warrants further investigation. The cardioprotective actions of the small heat shock proteins are by now well established (Chi and Karliner, 2004). However, how they confer protection is still unclear. Amongst others, they may act as chaperones (Georgopolous and Welch, 1993), stabilize the cytoskeleton (Larsen et al., 1997) or inhibit apoptosis (Rane et al., 2003).

1.4.4 The role of adenosine in mechanism of beta-adrenergic protection

The mechanism of β-AR has not been fully elucidated, but knowledge about it is evolving. It is conceivable that β-AR activation causes demand ischaemia, resulting in adenosine production and the downstream activation of its effectors. Indeed, Thornton et al. (1993) showed that induction of preconditioning by tyramine mediated release of endogenous catecholamines in rabbits was blocked by the non-selective adenosine blocker PD115,199, thus suggesting a role for adenosine in adrenergic mediated preconditioning.

Yabe et al. (1998) subsequently showed that β-PC elicited by isoproterenol was abolished by the PKC inhibitor polymyxin B. If this was true, one would expect β-AR to share another characteristic of adenosine mediated preconditioning, such as independence from activation of the mitoK_{ATP} channel.

1.4.5 Beta-adrenergic preconditioning and protection against apoptosis

The spectrum of protection against ischaemia elicited by ischaemic preconditioning was initially studied in the context of necrosis and dysrhythmias, and later found to include protection against apoptosis (Piot et al., 1999). The mechanism of the anti-apoptotic effect of IPC involve a host of factors, such as the generation of reactive oxygen species, an altered Bcl-2/Bax ratio and concomitant reduction in cytochrome c release from mitochondria, reduced activation of caspase activity and reduced ceramide production during ischaemia (Zhao and Vinten-Johansen, 2002). Beta-adrenergic stimulation per se is known to be pro-apoptotic (Patterson et al., 2004), an effect attributed to its activation of calmodulin kinase II (Zhu et al., 2001). In view of the finding that isoproterenol could mimic IPC, the question was asked whether a proapoptotic agonist could protect against apoptosis. Using PARP cleavage and caspace-3 activation as end-points, it was shown that β-PC with one cycle of 10^{-7} M isoproterenol for 5 minutes resulted in significantly less apoptosis at the end of reperfusion than in control hearts, and a reduced infarct size, accompanied by significantly less activation of p38 MAPK. To further evaluate the role of p38 MAPK activation, its antagonist SB203580 was administered 10 min prior to sustained ischaemia: this caused a significant reduction in p38 MAPK activation, which concurred with a marked anti-apoptotic effect as well as a reduction in infarct size (Moolman et al., 2006).

On the whole, results previously obtained on our laboratory showed that β-PC reduces both apoptosis and necrosis: these events are associated with attenuated activation of p38 MAPK during ischaemia and reperfusion. Whether this is the cause or the result of the cardioprotection still remains to be established.

1.4.6 Late preconditioning with pharmacological beta-adrenergic preconditioning

Whereas the mechanism of classic preconditioning involved rapid kinase activation, without the production of new peptides, it became clear that late preconditioning had a different mechanism.

Bolli (2000) elucidated the important role of NO as final common pathway in late preconditioning. Interestingly, it was found that very small doses of isoproterenol (4 x 0.0004 mg/kg), administered at 4 hourly intervals could elicit late preconditioning after 24 h (Moolman and Lochner, unpublished observations). Furthermore, co-administration of the NOS inhibitor L-NA completely abolished the effects of β-AR late preconditioning, suggesting a role for NO production in this scenario (unpublished observations), as is the case for late preconditioning elicited by ischaemia.

1.4.7 Summary and Conclusions

The role of β-adrenergic activation as mediator of ischaemic damage is undisputed. It is however becoming clear that activation of the β-adrenergic signal transduction pathway can elicit protective responses in the myocardium. Activation of the β-adrenergic signal transduction pathway occurs, and participates in the protective effect of ischaemic preconditioning, although ischaemic preconditioning is not solely dependent on this particular signal transduction. Attenuation of this pathway during sustained ischaemia is associated with cardioprotection, as reflected by a reduction in infarct size, apoptosis and an improvement in functional recovery during reperfusion. Pharmacological activation of the β-adrenergic signal transduction pathway *per se* can elicit both classical and late preconditioning.

In view of the above the rest of the literature survey will be devoted to a detailed description of the β-AR signaling pathway and the possible role players in β-PC.

1.5 β-adrenergic receptor (β-AR) subtypes

Sympathetic stimulation (via circulating catecholamines) of the β-adrenergic receptor (β-AR), a prototypical G protein-coupled receptor, regulates a wide range of biological processes from heart pacemaker activity, myocardial contractility and relaxation, vascular and bronchial smooth muscle tone, to metabolic regulation, such as glucose and lipid metabolism, cell growth, cell survival and cell death. Ahlquist (1948) was the first to differentiate the adrenergic receptors pharmacologically into α- and β-adrenergic receptors. Using appropriate agonists and antagonists, these early workers classified the adrenergic receptors into α_1-, α_2-, β_1-, and β_2-adrenergic receptor subtypes [Lands et al., 1967; Ablad et al., 1974]. The β_3-adrenergic receptor was indentified at a later stage [Tan et al., 1983; Emorine et al., 1989; Granneman et al., 1991].

Adrenergic receptors belongs to the superfamily of G protein-coupled receptors which shares the common feature of 7-transmembrane spanning domains and are involved in the response to neuro- and autocrine transmitters [Dixon et al., 1986; Yarden et al., 1986]. At least three human genes that express the β_1-, β_2- and β_3-AR subtypes have been identified using pharmacological and cloning methods [Emorine et al., 1989; Byland et al., 1995; Ihl-Vahl et al., 1996]. The existence of a fourth β-adrenergic receptor was suggested, based on the effects of nonconventional agonists observed in vitro in several species [Kaumann and Molenaar, 1997].

In the heart, β-AR stimulation by catecholamines (norepinephrine and epinephrine) serves as a powerful regulatory mechanism to enhance cardiac performance in response to stress, injury or exercise [Lohse et al., 2003; Hata et al., 2004]. Sympathetic adrenergic stimulation increases the release of norepinephrine and epinephrine (from the adrenal medulla) to all parts of the heart. Contrary to this, the parasympathetic nervous system or cholinergic system acts through the vagal nerve to release acetylcholine (Ach) which generally opposes the effects of the sympathetic stimulation to keep blood pressure within narrow limits [Opie, 1998].

The superfamily of G-protein coupled receptors is characterized by an extracellular glycosylated amino (N) terminus, an intracellular carboxyl (C) terminus region with serine and threonine residues that are potential phosphorylation sites and seven transmembrane domains (TD) linked by three extracellular loops and three intracellular loops [Dixon et al., 1986; Yarden et al., 1986].

Ligand binding induces a conformational change in the GPCR, which disrupts the ionic interactions between the third cytoplasmic loop and the sixth transmembrane segment and allows for coupling with the heterotrimeric guanine-nucleotide regulatory proteins (G-proteins) [Wess, 1997; Han et al., 1998].

The phosphorylation sites of PKA localized on the third intracellular loop of the receptor [Benovic et al., 1985], are believed to play a role in agonist promoted uncoupling, subsequent rapid desensitization and down regulation of the receptor [Benovic et al., 1985]. The C-terminal serine and threonine residues, when phosphorylated by the G-protein receptor kinase / β-adrenergic receptor kinase (GRKs / βARKs) also promote desensitization of the receptor [Benovic et al., 1985; Lohse et al., 1996].

Although, all three β-AR subtypes are found in variety of tissues they form an integral part of membrane proteins present in the heart of different species [Skeberdis et al., 1997; Bylund et al., 1998]. The β_1-AR is equally distributed in all parts of the heart [Brodde, 1991; Myslivecek et al., 2006]. Stimulation of the cardiac β_1-AR leads to an increase in automaticity, conduction velocity (chronotropy), excitability and contraction force (inotropy) [Kaumann, 1989; Bristow et al., 1990]. In the nonfailing heart, the β_1-AR group of receptors mediates the majority of the tensile responses to nonselective agonists [Brodde, 1991].

β_1-AR and β_2-AR functionally coexist in cardiomyocytes of many mammalian species including humans, with striking qualitative and quantitative differences in their functions and signaling mechanisms [Xiao et al., 1999]. In the human heart the β_1-AR is the predominate receptor [Brodde, 1991]: it expresses β_1- and β_2-adrenergic receptors at a ratio of about 70-80 % : 30-20 % in the ventricle and 60-70 % : 40-30 % in atria, both of which increase cardiac frequency and contractility.

The β_3-AR subtype was identified in a variety of tissues. This receptor subtype is found to a large extent in the coronary vascular bed [Strosberg, 1997] and in adipose tissue [Emorine et al., 1989] where it mediates thermogenesis in brown (BAT) and lipolysis in white adipose tissue (WAT) [Arch, 1989]. This receptor subtype reduces contractile force in human ventricular muscle [Gauthier et al., 1996] and stimulates L-type calcium current in human atrial myocytes [Skeberdis et al., 1999].

β_3-AR differs from β_1-AR and β_2-AR with regard to their molecular structure as well as their pharmacological profile [Bylund et al., 1994]. The β_1- and β_2-ARs show 48.9 % homology in their amino acid sequences, whereas β_3-AR exhibits 50.7 % and 45.5 % homology in amino acid sequences with the other two receptors, respectively [Dixon et al., 1986; Yarden et al., 1986; Emorine et al., 1989].

1.6 β-adrenergic receptor signaling

Ligand binding induces a conformational change in the GPCR, which disrupts the ionic interactions between the third cytoplasmic loop and sixth transmembrane segment and allows for coupling with the heterotrimeric guanine-nucleotide regulatory proteins (G-proteins) [Wess,1997; Han., et al., 1997] upon which the signals of ligand binding are relayed to the inside of the cell. These G protein-coupled receptors (GPCRs) activate a small but diverse subset of effectors, including the adenylyl cyclases (ACs), phospholipases and various ion channels [Gilman, 1987, 1990; Karoor et al., 1996].

The G-proteins are composed of Gαβγ heterotrimers [Gilman, 1987; Byland et al., 1998]. In the basal state, heterotrimeric G proteins have GDP bound to their catalytic site of GTPase on their Gα subunit. After interactions with the receptor, their activation requires association of GTP to the Gαβγ in exchange for GDP, leading to the dissociation of the complex into GTP Gα and Gβγ subunits. The dissociated Gα and βγ subunits subsequently either positively or negatively regulate a host of effector systems which result in changes in intracellular second messenger signaling [Northhup et al., Birnbaumer, 1992].

Hydrolysis of GTP to GDP by the complex results in the reassociation of the Gα and Gβγ subunits to commence the next cycle of activation [Helper and Gilman, 1992]. To date, at least 20 Gα, 5 Gβ and 11 Gγ subtypes of G proteins have been identified. The Gα subunits differ significantly from each other, whereas the Gβ and the Gγ subunits do not vary remarkably among the G proteins. The four primary families of Gα proteins [Gαs, Gαi, Gαq and $G\alpha_{11/12}$] [Rockman et al., 2002] diverge at this point with respect to downstream signaling molecules and subsequent physiological processes. Dissociated Gα subunits couple with an effector, an enzyme such as adenylyl cyclase (AC) and phospholipase Cβ, or an ion channel [Rockman et al., 2002].

Dissociated Gβγ subunits target a range of signaling pathways involved in desensitization, downregulation, apoptosis and ion channel activation (I_{Kach}) [Rockman et al., 2002; Lefkowitz, 1988; Krapivinsky et al., 1995].

1.7 The classical / traditional view of β-AR signaling and distinct β-AR subtype actions in the heart

β-AR subtypes have different affinities for different ligands. [Hoffmann et al., 2004]. The different subtypes expressed in the heart are important for normal cardiac function and in heart failure. β-AR function is of course dynamically regulated and is a component of normal physiological adaptation to maintain homeostasis.

Agonists (catecholamines) initiate β-AR signaling by binding to the receptor and cause it to undergo conformational change that results in activation of the classical Gs-adenylyl cyclase (AC)-cAMP-PKA signaling pathway. This, in turn, phosphorylates target proteins involved in metabolic regulation, growth control, muscle contraction and cell survival or death. In the heart, PKA phosphorylates a multitude of Ca^{2+} handling proteins, including sarcolemmal L-type Ca^{2+} channels [Zhao et al., 1994; Gerhardstein et al., 1999], sarcoplasmic reticulum (SR) membrane proteins, phospholamban (PLB) [Simmerman and Jones, 1998], ryanodine receptors [Marx et al., 2000], troponin I and C protein [Sulakhe and Vo,1995], myosin binding protein-C (MyBP-C) [Kunst et al., 2000] and protein phosphatase inhibitor-1 [Zhang et al., 2002]. This affects cardiomyocyte contractile behaviour by increasing Ca^{2+} influx into the sarcoplasmic reticulum (phospholamban / SERCA), and modulating myofilament Ca^{2+} sensitivity (troponin 1, MyBP-C) (Fig. 1.2).

Despite many similarities, β_1-AR and β_2-AR are genetically and pharmacologically distinct entities and couple to distinct signal transduction pathways to elicit different cellular responses with regard to G protein coupling, cAMP handling, target protein phosphorylation and most important, the modulation of cardiac EC coupling.

1.8 Coupling of β_1-AR to Gs versus the Dual coupling of β_2-AR to Gi as well as Gs regulatory proteins

Failure of the β_2-AR to produce a proportional contractile response in rodent and canine hearts is possibly due to differences in the manner in which these receptors are coupled to G proteins. Stimulation of β_2-AR but not β_1-AR activates Gi proteins in adult rat cardiomyocytes [Zhang et al., 1995], while both β-AR subtypes are able to stimulate the classic / traditional Gs-AC-cAMP-PKA signaling pathway [Xiao et al., 1995] as illustrated in HEK293 cells [Daaka et al., 1997] and in human heart [Kilts et al., 2000]. Gi coupling qualitatively and quantitatively modifies the outcome of Gs signaling and subsequently exhibit important cardiac protective effects (Fig. 1.2).

In many biological systems, regulatory Gs and Gi proteins engage in cross talk, which is mediated through different receptor families, e.g. activation of muscarinic or adenosine receptors, prototypic Gi-coupled receptors, markedly antagonizes positive inotropic effects of β-AR stimulation [Landzberg et al., 1994; Newton et al., 1996]. β_2-AR couples to both Gs and Gi proteins [Asano et al., 1984] and more recent studies, using a photoaffinity labeling technique illustrated such coupling of the β_2-AR in intact cardiomyocytes, as manifested by the incorporation of a photoreactive analogue, 32P GTP-azidoanilide, into α subunits of Gi_2 and Gi_3 in addition to Gs [Xiao et al., 1999]. Thus, β_2-AR signaling represents a unique mode of receptor – G protein interaction where a given receptor simultaneously activates more that one class of G protein giving rise to functionally opposing pathways.

Mechanisms underlying the differential coupling of β-AR subtypes to G proteins are not well understood but it has been shown that replacement of the cytoplasmic loop (for the binding of the G protein) of the muscarinic receptor with that of β_2-AR can induce Gs activation in response to muscarinic agonists [Wong, Parker and Ross, 1990]. In addition, it has been shown that the proline region in the third intracellular loop determines the different Gs coupling and sequestration of β_1-AR versus β_2-AR [Green and Liggett, 1994].

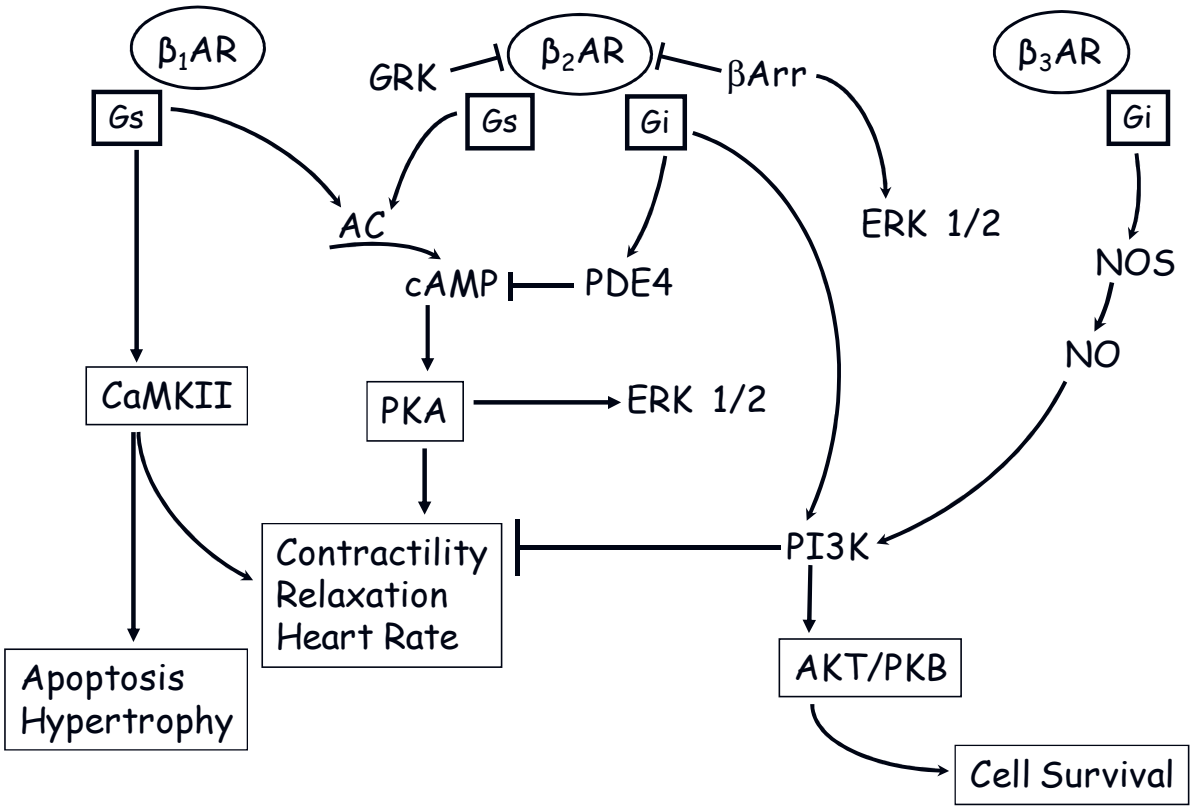


Fig. 1.2: Subtype-specific signaling pathways of cardiac β-ARs (Lohse, Engelhardt and Eschenhagen, 2003; Zheng et al., 2005)

1.9 β-AR subtypes differentially regulate Ca^{2+} handling and contractility

Cardiac EC coupling is initiated by a Ca^{2+} influx through the voltage-dependent sarcolemmal L-type Ca^{2+} channels during an action potential. However, this Ca^{2+} influx is insufficient to produce contraction, but triggers a larger Ca^{2+} release from the SR via ryanodine receptors through a Ca^{2+} induced Ca^{2+} release mechanism [Fabiato, 1985]. The resultant intracellular Ca^{2+} (Cai) transient activates contractile proteins, producing contraction; Cai is subsequently removed from the cytoplasm by the SR Ca^{2+} -ATPase (Ca^{2+}pump) and the Na^{+} -Ca^{2+} exchanger.

β-AR stimulation modulates most of these important components of the cardiac ECC cascade and therefore plays a prominent role in the regulation of cardiac performance.

However, there are several prominent physiological differences between β_1-AR and β_2-AR subtypes. In general, signaling cascades from β_1-AR and β_2-AR indicate important differences in their ability to affect downstream events, for example cAMP formation, PKA activation and PLB phosphorylation and camodulin kinase II (CAMKII) activation [Steinberg, 1999: Xiao et al., 1999].

In rat ventricular myocytes, stimulation of both β-AR subtypes increases L-type Ca^{2+} currents (Ica), Cai transients, and contraction amplitude (positive inotropic effect) but only β_1-AR stimulation markedly accelerates the Cai decay and contractile relaxation (positive lusitropic or relaxant effect) [Xiao and Lakatta, 1993]. This trend has also been observed in other mammalian species, e.g. cat and sheep [Lemoine and Kaumann, 1991; Borea et al., 1992]. In addition, increased SR Ca^{2+} uptake and decreased myofilament Ca2+ sensitivity contribute to the relaxant effect after stimulation of β_1-AR but not β_2-AR. Only β_1-AR stimulation increases the resting cytosolic Ca^{2+} oscillations in several mammalian species [Xiao and Lakatta, 1993; Cerbai et al., 1990; Parratt, 1988], indicating that β_1-AR may be more prone than β_2-AR to elicit Ca^{2+} -dependent arrhythmias.

As stated previously, β_1-AR stimulation affects the phosphorylation of sarcolemmal L-type Ca^{2+} channels and the aforementioned regulatory proteins remote from the sarcolemma, thus increasing the multitude and kinetics of intracellular Ca^{2+} transients and conduction [Xiao and Lakatta, 1993; Kuschel et al., 1999; Xiao, 2001]. In contrast, a large body of evidence has demonstrated that β_2-AR stimulation specifically modulates sarcolemmal L-type Ca^{2+} handling without affecting the above intracellular regulatory proteins in cardiomyocytes from mammalian species, including rat and dog [Kuschel, et al., 1999; Xiao, 2001].

Although β_2-AR stimulation in the human heart is able to increase cAMP formation or PKA-dependent phosphorylation of intracellular regulatory proteins (PLB,TnI and C protein), for a given elevation in cAMP production or PKA activation, the positive inotropic effect of β_2-AR stimulation is significantly smaller compared to that induced by β_1-AR stimulation [Kaumann et al., 1996, 1998, 1999].

In rat and canine cardiomyocytes, the β_2-AR mediated increase in cAMP is dissociated from its contractile response [Xiao et al., 1994; Altschuld et al., 1995; Zhou et al., 1997]. Also, in mouse cardiomyocytes, β_2-AR induced cAMP formation results in a minor positive inotropic effect [Xiao et al., 1999]. In rat ventricular myocyte preparations, the dose-response curve of total cAMP accumulation induced by β_1-AR stimulation with norepinephrine overlaps with that induced by β_2-AR stimulation with zinterol [Xiao et al., 1994]. However, the maximal increase in the particulate cAMP induced by the β_2-AR agonist, zinterol is about 50 % of that caused by β_1-AR stimulation, suggesting differential compartmentalization of cAMP depending on β-AR subtype stimulation [Xiao et al., 1994].

In human paced atrial strips, stimulation with zinterol caused a positive inotropic and lusotropic effects with EC50 values of 3 and 2 nM, respectively [Kaumann et al., 1996]. The zinterol-evoked effects were unaffected by β_1-AR selective antagonists, but were significantly blocked by the β_2-AR selective antagonist ICI 118551 [Kaumann et al., 1996]. Contrary to the belief that zinterol is a selective agonist for the β_2-AR, recent work investigating the action of this drug at β_3-AR level, in mouse primary adipocytes and Chinese hamster ovary cells (CHO-K1) expressing human β_1-AR and β_3-AR only, revealed that it significantly increased cAMP levels, an effect which was totally abolished in adipocytes from β_3-AR knock-out mice.

In CHO-K1 cells expressing human β_3-AR, zinterol and L755507 (selective β_3-AR agonist) caused a robust concentration-dependent increase in cAMP accumulation which was enhanced after pretreatment with PTX, indicating that human β_3-AR couples to both Gi and Gs regulatory proteins [Sato et al., 2006]. Zinterol is therefore one of few beta-adrenergic agonists with high potency and efficacy for the human β_3-AR [Huchinson et al., 2006].

1.10 Compartmentalized / Localized cAMP signaling during cardiac β_2-AR stimulation

Observations thus far demonstrating that the effect of the β_1-AR is more widespread while β_2-AR signaling modulates intracellular Ca^{2+} but cannot phophorylate regulatory proteins remote from the cell surface membrane, suggesting that β_2-AR is tightly localized near the subsarcolemmal

microdomain, in the vicinity of the L-type Ca^{2}_{+} channels. More direct evidence supporting localized β_2-AR signaling has emerged from cell patch-clamp single L-type Ca^{2+} channel recordings: the L-type Ca^{2+} channel responds only to local (agonist in pipette solution) but not to remote (agonist added to bathing solution) β_2-AR stimulation [Sham et al., 1998].

These results are in general agreement with the observation that in frog cardiomyocytes, in which the β_2-AR predominates, β-AR stimulation by isoproterenol applied to one end of the cell has little stimulatory effect on remote L-type Ca^{2+} channels [Jurevicius and Fischmeister, 1996]. These results evoked doubts as to whether the β_2-AR cardiac response is mediated by a cAMP-dependent signaling pathway. However, accumulating evidence indicates that the effect of β_2-AR stimulation on intracellular Ca^{2+} is mediated exclusively by a cAMP-dependent mechanism [Kuschel et al., 1999; Hartzell et al., 1991; Xiao et al., 1999]. To delineate a role of cAMP-dependent PKA activation in β-AR subtype signaling, specific inhibitors of PKA, including Rp-cAMP, H-89 and a peptide PKA inhibitor (PKI) have been used.

Most studies have demonstrated that PKA inhibitors (for example, Rp-cAMP and H-89) not only block the effect of β_1-AR stimulation but also completely reverse the effects of β_2-AR [Kuschel et al., 1999; Zhou et al., 1997; Xiao et al., 1999]. Similarly, in human and frog cardiac myocytes, the β_2-AR-induced augmentation of intracellular Ca^{2+} is totally prevented by PKI [Skeberdis et al., 1997]. Collectively, several lines of evidence support the idea that cAMP-dependent PKA activation is mandatory for β_2-AR mediated cardiac responses, but in some species this is highly localized to the surface membrane.

1.11 The involvement of PKA; RhoA / Rho-kinase signaling pathways in Cardioprotection

Protein kinase A (PKA) is a ubiquitous cellular multi-kinase that phosphorylates serine and threonine residues in response to adenylyl cyclase (AC)-mediated cAMP [Niswender et al., 1975]. The widespread expression of PKA subunit genes, coupled with the many mechanisms by which cAMP is regulated within the cell, suggest that PKA signaling is of extreme importance in cellular function.

In the heart, PKA is a key regulatory enzyme in the catecholamine-mediated control of excitation-contraction coupling as well as in many other functions including activation of transcription factors and control of metabolic enzymes.

PKA consist of two catalytic (C) subunits and a regulatory (R) subunit [Corbin et al., 1988; Taylor, Beuchler and Yonemoto, 1990]. When the regulatory subunits are activated by cAMP, the catalytic units are released as active haloenzymes [Taylor, Buechler and Yonemoto, 1990]. In general, Cα is expressed ubiquitously expressed in all tissues, while Cβ shows a more restricted pattern of expression in the brain [Brandon et al., 1998], liver [Enns et al., 2009b] and hematopoietic cells. In addition to having different tissue-specific expression patterns, the Cα and Cβ subunits of PKA are believed to have unique functions [Gamm et al., 1996] and are known to phosphorylate different downstream targets [Yu et al., 2004].

It has been shown that the AKAPs (A kinase anchoring proteins), a family of proteins that act as molecular scaffolds to anchor PKA in the vicinity of specific substrate molecules [Wong and Scott, 2004], contribute largely to the specificity of PKA thus directing PKA activity toward relevant substrate molecules. Spatial control of cAMP formation may represent another mechanism which could contribute to the specificity of PKA [Zaccolo and Pozzan, 2002]. Cardiac myocytes express all four types of PKA, PKA-RIα, PKA-RIIα, PKA-RIβ and PKA-RIIβ [Scholten et al., 2007]. These isoforms have different subcellular localizations, with PKA-RII being mainly associated with the cellular particulate fraction, whereas PKA-RI has been found mainly in the cytosol [Corbin et al., 1977; Brunton, Hayes and Mayer, 1981]. Even though PKA isoforms have distinct biochemical properties and specific subcellular localizations, it is still not established as to how the individual PKA isoforms serve to deliver a specific response.

The importance of β-adrenergic receptor stimulation and presumeably PKA in the pathogenesis and treatment of heart failure (HF) is well accepted [Lohse et al., 2003]. Excessive β-adrenergic receptor stimulation in HF that could lead to adverse effects on myocardial function as has been shown in the context of ischaemia / reperfusion [Rona, 1985; Waldenstrom, Hjalmarson and Thornell, 1978]. Myocardial ischaemia / reperfusion is characterized by cAMP accumulation and activation of PKA [Sakai, Shen and Pappano, 1999], increased phosphorylation and opening of L-type Ca^{2+} channels and consequently the development of cytosolic Ca^{2+} overload [Shine and Douglas, 1983; Du Toit

and Opie, 1992]. The role of PKA in the cardioprotection elicited by IPC, still needs to be evaluated. Makaula and coworkers (2005) showed that inhibition of PKA prior to sustained ischaemia, enhanced the cardioprotection of IPC, suggesting a harmful role for this kinase in this regard. However, activation of the β-adrenergic pathway is essential for cardioprotection, since β-adrenergic blockade during a preconditioning protocol abolishes protection [Lochner et al., 1999].

It was also demonstrated that transient preischaemic activation of PKA reduces infarct size through Rho-kinase inhibition during sustained ischaemia, implicating a novel mechanism for cardioprotection by IPC independent of PKC [Sanada et al., 2004]. It was demonstrated that increased cAMP levels followed by PKA activation cause temporary inhibition of the small GTPase RhoA [Dong et al., 1998; Manganello et al., 2003] and its downstream kinase Rho-kinase [Dong et al., 1998]. Apart from its protective effects [Sanada et al., 2004], the RhoA / Rho-kinase mediated pathway plays an important role in vascular smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, cytokinesis and gene expression, all of which may be involved in the pathogenesis of atherosclerosis [Shimokawa, 2002].

1.12 The role of β_2-AR/Gi coupling in localized control of β_2-AR stimulated cAMP signaling

During acute receptor stimulation, the β_2-AR-Gi coupling activates phosphoinositide 3-kinase (PI3-K), which in turn mediates compartmentalization of the concurrent Gs-cAMP signaling [Kuschel et al., 1999; Jo et al., 2002] (Fig.1.2). Inhibition of phoshatidylinositol 3-kinase (PI3-K), enables β_2-AR-activated cAMP/PKA signaling to reach intracellular substrates, as indicated by the marked increase in PLB phosphorylation, significant relaxant and greater positive inotropic effects [Jo et al., 2002]. Blocking Gi or Gβγ signaling completely, prevents the potentiating effects of PI3-K inhibition, suggesting that the pathway restricting β_2-AR-cAMP signaling sequentially involves Gi, Gβγ and PI3-K [Jo et al., 2002], despite early studies showing that PTX pre-treatment had no significant effect on β_2-AR-mediated global cAMP accumulation or PKA activation [Zhou et al., 1997; Kuschel et al., 1999].

PTX pre-treatment eliminates the role of Gi proteins and permits β_2-AR stimulation to induce a dose-dependent increase in PLB phosphorylation, associated with significant contractile relaxation and positive inotropic effects [Xiao, Ji and Lakatta, 1995; Xiao et al., 1998; Gupta, Neumann and Watanabe, 1993], very similar to those induced by β_1-AR stimulation which is insensitive to PTX pre-treatment [Xiao, Ji and Lakatta, 1995; Kuschel et al., 1999], showing that Gi proteins are not involved.

Thus, in addition to its very important role in cell growth and cell survival (Fig.1.1), PI3-K constitutes a key downstream event of acute β_2-AR-Gi signaling that confines and counteract the concurrent β_2-AR/Gs-mediated cAMP signaling [Zheng et al., 2004]. These studies indicate that in rat cardiomyocytes the β_2-AR/Gi coupling underlies the functional compartmentalization of the β_2-AR/Gs – directed cAMP/PKA signaling, which may largely account for the qualitative and quantitative differences between β_1-AR and β_2-AR mediated cardiac responses [Xiao et al., 1999].

1.13 Switch from PKA to calmodulin-dependent protein kinase II-dependent signaling during sustained β_1-AR activation

As summarized in the preceding sections, stimulation of both β_2- and β_1-AR mediates increases in contractility via the Gs-dependent adenylyl cyclase- cAMP- PKA pathway [Xiao and Lakatta, 1997]. The β_2-AR is also coupled to the pertussis toxin (PTX)-sensitive signaling pathway mediated by the Gi regulatory protein which qualitatively and quantitatively modifies the outcome of Gs signaling [Xiao et al., 1995; Daaka, Luttrell and Lefkowitz, 1997; Communal et al., 1999]. It was thought that the functional consequences of either acute or chronic β_1-AR activation might be exclusively mediated by the classic Gs- AC- cAMP- PKA signaling pathway. This idea has been challenged by more recent studies, which illustrated that persistent β_1-AR activation augments myocyte contractility and intracellular Ca2+ transients via Ca2+ / Calmodulin-dependent protein kinase II (CaMK II) signaling which is independent of the cAMP-PKA pathway in adult rat cardiac myocytes (Fig.1.2).

Inhibition of the PKA pathway could not block prolonged β_1-AR stimulation-mediated increases in myocyte contraction and Ca^{2+} transients, whereas the inhibition of CaMK II activation, fully

abolished the effects of sustained β_1-AR stimulation without affecting those targets excited by acute β_1-AR stimulation [Wang et al., 2004]. In contrast, inhibition of PKA but not CaMK II prevents transient β_1-AR signaling-mediated positive inotropic and lusitropic effects [Wang et al., 2004]. In addition, progressive and consistent CaMK II activation is accompanied by rapid desensitization of the cAMP-PKA signaling [Hausdorf et al., 1990] indicating that β_1-AR signaling undergoes a time dependent switch from the PKA dominant pathway to the CaMK II dominant pathway after receptor stimulation [Wang et al., 2004].

Thus, the time dependent β_1-AR signaling switch from PKA to the CaMK II dominant pathway may be clinically relevant and CaMK II inhibition may be a potential target to prevent adverse cardiac remodeling, particularly myocyte hypertrophy and apoptosis in the context of enhanced β_1-AR signaling, which is characteristic of CHF.

1.14 Coupling of the β_3-AR to regulatory Gs and / or Gi protein

During the 1980s, the classification of β-adrenergic receptors into two types (β_1- and β_1-AR) [Lands et al., 1967] was challenged. It is now known that 3 different subtypes, β_1- , β_2- , and β_3- may participate in the regulation of cardiovascular function. β_3-AR differs from the other two subtypes by its molecular structure and pharmacological profile. The gene encoding human β_3-AR was cloned in 1989 [Emorine et al., 1989]. Since then, the gene has been identified in the rat, mice, bovine, monkey, dog [for review, see Strosberg, 1997], sheep and goat [Forrest and Hickford, 2000; for review, see Moens et al., 2010].

The β_3-AR differs from classical β_1- and β_2-AR in their regulatory properties. It is known that desensitization of β_1- and β_2-AR responses upon agonist stimulation involves phosphorylation of the occupied receptor, uncoupling and internalization [Summers et al., 1997]. Both β_1- and β_2-AR have serine and threonine residues in the intracellular C-terminus tail that act as substrates for G protein-coupled receptor kinases and for phosphorylation by cAMP-dependent protein kinase (PKA). The β_3-AR lacks a PKA phosphorylation site and has fewer serine and threonine residues in the C-terminus tail which may explain the resistance of the β_3-AR to short term agonist-promoted desensitization [Liggett et al., 1993].

Furthermore, the β_3-AR is activated at higher concentrations of catecholamines than the β_1- and β_2-adrenergic receptors [Lafontan et al., 1994] and once activated the receptor would deliver a more sustained intracellular signal [Granneman, 1995]. Together these data suggest that following prolonged activation by the sympathetic nervous system, the β_3-AR-mediated response might be preserved, whereas the β_1- and β_2-adrenergic mediated responses are diminished.

β_3-ARs are pharmacologically characterized by a set of criteria that include (1) high affinity for selective agonists such as BRL 37344 and SR 58611A [Arch and Kaumann, 1993; Emorine et al., 1994]; (2) partial agonistic activity for several β_1- and/or β_2-AR antagonists, such as CGP 12177A [Liggett, 1992; Blin et al., 1993], bucindolol [Blin et al., 1993] and pindolol [Blin et al., 1993]; (3) atypically low affinity for conventional β-AR antagonists such as propranolol and nadolol. β_3-ARs are blocked by nonselective β-AR antagonists such as bupranolol [Langin et al., 1991] and the selective β_3-AR antagonist SR 59230A [Kaumann and Molenaar, 1996; Arch, 2002].

Recent studies using the β_2-AR agonist, zinterol showed that in primary adipocytes, zinterol was a full agonist at increasing cAMP levels and this effect was totally abolished in adipocytes from β_3-AR knock-out mice. It was also shown that human β_3-AR couples to both Gs and Gi [Sato et al., 2007].

In the heart, β-AR pathways are the primary means of increasing cardiac performance response to acute or chronic stress. However, depending on the tissue, β_3-AR stimulation leads to either opposite or comparable functional effects to those produced by stimulation of β_1- and β_2-ARs, e.g. β_3-AR activation produces a negative inotropic effect in human ventricles [Gauthier et al., 1998] and they are capable of exerting positive inotropic effects in isolated atria [Emorine et al., 1994; Skeberdis VA et al., 1999]. However, their actual contribution to cardiac contractile function has yet to be defined more accurately.

The β_3-AR was shown to possess the same intracellular signaling pathways as β_1- and β_2-ARs, i.e. activation of adenylyl cyclase and cAMP-dependent phosphorylation, e.g. in human atrial myocytes the activation of β_3-AR leads to the phosphorylation of calcium channels and increase of intracellular Ca^{2+} [Emorine et al., 1989; Skeberdis et al., 1999; 2008], suggesting, that the β_3-AR may be coupled to the stimulatory Gs protein as well.

The linkage of the β_3-AR to Gi proteins, may explain why stimulation of this receptor inhibits cardiac contraction and relaxation [Gauthier et al., 1998; Cheng et al., 2001; Gauthier et al., 2000; Kitamura et al., 2000]. Tavernier et al. (2003), found that cardiac overexpression of human β_3-AR in mice reproduces negative inotropic effects. Although the exact physiological and pathophysiological role of the β_3-AR remains uncertain, recent reports propose that in normal heart, β_3-AR participate in NO-mediated negative feedback control over contractility [Gauthier et al., 1998] (Fig 1.2).

It has been shown that β_3-AR in human ventricular muscle stimulated the production of nitric oxide (NO) through the activation of eNOS present in ventricular myocytes as well as endothelial cells [Gauthier et al., 1998; Brunner et al., 2001]. NO causes the generation of cGMP and inhibition of phosphodiesterase 3 (PDE3) and/or activation of PDE2, which can reduce cAMP induced stimulation of contractility (Fig. 1.2). However, β_3-AR agonist activation mechanisms in the cardiovascular system are complex. The response to β_3-AR stimulation differs not only among species but also among different anatomical regions within the myocardium [Gauthier et al., 2000]. For example, a study in isolated rodent atria, illustrated that both cAMP and NO-cGMP (via nNOS) are involved in the β_3-AR mediated positive chronotropic effect of agonists [Sterin-Borda et al., 2006].

It was also demonstrated that modulation of eNOS activity and an increase in NO formation after the application of BRL 37344 is specifically coupled to a stimulation of the cardiac β_3-AR. On the other hand activation of eNOS, either via translocation or via phosphorylation, was absent in $\beta3^{-/-}$ mice after the application of BRL 37344.

β_3-AR coupled NO production via nNOS has recently been demonstrated in diabetic and aged rat hearts [Maffei et al.,2005; Birenbaum et al., 2008]. These two studies suggest that while β_3-AR's functional significance may not be apparent in healthy subjects, it has the capability to signal through nNOS and can become important in altering contractile response to β-AR stimulation in conditions with increased β_3-AR expression.

There is very limited information available on β_3-AR's association with iNOS. The β1-blocker, nebivolol, which is also a β_3-AR agonist, induces NO via an iNOS-dependent manner, not eNOS nor nNOS [Maffei et al.,2005].

In a recent publication, Maffei et al. demonstrated that β3-antagonist SR 59230A inhibits nebivolol-induced NO in an in vitro Langendorff model, suggesting a possible role for β_3-adrenergic receptors in regulating iNOS-dependent NO [Maffei et al.,2005]. There is a need for more research pertaining to this area of study.

1.15 β-AR desensitization and down regulation

The mechanisms involved in receptor desensitization, may be divided into acute (uncoupling) and chronic responses (internalization and downregulation). This phenomenon has been almost exclusively investigated for the β_2-AR. However, the mechanisms can probably be extrapolated to other G-protein coupled receptors regulated by phosphorylation [Hoebeke, 1996]. Mechanisms contributing to desensitization of β-adrenergic receptors include (i) rapid functional uncoupling of the β-AR from the Gsα-protein; (ii) rapid sequestration of receptors away from the cell surface into an as yet ill-defined membrane compartment; (iii) a slow reduction of the total cellular receptor complement, a process that is called down-regulation.

Desensitization of β-adrenergic receptors is an agonist induced process and is often divided into a homologous and heterologous form. Homologous desensitization refers to phenomena which are agonist-specific, i.e. changes which affect only the β-AR. Heterologous desensitization, on the other hand, is a process whereby activation of one type of receptor causes desensitization of other types as well. It has been shown that both PKA-dependent [Harden, 1983; Benovic et al., 1988] and PKA-independent [Shear et al., 1976; Harden, 1983; Benovic et al., 1988] mechanisms contribute to agonist-induced desensitization of β-adrenergic receptors in intact cells. It has been suggested that PKA, which phosphorylates many other proteins and not exclusively the β-adrenergic receptors, plays a major role in heterologous desensitization, whereas βARK and receptor sequestration are more associated with homologous desensitization.

Ligand binding to / stimulation of GPCRs activate G proteins and promotes the dissociation of G proteins into Gα and Gβγ subunits, both of which activate target effectors [Clapham and Neer, 1993; Xiao, 2001]. Gβγ-dependent targets include activation of certain isoforms of adenylyl cyclase [Tang and Gilman, 1991], PI3-K [Naga Prasad et al., 2000] and β-adrenergic receptor kinase (βARK1 or

GRK2) [Koch et al., 1993]. Rapid functional uncoupling of receptors occurs after binding of the agonist to its specific receptor and subsequent phosphorylation of the receptor on its cytosolic domains by protein kinases. This agonist induced process is initiated within seconds to minutes and can be triggered by the phosphorylation of receptors either by cAMP-dependent PKA or by β-adrenergic receptor kinase (βARK1 or GRK2), a specific cytosolic kinase that phosphorylates only the active, agonist-occupied form of the G-protein –coupled receptors [Benovic et al., 1986; Benovic et al., 1990]. Receptor phosphorylation by βARK promotes the binding of another protein, β-arrestin, which prevents the coupling of the Gsα to the adenylyl cyclase, blocking signal transduction [Rapacciuolo and Rockman, 1999].

Recent work suggests that homologous desensitization of GPCRs, triggered by βARK1 phosphorylation and β-arrestin binding, targets receptors to endosomes through an internalization process [Kohout et al., 2003] and that PI3-K and phophoinositide products play a critical role this process [Gaidarov and Keen, 1999]. There is increasing evidence that in addition to leading to receptor desensitization, the βARK1 / β-arrestin complex can lead to the activation of mitogen-activated protein kinase (MAPK) [McDonald et al., 2000].

A final step which is directly related to downregulation of β-adrenergic receptors is the cAMP-dependent destabilization of the receptor mRNA and decreasing synthesis of the receptor molecule [Hadcock, Ros and Malbon, 1989].

1.16 The involvement of PKB/Akt and the mitogen activated protein kinases (MAPK) in cardiac function and protection

1.16.1 PI3-K - PKB/Akt

Phosphoinositide 3-kinase (PI3-Ks) is a family of enzymes that have the unique capacity to function as lipid and protein kinases. Mammalian PI3-Ks have been divided into three classes (I, II and III) based on their substrate specificity, mode of action and molecular structure [Oudit et al., 2004]. Activated PI3-Ks convert phosphatidylinositol-4,5-biphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3), which acts as a second messenger by recruiting various downstream effectors, such as phosphoinositide-dependent kinase-1 (PDK1/2) which phosphorylates and activates a number of kinases including protein kinase B (PKB/Akt) [Burgering and Coffer, 1995] (Fig. 1.3).

Class I PI3-K can be divided into two subclasses. Class IA PI3-K are heterodimers of a 110-kDa catalytic subunit (p110α, p110β, p110δ) and a regulatory subunit of 85 or 55 kDa (p85/p55), whereas class IB PI3-K (PI3-Kγ) comprises of a p110γ catalytic subunit and a p101 regulatory subunit. Subclasses 1A and 1B are the main isoforms expressed in cardiomyocytes [Naga Prasad et al., 2003]. Class 1A PI3-Ks are activated by receptor tyrosine kinase pathways and regulate cardiac growth [Vanhaesebroeck et al., 2001; Luo et al., 2005]. Class 1B (PI3-Kγ) is coupled to GPCR and has emerged as an important regulator of cardiac contractility [Crackower et al., 2002; Nienaber et al., 2003; Patrucco et al., 2004] because of its ability to modulate cAMP metabolism in compartmentalized microdomains within ventricular myocytes [Kerfant et al., 2005]. PI3-Kγ has also been shown to be a critical regulator for the induction of hypertrophy, fibrosis and cardiac dysfunction in response to long term β-AR stimulation in an in vivo mouse model. Thus, PI3-Kγ may represent a novel therapeutic target for the treatment of decreased cardiac function in heart failure [Oudit et al., 2003].

Insulin or IGF-1 signaling is mediated in part via tyrosine kinase phosphorylation of the insulin receptor substrate -1 (IRS-1) and IRS-2 which activates PI3-K and as previously mentioned, PI3-K in turn stimulates the formation of PIP_3 as well as activation of PKB/Akt. In addition, PI3-K can also be stimulated by the platelet-derived growth factor receptor (PDGFR) [Ishii et al., 1994] and epidermal growth factor receptor (EGFR) [Kamohara et al., 1995] (Fig. 1.3).

The convergence of GPCRs and RTK signaling pathways in the activation of PI3-K is supported by observations that at least three RTKs, those for platelet-derived growth factor receptor (PDGFR) [Linseman et al., 1995], epidermal growth factor receptor (EGFR) [Daub et al., 1996] and insulin-like growth factor (IGF-1) [Rao, 1995], become tyrosine phophorylated after GPCR activation. The proximal mediators of GPCRs / RTK transactivation are largely undefined. However, the cytosolic non-receptor tyrosine kinase, Src kinase was shown to play a key role in this transactivation process [Maulik et al., 1996; Johnson et al., 2000].

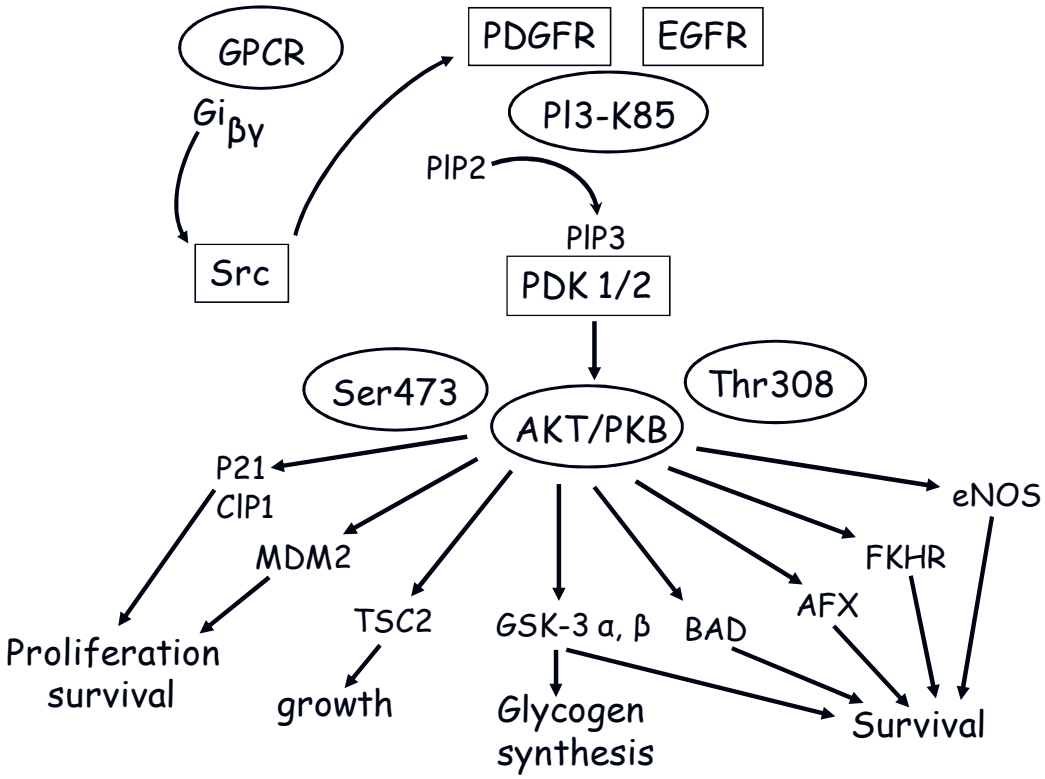


Fig. 1.3: The PI3-K / PKB / Akt signaling cascade with respect to other signaling pathways to deliver cellular regulation (Hawkins and Stephens, 2007)

The serine / threonine kinase Akt (PKB) plays a central role in the regulation of cellular growth, survival and metabolism across many species. In mammalian cells there are 3 distinct Akt isoforms (Akt1,-2, and -3; also known as PKB α, -β, -γ), which are the products of distinct genes [Bellacosa et al., 2004]. Akt isoforms can be activated in response to various growth factors and hormones, including insulin, insulin growth factor-1 (IGF-1), VEGF and β-adrenergic receptor stimulation. In the cardiovascular system, PKB/Akt plays an important role in the regulation of cardiac hypertrophy, angiogenesis and apoptosis [Oudit et al., 2004] and the observation that transient activation of PKB/Akt in cardiomyocytes in vivo and in vitro protects against apoptosis after ischaemia / reperfusion injury, raised hopes that PKB/Akt activation could be an important therapeutic strategy for limiting myocardial injury [Matsui et al., 2001].

Pharmacological evidence suggested that β_1-AR and β_2-AR may exert different effects on cardiac apoptosis [Communal et al., 1999; Zaugg et al., 2000]. This was highlighted in the following study in which it was revealed that stimulation of a single class of receptors, β_2-AR, elicits concurrent apoptotic and survival signals in cardiac myocytes which was shown to be mediated via the Gi-Gβγ- PI3-K- PKB/Akt signaling pathway [Zhu et al., 2001]. Also, the importance of β-AR transactivation of PI3-K- PKB/Akt in cardioprotection was clearly illustrated when transient β-AR stimulation with isoproterenol resulted in increased tyrosine kinase-associated PI3-K activity and phosphorylation of PKB/Akt and p70S6K in cardiomyocytes. This was shown to be mediated via β-AR-mediated transactivation of cardiac PI3-Kα and the sequential involvement of Gαi- Gβγ, Src and PDGFR [Yano et al., 2007; Yano et al., 2008].

Because PKB/Akt is a serine / threonine kinase, which can be activated by β-AR as well as insulin receptor stimulation, it was hypothesized and shown that after β-AR stimulation, PKB/Akt phosphorylates the β-subunit of IRS-1 [Sandra et al., 1979]. Also, β-AR stimulation has a biphasic effect on insulin-stimulated glucose uptake and short-term stimulation induces an additive effect on insulin-induced glucose uptake [Morisco et al., 2005; Liu et al., 1997], which was shown to be PI3-K independent, but occurs via PKA and CaMK.

1.16.2 PI3-K- PKB/Akt signaling in cardioprotection

It has been demonstrated in the myocardium that the activation of this pathway by procedures such as ischaemic pre- or postconditioning or by the administration of pharmacological agents is crucial for the salvage of the ischaemic / reperfused myocardium. It has also been shown that activation of the PI3-K/Akt pathway either before the lethal ischaemic insult [Tong et al., 2000; Mocanu et al., 2002] or at reperfusion following a sustained ischaemic period [Hausenloy et al., 2004] is associated with cardioprotection.

As expected, a large number of pharmacological agents, which are known to activate PI3-K/Akt signaling pathway, have also been shown to protect against myocardial infarction. In this regard, insulin [Jonassen et al., 2001], urocortin [Brar et al., 2001], atorvastatin [Bell and Yellon, 2003a], bradykinin [Bell and Yellon, 2003b], erythropoietin [Tramontano et al., 2003] and glucagon-like peptide 1 [Bose et al., 2005] have all been shown to reduce infarct size following a lethal ischaemic insult. These observations support the hypothesis that pharmacological manipulation and upregulation of this pro-survival kinase is essential to protect the myocardium from ischaemia / reperfusion-induced cell death [Hausenloy and Yellon, 2004].

PKB/Akt once activated, may induce its antiapoptotic effects via the phosphorylation of two types of substrate: (a) the proapoptotic substrates such as glycogen synthase kinase-3-beta (GSK-3β) [Nishihara et al., 2006] or Bad [Jonassen et al., 2001], which, after phosphorylation exhibits an increased affinity for the cytosolic 14-3-3 proteins and becomes inactive by binding to them or (b) the antiapoptotic substrates such as p70s6 kinase [Jonassen et al., 2001] or eNOS (endothelial nitric oxide synthase) [Bell and Yellon, 2003b], which, after phosphorylation become activated and stimulate cellular processes essential for an increased survival.

However, it must also be noted that the chronic activation of this pathway may lead to hypertrophy. As such there appears to be a fine balance between the potentially beneficial effects of activating this signaling pathway acutely and the potentially harmful effects of sustained activation of this pathway [Franke et al., 2003]. The principal factor protecting against the long-term activation of the PI3-K/Akt pathway in normal cells is PTEN (phosphatase and tensin homolog deleted on chromosome ten), a unique dual protein–lipid phosphatase [Leslie and Downes, 2004] which dephosphorylates PKB/Akt.

1.16.3 Mitogen-activated protein kinases (MAPK)

All eukaryotic cells possess multiple MAPK pathways, which coordinately regulate diverse cellular activities, including gene expression, mitosis, differentiation, metabolism, motility, cell survival and apopotosis [Roux and Blenis, 2004]. The following distinct groups of MAPK have been characterized in mammals: extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK 1/2), c-Jun amino-terminal kinases (JNKs) 1, 2 and 3, p38 isoforms α, β, γ and δ , ERKs 3, 4 and 5 [Chen et al., 1992; Kyriakis and Avruch, 2001]. MAPKs can be activated by a variety of stimuli, but in general, ERK1 and 2 are preferentially activated in response to growth factors and phorbol esters, while JNK and p38 MAPK are more responsive to stress stimuli ranging from osmotic shock, ionizing radiation to cytokine stimulation [Enslen and Davis, 2001] (Fig. 1.4).

Each family of MAPKs is composed of a set of three conserved sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). The MAPKKKs, which are serine / threonine kinases, are often activated through phosphorylation and /or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli [Kolch, 2000]. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which in turn stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues. Once activated, MAPKs phosphorylate target substrates on serine or threonine residues followed by proline. Importantly, MAPK cascade specificity is also mediated through interaction with scaffolding proteins which organize pathways in a manner that binds several components simultaneously. A wide range of functions of the MAPKs are mediated through phosphorylation of several substrates, including phospholipases, transcription factors and cytoskeletal proteins. MAPKs also catalyze the phosphorylation and activation of several protein kinases, termed MAPK-activated protein kinases (MKs), which represents an additional enzymatic amplification step in the MAPK catalytic cascades.

The MK family comprises the ≈90-kDA ribosomal S6 kinases (RSKs), the mitogen- and stress-activated kinases (MSKs), the MAPK-interacting kinases (MNKs), MAPK-activated protein kinases 2 and 3 (MK2 and -3, formally termed MAPKAP-K2 and -3) and the MAPK-activated protein kinases 5 (MK5, formally termed MAPKAP-K5). The MKs are related kinases that mediate a wide range of biological functions in response to mitogens and stress stimuli (Fig. 1.4).

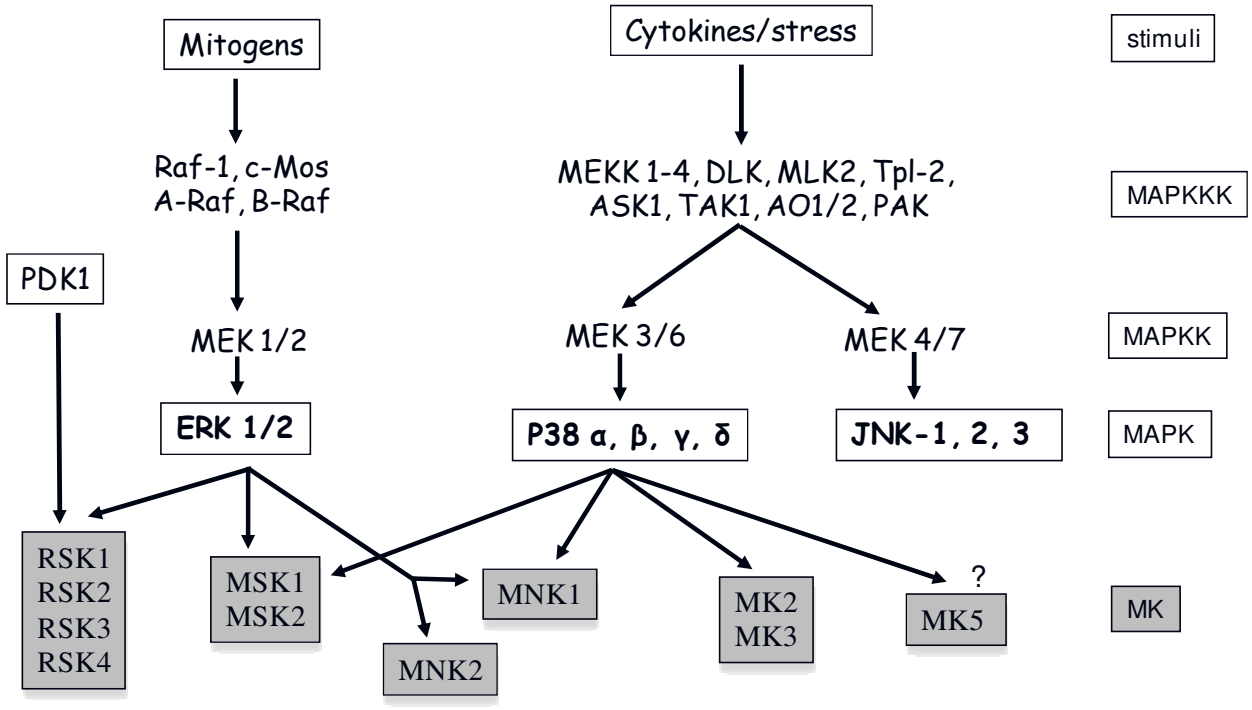


Fig. 1.4: Signaling cascades leading to the activation of MAPKs, subtrate kinase and transcription factors (Armstrong, 2003; Roux and Blenis, 2004)

1.16.3.1 ERK 1/2 or ERK p44/p42 MAPK

The mammalian ERK 1/2 cascade is also known as the classical mitogen kinase cascade and consists of the MAPKKKs A-Raf, B-Raf and Raf-1, the MAPKKs MEK1 and MEK2, and the MAPKs ERK 1 and ERK 2 (Fig. 1.4).

ERK 1 and ERK 2 have 83% amino acid homology and are expressed to various extents in all tissues [Chen et al., 1992]. They are strongly activated by growth factors, serum and phorbol esters and to a lesser extent by ligands of the heterotrimeric GPCRs, cytokines, osmotic stress and microtubule disorganization [Lewis et al., 1998]. Typically, cell surface receptors such as tyrosine kinases (RTK) and GPCRs transmit activating signals to the Raf/MEK/ERK cascade through different isoforms of the small GTP-binding protein Ras [Neufeld et al., 2000; Campbell et al., 1998].

Activation of membrane-associated Ras is achieved by the recruitment of SOS (son of sevenless), a Ras-activating guanine nucleotide exchange factor, allowing Ras to interact with a wide range of downstream effector proteins, including isoforms of the serine/threonine kinase Raf [Geyer and Wittinghofer, 1997]. The exact mechanism of Raf activation is still elusive but it is known to require Ras binding as well as multiple phosphorylation events at the membrane [Chong et al., 2003]. Activated Raf binds to and phosphorylates the dual specificity kinases MEK1 and 2, which in turn phosphorylate ERK 1/2 [Hallberg et al., 1994] (Fig. 1.4).

ERK 1/2 are distributed throughout quiescent cells but upon stimulation a significant proportion accumulates in the nucleus [Gonzalez et al., 1993; Lenormand et al., 1993]. The mechanisms involved in nuclear accumulation of ERK 1/2 are still unclear but nuclear retention, dimerization, phosphorylation and release from the cytoplasmic anchors have been shown to play a role [Pouyssegur et al., 2003]. Activated ERK1/2 phosphorylate numerous substrates in all cellular compartments, including various membrane proteins (CD 12a, Syk and calnexin), nuclear substrates (Src-1, Pax6, NF-AT, Elk-1, MEF2, c-Fos, cMyc and STAT3), cytoskeletal proteins (neurofilaments and paxillin) and several MKs [Chen et al., 2001].

1.16.3.2 p38 MAPK

The p38 cascade consists of several MAPKKKs, including MEKKs 1 to 4, (MEK1-4), MLK2 and -3, DLK, ASK1, Tpl2 (Cot) and Tak1, the MAPKKs, MEK3 and MEK6 (MKK3 and MKK6) and the four known p38 isoforms (α, β, γ and δ) [for review see Kariakis and Avruch, 2003] (Fig. 1.4). In mammalian cells, p38 isoforms are strongly activated by environmental stresses and inflammatory cytokines but not significantly by mitogenic stimuli. Most stimuli that activate p38 also activate JNK, but only p38 is inhibited by the anti-inflammatory drug SB203580, which has been extremely useful in delineating its function [Lee et al., 1994].

MEK3 and MEK6 are activated by a plethora of MAPKKKs which become activated in response to various physical and chemical stresses, such as oxidative stress, UV radiation, hypoxia, ischaemia and various cytokines, such as interleukin-1 (IL-1), and tumor necrosis factor alpha [Chen et al., 2001]. MEK3 and MEK6 show a high degree of specificity for p38, since they do not activate ERK 1/2 or JNK. MEK4 is a known JNK kinase that possess some MAPKK activity toward p38, suggesting that MEK4 represents a site of integration for p38 and JNK pathways [Meier et al., 1996]. While MEK6 activates all p38 isoforms, MEK3 is somewhat selective, since it preferentially phosphorylates the p38α and p38β isoforms. This kinase was shown to be present in both the nucleus and cytoplasm of quiescent cells but upon stimulation the distribution of p38 is not well understood but some evidence suggests that p38 translocates to the nucleus after activation [Ben-Levy et al., 1995]. Some other data show that p38 is also present in the cytoplasm under these conditions [Raingeaud et al., 1995]. p38 activity is critical for normal immune and inflammatory responses [Ono and Han, 2000].

Even though the exact mechanism in p38 in immune responses is only starting to emerge, activated p38 has been shown to phosphorylate several targets, including cytosolic phospholipase A2, the microtubule-associated protein Tau, and the transcription factors ATF1 and -2, MEF2A, Sap-1, Elk-1, NF-κB, Ets-1 and p53 [Kyriakis and Avruch, 2001].

1.16.3.3 JNK MAPK

JNK1, JNK2 and JNK3 (also known as SAPKα, SAPKβ, SAPKγ respectively) exist in 10 or more different spliced forms and are ubiquitously expressed, although JNK3 is primarily expressed in the brain. The JNKs are strongly activated in response to cytokines, UV irradiation, growth factor deprivation, DNA-damaging agents and to a lesser extent by some G protein-coupled receptors, serum and growth factors [Kyriakis and Avruch, 1990]. Like ERK 1/2 and p38, JNK activation requires dual phosphorylation on tyrosine and threonine residues within a conserved Thr-Pro-Tyr (TPY) motif. The MAPKKs that catalyze this reaction are known as MEK4 and MEK7, which are phosphorylated and activated by several MAPKKKs, including MEKK1-4, MLK2 and -3, TPL-2, DLK, TAO1 and -2, TAK1, ASK1 and -2 ([Kyriakis and Avruch, 1990] (Fig. 1.4).

1.16.3.4 The role of MAPKs in cardioprotection

A cardiac protective role of the ERK cascade in the mechanisms of cell survival is supported by the observations that several growth factors such as insulin-like growth factors [Buerke et al., 2001], fibroblast growth factors [Htun et al, 1998], cardiotrophin-1 [Gosh S et al., 2000; Kuwahara et al., 2000] that activate ERKs [Kuwahara et al., 2000], also exert anti-apoptotic effects [Parrizas et al., 1997; Stephanou et al., 1998] or limit ischaemia / reperfusion injury [Buerke et al., 2001; Vogt A et al., 1997; Stephanou et al., 1998].

Also, a key cardioprotective role for ERK-1 (ERK p42) was documented in opioid-induced cardioprotection in rats [Freyer et al., 2001]. This cascade was shown to be activated during ischaemia in the in vivo pig [Barancik et al., 1997; Araujo et al., 2001] and rat [Miyakawa et al., 2001] models, in neonatal rat cardiomyocytes [Yue et al., 2000], as well as in human hearts [Talmor et al., 2000]. ERK activation was also observed during ischaemia and reperfusion in human, bovine, rat and guinea pig hearts by several [Talmor et al., 2000; Yue et al., 2000; Araujo et al., 2001], but not by all investigators [Clerk et al., 1998; Bogoyevitch et al., 1996].

Numerous studies demonstrated the involvement of various MAPK cascades in the cardioprotective mechanisms of ischaemic preconditioning (IPC) [Cohen, Baines and Downey, 2000].

In this context the ERK pathway was shown to be involved in IPC-mediated cardioprotection in pig myocardium, when inhibition of this pathway during the IPC caused inhibition of the infarct size limiting effect of IPC as well as inhibition of the ERK MAPK [Strohm et al., 2000]. Other studies also confirmed the cardioprotective role of ERKs in regulation of both the 'classic' early [Freyer et al., 2002] and late [Ping et al., 1999] phase of IPC-mediated cardioprotection. Essentially ERK MAPK forms part of the prosurvival kinases (PKB/Akt and ERK MAPK) also referred to as the Reperfusion Injury Salvage Kinases (RISK) pathway [Hausenloy and Yellon, 2004]. Pharmacological manipulation and up-regulation of these survival cascades recruited at the time of reperfusion in response to IPC, protect the myocardium from lethal reperfusion-induced cell death and may provide a novel strategy to salvage viable myocardium and limit infract [Hausenloy and Yellon, 2004].

The p38 MAPK pathway belongs to the most investigated, but also the most controversial signaling pathway in the study of myocardial responses to ischaemia / reperfusion. Many studies have shown that activation of p38 MAPK during ischaemia increases myocardial damage [Barancik et al., 2000] and inhibition this kinase proved to be cardioprotective [Pombo et al., 1994; Ma et al., 1999; Mackay and Mochly-Rosen, 1999; Martin et al., 2001]. However, studies of IPC suggest that p38 MAPK confers myocardial protection [Zechner et al., 1997]. It is established that IPC activates p38 MAPK in the isolated rat heart [Maulik et al., 1998; Alkhulaifi et al., 1997] and inhibition of this kinase with SB203580 during IPC abolished cardioprotection, suggesting that p38-MAPK activation contributes to its protective effects. The importance of P38 MAPK activation in IPC was further established when it was shown that raised p38 MAPK activity during ischaemia was associated with increased cardioprotection, which suggested a crucial role for p38-MAPK in IPC [Mocanu et al., 2000].

These controversial results may be due to the fact that four isoforms of the kinase exists. Of the four isoforms, p38α MAPK and β are the most prevalent in the heart, and are similar in structure but have essential functional differences. Opposing functions of p38α and β isoforms were demonstrated in a study using adenoviral-mediated co-expression of p38α- and p38β MAPK in neonatal rat cadiomyocytes. In this study p38α MAPK was shown to have pro-apoptotic effects whereas overexpression of the β isoform results in hypertrophic effects [Wang et al., 1998], whereas others have reported a protective role for p38β MAPK [Saurin et al., 2000; Schulz et al., 2002; Martindale

et al., 2005]. In addition, blocking of the α, but not β isoform, led to an increase in cell viability and cardioprotection [Saurin et al., 2000]. Also in our laboratory, we demonstrated a down-regulation of p38 MAPK during a multi-cycle IPC protocol [Marais et al., 2001] with a subsequent further decrease of p38 MAPK activation during sustained ischaemia [Marais et al., 2001]. The evidence presented to date definitely supports the concept of differential activation of p38-MAPK isoforms by IPC [Saurin et al., 2000].

In contrast to the p38-MAPK pathway, the SAPK / JNK signaling pathway shows a different pattern of activation. Several studies showed that this kinase pathway is moderately or not activated during ischaemia, however, a stronger activation of JNKs was found during reperfusion after a brief ischaemic stimulus [Barancik et al., 1997; Knight and Buxton, 1996; Bogoyevitch et al., 1996]. The precise role of SAPK / JNK in pathophysiology of ischaemic injury remains unresolved.

Recently, it was demonstrated in the in vivo rat model that SAPK/JNK activation is an important component of IPC- or opioid receptor-mediated reduction of infarct size [Fryer et al., 2001]. This is strengthened by the observation that IPC increased SAPK / JNKs activities [Ping P et al., 1999]. Moreover, pharmacological preconditioning with protein synthesis inhibitor anisomycin conferred the IPC-like anti-infarct protection in pigs [Barancik et al., 1999], rabbits [Weinbrenner et al., 1997; Nakano et al., 2000] and rats [Sato et al., 2000] and was found to be accompanied by an activation of SAPK/JNKs only [Barancik et al, 1999], or both SAPK/JNK and p38-MAPK cascades [Weinbrenner et al., 1997; Nakano et al., 2000]. Although the JNK pathway, similar to p38-MAPK pathway, is generally implicated in apoptotic processes, the effects of its activation could be isoenzyme specific and depend on the extent, intensity and timing of JNKs activation [Wang et al., 1998].

1.17 Adenosine (Ado)

Under normal conditions adenosine, an ubiquitous nucleoside, is continuously produced intracellularly and extracellularly and maintained at low intracellular levels (100-300 nM) by the enzymes adenosine kinase and adenosine deaminase. Adenosine is an intermediate metabolite with a short half-life (less than 1,5 seconds) [Honey, Ritchie and Thompson, 1930]. When released by metabolically active or stressed cells (oxygen depletion), the extracellular adenosine levels can rise up to 10 μM [Zetterstrom et al., 1982; Hagberg et al., 1987]. Adenosine was shown to play a major role in the cardiovascular system [Baines et al., 1999], the central nervous system [Fredholm et al., 1995], the gastrointestinal tract [Linden, 1994; Marquardt, 1998], the immune system [Cronstein, 1994], cell growth, proliferation and apoptosis [Burnstock, 2002; Abbracchio et al., 1997; Ohana et al., 2001] under basal conditions or in emergency situations. It also acts as an endogenous modulator of pain [Sawynok, 1998 and 1999].

In the heart, adenosine has potent electrophysiological effects [Drury and Szent-Gyorgi, 1929; Belardinelli, Linden J and Berne, 1989]: the transient, reversible slowing of heart rate (negative chronotropic effects), impairment of atrioventricular conduction (negative dromotropism) and antiarrhythmic effects of adenosine on mammalian hearts were first reported in 1929 by Drury and Szent-Gyorgi. Adenosine-mediated actions in the heart are essentially of two types, those that are cAMP independent (direct effects) and others that are cAMP dependent (indirect effects). The electrophysiological actions of adenosine was found to be directed at the activation of the inward rectifying potassium current, $I_{KACH,Adenosine}$, referred to as $I_{KAdenosine}$, and is thought to underlie the direct effects of adenosine that are observed in the sinoatrial node (SA), atrium, and atrioventricular node (AV) [Belardinelli and Isenberg, 1983; Belardinelli, Giles and West, 1988]. Adenosine also causes a small (12-18%) inhibition of nonstimulated, basal inward current (Ica) in atrial myocytes [Cerbai et al., 1988; Visentin et al., 1990]. These events explain and form the basis of the antiarrhythmic properties of adenosine and were shown to 1) shorten the action potential in atrial cells [Belardinelli, Linden and Berne, 1989] 2) cause sinus slowing and hyperpolarize SA nodal cells to the potassium equilibrium potential [Belardinelli, Giles and West, 1988; West and Belardinelli, 1985] 3) depress the action potential in AV cells [Clemo and Belardinelli, 1986].

Adenosine has an important role in regulating the myocardial oxygen supply – demand balance. This is achieved by increasing oxygen supply through coronary vasodilation and reducing oxygen demand by decreasing myocardial contractility, antagonizing the effects of catecholamines, and in so doing, depressing conduction within the sinoatrial (SA) and atrioventricular (AV) nodes, atrial contractility and ventricular automaticity, respectively [Belardinelli, Linden and Berne, 1989]. Adenosine also attenuates the release of norepinephrine from nerve terminals [Belardinelli, Linden and Berne, 1989; Pelleg, 1985], inhibits oxygen metabolite generation by activated neutrophils and stimulates glycolysis [Belardinelli, Linden and Berne, 1989; Pelleg, 1985]. Potassium conductance studies revealed that the cardiac actions of adenosine were remarkably similar to that of acetylcholine (ACh) [Belardinelli and Isenberg, 1983]. It appears therefore that ACh and adenosine, in addition to their direct effects, function in parallel to oppose the cardiac stimulatory actions of sympathetic stimulation.

1.17.1 The pathways of normoxic and anoxic mediated intracellular and extracellular adenosine production and transport

Three pathways are involved in the production of adenosine, the **first** is from the breakdown of intracellular ATP to AMP and the subsequent dephosphorylation of AMP to adenosine by a cytosolic 5'-nucleotidase [Phillips and Newsholme, 1979] (Fig. 1.5). Adenosine is subsequently released from the cell by a nucleoside transporter [Meghii et al., 1985]. A **second** pathway involves an extracellular membrane bound 5'-nucleotidase that produces adenosine from extracellular sources of ATP / AMP [Bontemps et al., 1993]. Adenine nucleotides released from platelets and endothelial cells are potential sources of adenosine, while extracellular cyclic AMP (cAMP) may also be an important source of the nucleoside during β-adrenergic stimulation [Olsson et al., 1973]. A **third means** of adenosine formation is by the transmethylation pathway. This pathway converts methionine to cysteine and propionyl CoA. The first enzyme uses methionine as a substrate along with ATP. This is followed by the hydrolysis of S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase to produce adenosine and homocysteine as its end products. The relative contribution of each metabolic pathway to the formation of adenosine varies according the experimental condition.

For example, in normoxia a significant fraction of adenosine is derived from the transmethylation / SAH-pathway, whereas during hypoxia the ATP pathway is the main source of adenosine formation [Lloyd et al., 1988] (Fig. 1.5). However, during anoxia this enzyme is completely suppressed. Therefore, during anoxia the increase in adenosine is mainly due to a decreased recycling of adenosine rather than an increase in its formation [Bontemps F et al., 1983].

The elimination of intracellular adenosine occurs via the action of two enzymes (Fig. 1.5). Adenosine can be rephosphorylated into AMP by adenosine kinase, or catabolized by adenosine deaminase into inosine [Bontemps et al., 1993]. Inosine can be degraded to hypoxanthine, xanthine and uric acid or allontoin which can then be excreted out of the cell. Under normoxic conditions, adenosine is completely rephosphorylated to AMP by adenosine kinase and not converted to waste products. Inhibition of adenosine deaminase during anoxic stress also appears to be important in the accumulation of adenosine under these conditions [Phillis et al., 1988].

Ischaemia increases cytosolic calcium levels [Roy and McCord, 1983], which activates Ca^{2+} dependent proteases and subsequently converts xanthine dehydrogenases to xanthine oxidase [Mc Cord, 1984]. On reperfusion, with the readmission of molecular oxygen, the presence of high concentrations of hypoxanthine, the other substrate of xanthine oxidase, may result in a burst of superoxide formation (O-) and other oxygen derived free radicals (Fig. 1.5).

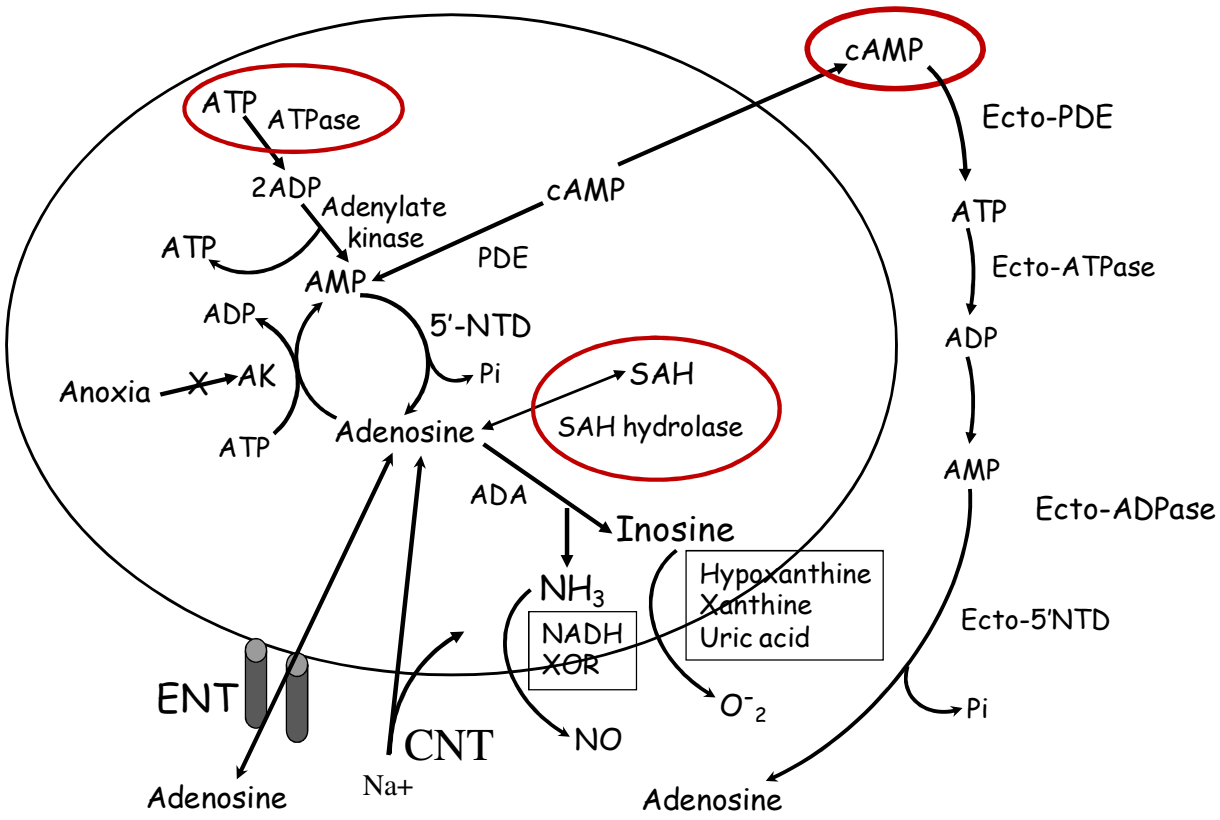


Fig. 1.5: The pathways of normoxic and anoxic mediated intracellular / extracellular adenosine production and transport [lerman and Belardinelli, 1991; Buck and Shin 2003; Buck, 2004]

1.17.2 Adenosine receptors

It is now well established that adenosine acts as an important regulatory molecule through activation of cell surface receptors namely A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (AdoRs), all of which belong to the G-protein-coupled superfamily of receptors [Stiles, 1992; Fredholm et al., 2001; Olah and Stiles 1995; Auchampach and Bolli, 1999]. Collectively, these receptors are widespread in virtually every organ and tissue and represent promising drugs targets for pharmacological interventions in many pathophysiological conditions that are associated with changes in adenosine levels such as asthma, neurodegenerative disorders, chronic inflammatory diseases and cancers [Pierce et al., 2002].

While there is evidence that all of these receptors are expressed in different cell types of the heart and vessels, it should be noted that the A_1 and A_{2A} adenosine receptors are expressed in ventricular myocytes [Marala and Mustafa, 1998; Kilpatrick et al., 2002]. Also, the effects of the adenosine receptors on myocardial responses to ischaemia may not necessarily reflect direct myocyte responses but also indirect actions of other cell types [Peart and Headrick, 2007].

Initially, these receptors were pharmacologically categorized based on their effect on adenylate cyclase (AC) and on their selectivity for agonists and antagonists [Mubagwa and Flemming, 2001]. They have also been differentiated on the basis of amino acid sequences and molecular weights. Subtle interspecies differences exist in the primary structure of each receptor subtype [Linden, 1994; Ralevic and Burnstock, 1998].

The A_1-AdoRs were shown to be coupled to $Gi_{1,2,3}$ and Go [Freissmuth et al, 1991; Jockers et al., 1994] , while the A_3 receptors were shown to couple to $Gi_{2,3}$ and possibly, $Gq_{/11}$ [Palmer TM et al., 1995]. The A_{2A}-AdoRs are generally known to be coupled to the Gs protein, but recent reports [Kull B et al., 2000] show that these receptors can also be coupled to Go_{1f}, $G_{12/13}$ [Sexl et al., 1997] or G15/16 [Offermanns and Simon, 1995], depending on the tissue type.

The A_{2A}-AdoR, referred to as the low affinity receptor [Beukers et al., 2000] is generally coupled to the Gs protein but several other studies implicated signaling via Gq/11, mainly because of this receptor's effects on inositolphosphate production and intracellular calcium, which were sensitive to inhibition by phospholipase C (PLC) blockers, but not to pertsussis toxin (PTX) [Yakel et al., 1993; Feoktistov et al., 1994].

Adenosine receptor signaling can both enhance and inhibit proliferation of various cell types. The outcome of adenosine receptor stimulation with regard to mitogenicity and intracellular signaling may depend on the particular subtype present on the cell under investigation, the strength and duration of stimulation and many more. Indeed, all adenosine receptors are heterologously expressed in different cell types and were shown to interact with MAPK activation at various levels [Schulte and Fredholm, 2003].

1.17.2.1 A_1-AdoR

The A_1-AdoRs are the most extensively studied and well characterized adenosine receptor subtype in relation to cardiac protection. However, there remain some controversies regarding the signaling cascades as well as the A_1-AdoR mediated responses in the heart [Peart and Headrick, 2007]. Initially, it was thought that adenosine, via this receptor type induces myocardial protection through the preservation of ATP (and improved nucleotide repletion on reperfusion), stimulation of glycolysis and normalization of the oxygen supply / demand ratio [Ely and Berne, 1992; Mentzer et al., 1993].

Subsequent investigations have identified essential protein kinase signaling cascades together with putative end-effectors facilitating cardioprotective phenomena such as preconditioning. For the most part, the transduction cascades induced by A_1-AdoR agonism, follow those of several other GPCR systems [Gutkind, 1998; Marinissen and Gutkind, 2001].

As stated previously in (1.3.2), the A_1 and A_3 subtypes are coupled to G proteins (Go, Gi) mediating inhibition of AC- cAMP and its downstream targets. In the avian heart A_1-AdoR stimulation causes a concomitant rise in phosphoinositides indicating that these receptors also activate phospholipase C [Mubagwa and Flameng, 2001]. Subsequent investigations identified signaling cascades, generally linking the A_1-AdoR agonism to the PLC, DAG, IP3 cascade and to several kinase systems, including PKC, MAPKs, PI3-Kinase and PKB/Akt [Mubagwa and Flemming, 2001; Headrick et al., 2003; Buck, 2004], which are shown in Fig. 1.6.

The mitoK_{ATP} channel and PKC can contribute additively to acute adenosine receptor mediated protection [Peart et al., 2003; Peart and Headrick, 2003]. In the ischaemic myocardium PKC translocation was shown to be blocked by A_1-AdoR antagonism [Borst et al., 1999] and on the other hand, PKC inhibitors indicated the involvement of this kinase in A_1-AdoR mediated cardioprotection [Dana et al., 2000; Peart and Headrick, 2003]. Cardioprotection via this receptor subtype was shown to trigger both PKB/Akt and Erk p44/p42 MAPK activation [Germack et al., 2004] and a p38 MAPK pathway has also been identified in the in vivo porcine myocardium [Yoshimura et al., 2004].

These cardioprotective responses / signaling pathways converge on effectors such as the release of Ca2+ from the endoplasmic reticulum via an IP3-gated Ca2+ channel, mitochondrial targets such as the mitochondrial permeability pore (mPTP) [Pepe, 2000; Hausenloy et al., 2002; Murphy, 2004], mediating a reduction in heart rate, contractility and an attenuation of catecholamine stimulation in the heart [Huchinson and Scammells, 2004].

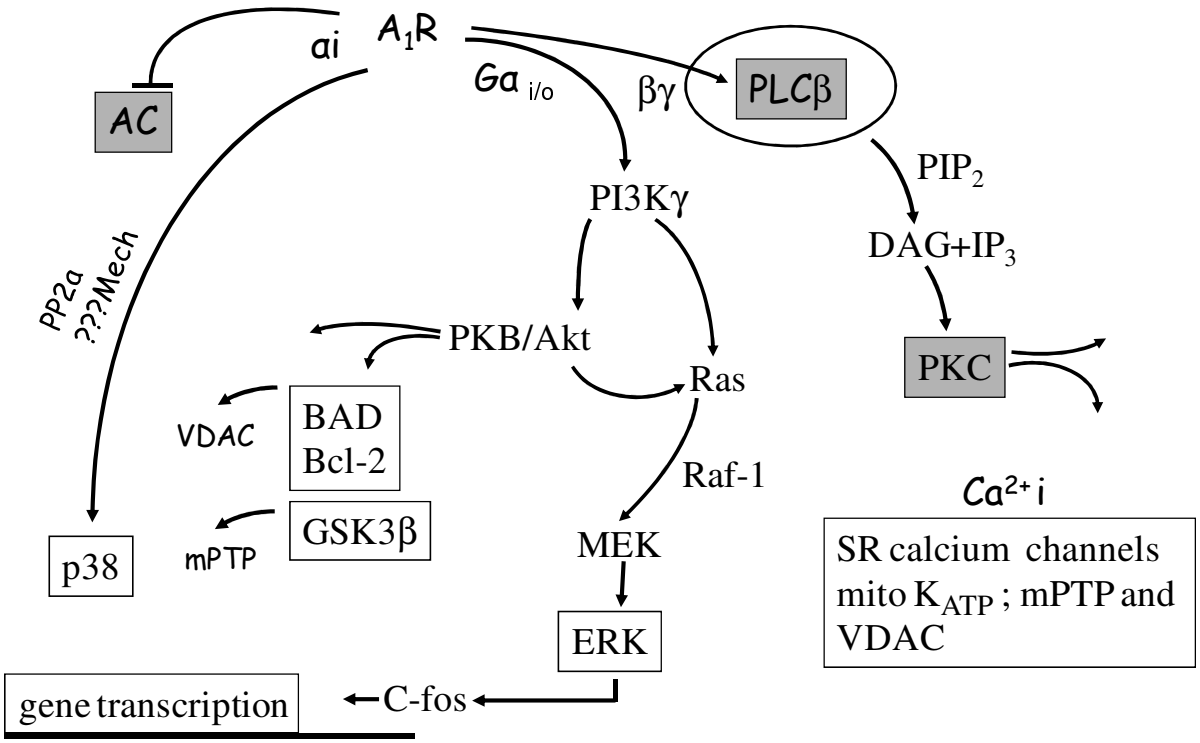


Fig. 1.6: The diagram summarizes possible pathways from the A_1-AdoR to several kinase systems and possible end effectors of cardioprotection [Mubagwa and Flemming, 2001; Headrick et al., 2003; Buck, 2004; Pepe 2000; Hausenloy et al., 2002]

A_1-AdoR-mediated cardioprotection

Cardioprotection due to A_1-AdoR agonism has been observed in all species examined [Reichelt et al., 2005; Morrison et al., 2006]. It is important to note that ischaemia itself can significantly inhibit A_1-AdoR gene expression [Ashton et al., 2003] an effect which may impact on postischaemic outcomes. Involvement of this receptor subtype in IPC was demonstrated in the mouse A_1-AdoR knockout model [Shulte et al., 2004]. Controversy still exists regarding the role of the A_1-AdoR in different protective phenomena, including ischaemic preconditioning (IPC). For example Auchampach et al. (2004) indicated that A_1-AdoR blockade does not modify protection with multiple cycle IPC in canine hearts although they previously demonstrated a role for these receptors with a single cycle IPC in dogs [Auchampach and Gross, 1993]. However, DPCPX, a highly selective A_1-AdoR antagonist failed to abort IPC in the isolated rabbit heart [Liu et al., 1994], which suggested the involvement of another adenosine receptor subtype in the cardioprotective effects of IPC.

The majority of current evidence suggests that the A_1-AdoR mediated reduction in infarct size occurs via activation of PKC and possibly p38 MAPKs with subsequent phosphorylation of the mitochondrial K_{ATP} channel [Baines, Cohen and Downey, 1999; Miura et al., 2000]. However, adenosine can also elicit IPC without involvement of the K_{ATP} channel [for review see Downey et al., 2008]

1.17.2.2 A_{2A}-AdoR

Although, all the adenosine receptor subtypes appear to be expressed in cardiomyocytes [Auchampach and Boli, 1999], the A_{2A}-AdoRs are known to be most prevalent in the coronary vessels. Generally, this receptor subtype is located on smooth muscle and endothelial cells of blood vessels mediating vascular effects of adenosine [Li and Fredholm, 1985]. However, A_{2A}-AdoR mRNA and a functional coupling of this receptor to cAMP accumulation and positive inotropy have been demonstrated in cardiac myocytes [Xu, Stein and Liang, 1996].

The A_{2A}-AdoRs are coupled to the AC- cAMP- PKA pathway via the stimulatory Gs protein. The catalytic subunit of PKA phosphorylates a range of substrates within the myocyte. In the heart, PKA functions to modulate contractilty (cardiac-excitation-contraction coupling - ECC) via phosphorylation of myocyte proteins including the voltage-gated L-type Ca2+ channel, the cardiac ryanodine receptor (RyR2), phospholamban and troponin I [Cannell et al., 1995; Lindemann et al., 1983; Zhang R et al., 1995]. Signaling pathways leading from the adenosine A_{2A} receptor to the positive modulation of ERK p44/p42 MAPK activation [Schulte and Fredholm, 2000; Klinger et al., 2002] are summarized in Fig. 1.7. The pathway from the A_{2A}-AdoR to ERK p44/p42 MAPK is Ras and MEK dependent and was reported to be independent of the Gs- cAMP- PKA -calcium and EGF transactivation pathway [Sexl et al., 1995] with coupling of the receptors to the $G_{12/13}$ protein instead of Gs [Sexl et al., 1992].

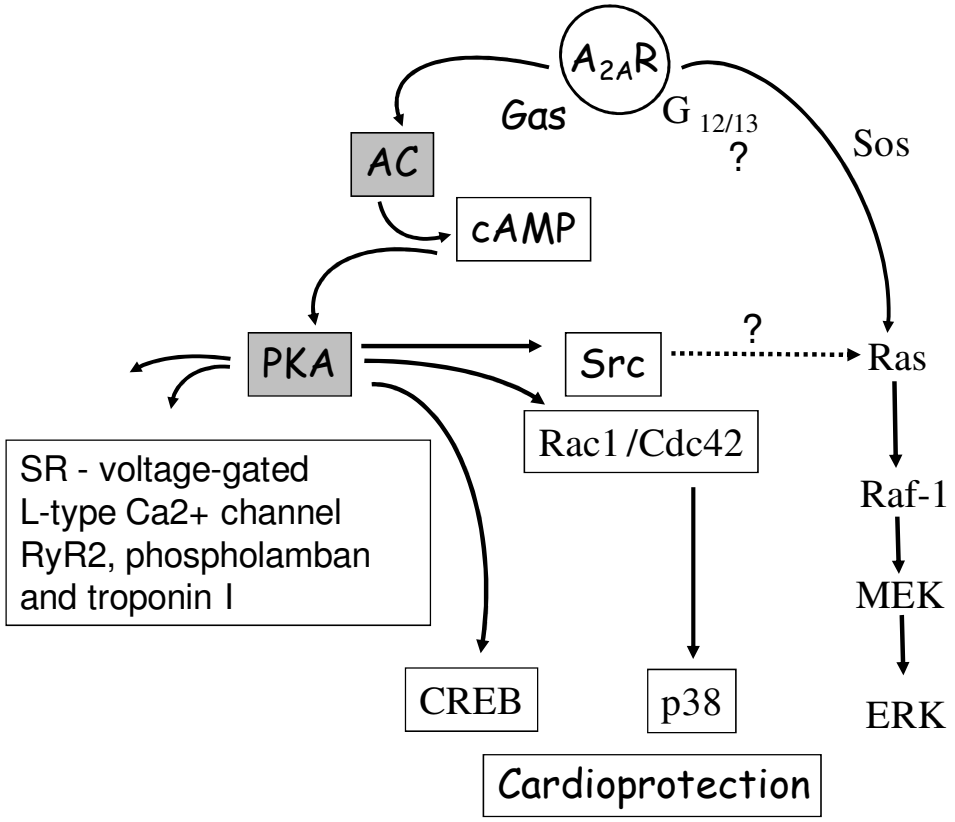


Fig. 1.7: Summary of signaling pathways leading from the A_{2A}-AdoR to the positive or negative modulation several kinase systems and possible end effectors of cardioprotection (Mancusi et al., 1997; Schulte and Fredholm 2000; Klinger et al., 2002)

A_{2A}-AdoRs in cardioprotection

The A_{2A}-AdoRs regulate inflammatory / immune responses in a variety of organs [Cronstein, 1994; Vinten-Johansen et al., 1999; Lukashev et al., 2004] and since inflammation is important in early and late aspects of tissue damage and remodeling with ischaemia and reperfusion, it seems likely that this receptor subtype exerts protective effects in this scenario [Peart and Headrick, 2007].

Mechanisms implicated in A_{2A}-AdoR dependent cardioprotection include inhibition of leukocyte-dependent inflammatory processes [Visser et al., 2000; Sullivan et al., 2001] and direct inotropic actions [Dobson and Fenton, 1997].

An A_{2A}-AdoR-dependent reduction in ischaemia-reperfusion injury has been documented in vivo [Jordan et al., 1997; Lasley et al., 2001] which is consistent with evidence that the these receptors can limit the injurious effects of neutrophil activation [Zhao et al., 2001]. Furthermore, these receptors has also been implicated in the cardioprotective phenomenon of postconditioning [Kin et al., 2005], evident in both the presence and absence of blood cells [Zhao et al., 2003; Yang et al., 2005]. Apart from modification of infarction and inflammation the A_{2A}-AdoRs are also implicated in the process of angiogenesis, which may facilitate postischaemic recovery [Montesinos et al., 2002].

Enhanced vasodilation during reperfusion induced by A_{2A}-AdoR agonism in an experimental setting [Maddock et al., 2001; Peart et al., 2002] may be beneficial as was shown in guinea-pig, pig and murine hearts [Belardinelli et al., 1998; Lew and Kao 1999]. The A_{2A}-AdoR was also indicated to be a key role player in vascular relaxation of porcine coronary smooth muscle, possibly via the p38 MAPK pathway [Teng, Ansari and Mustafa, 2005]. Adenosine A_{2A} and A_{2B} receptor agonists was shown to enhance cGMP through NO generation in coronary endothelial cells [Olanrewaju and Mustafa, 2000].

A recent study highlights another form of receptor cross-talk [Lasley et al., 2006]. In this study the A_{2A}-AdoRs and / or A_{2B}-AdoRs appear to be essential for expression of cardioprotection with preischaemic adenosine A_1 agonism. A_{2A}-AdoR and / or A_{2B}-AdoR antagonism during reperfusion blocks cardioprotection triggered by A_1-AdoR agonist pretreatment. However, the basis for this cross-talk is still unclear [Lasley et al., 2006].

A_{2A}-AdoR stimulation during reperfusion in isolated rabbit heart has been shown to limit infarct size and this cardioprotection was linked to downstream kinase activation such as Erk p44/42 MAPK [Kis, Baxter and Yellon, 2003]. As previously mentioned, ERK p44/p42 MAPK forms part of the prosurvival kinases (PKB/Akt and ERK MAPK) [Hausenloy and Yellon, 2004] activated at the time of reperfusion in response to IPC and which protect the myocardium from lethal reperfusion-induced cell death as previously described.

1.17.2.3 A_{2B}-AdoRs

A_{2B}-AdoRs show a ubiquitous distribution, the highest levels being present in the colon, bladder, followed by blood vessels, lung, eye and mast cells [Puffinbarger et al., 1995; Ralevic and Burnstock, 1998]. The A_{2B} receptor subtype has been implicated in the modulation of inflammatory processes involved in asthma, tumor growth, tissue injury, ischaemia and pain [Holgate, 2005; Abo-Salem et al, 2004; Strohmeier et al., 1995]. However, controversy also exists regarding the presence and function of these receptors in mammalian cardiomyocytes, but functional evidence for their presence has been obtained in avian myocytes [Liang and Haltiwanger, 1995].

Adenosine A_{2A} and A_{2B} receptors were first identified and differentiated by their ability to stimulate cAMP production in brain slices at low (0.1-1uM) and high (>10 uM) adenosine concentrations, respectively [Schulte and Fredholm, 2003]. A_{2B}-AdoRs were only stimulated at high adenosine concentrations and are therefore known as the low affinity adenosine receptor.

A_{2B}-AdoRs are thought to be coupled to AC via the stimulatory Gs protein and to the phosphoinositide metabolism via Gq [Yakel et al., 1993; Feoktistov et al., 1994; Feoktistov and Biaggioni, 1995; Feoktistov and Biaggioni, 1998]. Coupling of the this receptor type to the Gs protein was demonstrated in a study done on guinea pig coronary artery showing adenosine induced hyperpolarization. This effect is mediated through stimulation of AC, resulting in increased cAMP, activation of PKA, phosphorylation and increased K_{ATP} channel conductance [Mutafova-Yambolieva and Keef, 1997], which in turn modulates contractility.

The possible signaling pathways leading from the A_{2B}-AdoR to activation of MAPKs are summarized in the schematic drawing in Fig. 1.8. The results were obtained with different cellular systems, such as receptor-transfected CHO cells [Schulte and Fredholm, 2000], HEK293 cells [Gao et al., 1999], human retinal endothelial cells [Grant et al., 2001] etc. The A_{2B}-AdoRs are involved in proliferation in many cell types and activate all three families of MAPK: ERK p44/p42, p38, and JNK [Gao et al., 1999; Schulte and Fredholm, et al 2000; Schmitt and Stork, 2002]. The different kinetics of these protein phosphorylations, imply that they may be regulated by different upstream events. For example, the A_{2B}-AdoRs in HEK293 cells were shown to activate MAPK ERK p44/p42

via PLC- Ras [Feig and Cooper, 1988] -MEK [Gao et al., 1999] signaling and the involvement of PKC was excluded because of the lack of effect of PKC inhibitors.

In the human retinal endothelial cells studied, A_{2B}-AdoR stimulation leads to a H89-sensitive increase in CREB phosphorylation [Grant et al., 2001]. Another recent study [Schulte and Fredholm, et al 2002] indicates that A_{2B}-AdoRs expressed in CHO cells mediate ERK p44/p42 phosphorylation in a cAMP – dependent but PKA–independent manner involving signaling via PI3-K. On the other hand, both CREB and p38 phosphorylation, which are activated with similar potency as ERK p44/p42 phosphorylation, are dependent on cAMP and PKA but independent of PI3-K. This indicates that Gs-dependent activation of MAPK can occur via A_{2B}-AdoRs as well [Gao et al., 1999; Feoktisov I, Goldstein and Biaggioni, 1999].

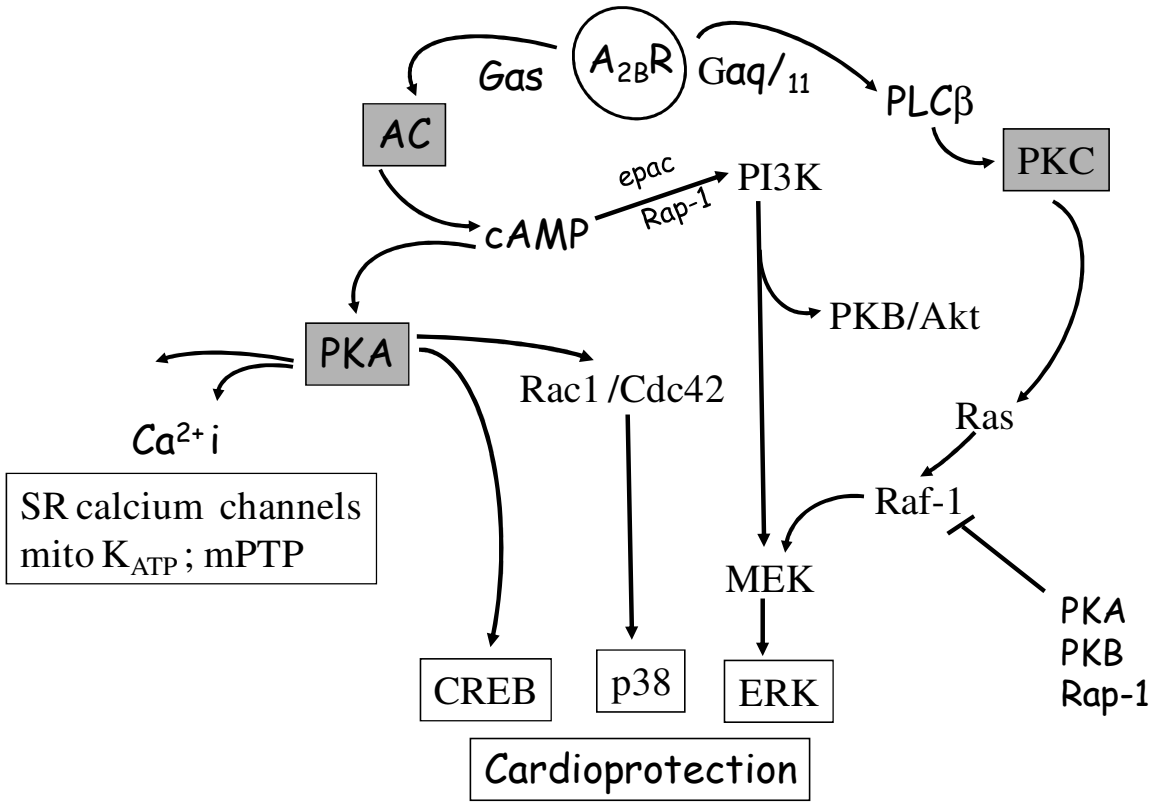


Fig. 1.8: The possible signaling pathways leading from A_{2B}-AdoRs to MAPKs activation (Schulte and Fredholme, 2000; Gao et al., 1999; Grant 2001)

A_{2B}-AdoRs in cardioprotection

The A_{2B}-AdoRs are even less well studied and understood than the A_{2A}-AdoRs in the context of myocardial ischaemia-reperfusion. In addition, there is no direct evidence of the presence of A_{2B}-AdoRs in ventricular myocytes [Marala and Mustafa, 1998; Kilpatrick et al., 2002]. Proposed protective effects of this receptor type in ischaemic – reperfused hearts may stem from responses mediated by other cell types, e.g. the adenosine A_{2B} subtype is known to activate angiogenic factors [Feoktistov et al., 2002] and trigger coronary endothelial growth [Dubey et al., 2002].

Thus, this subtype may play a key role in modulation of vascular growth and tissue remodeling [Chen et al., 2004; Dubey et al., 2001] and consequently play a role in the progression of postischaemic changes, limiting potential fibrosis and facilitating angiogenic growth. Molecular targeting of the A_{2B}-AdoRs impairs neovascularization in noncardiac tissue [Afzal et al., 2003].

In terms of acute cardiac effects, the A_{2B}-AdoRs have been implicated in the infarct limiting effects of postconditioning in rabbit myocardium [Philipp et al., 2006] with the protection based on the involvement of PKC. An important issue of the A_{2B}-AdoR is its apparent low sensitivity to adenosine, which would limit its activation to periods of only excessive adenosine accumulation [Peart and Headrick, 2007]. Thus, based on this known sensitivity, significant A_{2B} receptor activation will occur at times of excessive adenosine accumulation during and following ischaemia. The role of the A_{2B}-AdoR at reperfusion following an IPC protocol was further established when blocking of this receptor at reperfusion abolishes IPC [Solenkova et al., 2005].

1.17.2.4 A_3-AdoR

The A_3-AdoRs are found in high levels in testes [Zhao et al., 1992], kidneys, lungs, heart [Carruthers and Fozard, 1993] and vascular smooth muscle cells [Zhao et al., 1997]. This subtype has almost uniformly been shown to mediate cardioprotective effects in multiple species and models, including man [Jacobson et al., 1998; Linden et al., 2001; Peart and Headrick, 2007; Armstrong and Genote, 1994; Auchampach et al., 1997; Tracey et al., 1997]. The role of the A_3-AdoRs in myocardial protection relates to the cellular location of the receptor [Headrick and Peart, 2005]. On the other hand, prolonged and / or extreme levels of A_3 agonism [Jacobson et al., 1998] has a paradoxical pro-death effect.

Interestingly, the adenosine A_1; A_{2A} and A_{2B} receptors bind to and are antagonized by methylxanthines, such as caffeine, theophylline or enprofyllin, whereas the adenosine A_3 subtype is pharmacological distinct in being insensitive to xanthine based antagonists [Fredholm, 1995; Fredholm et al., 2001].

Although expression of the adenosine A_3 subtype appears to be exceedingly low in the murine myocardium [Black et al., 2002] it was shown to be present in dog, rabbit and rat cardiac tissues [Auchampach et al., 1997; Takano et al., 2001]. Given that the adenosine A_3 receptor itself has not been unequivocally isolated from cardiac tissues [Dixon et al., 1996], myocardial effects of stimulation this receptor may be indirect. The pronounced pharmacological heterogeneity of A_3-AdoR agonist and antagonists across species, complicates the interpretation of experimental findings [Muller, 2003]. Cellular location of the A_3-AdoR raises another issue: A_3 receptor mediated signaling in mast cells (non-cardiac cells) may be detrimental to the myocardium via pro-inflammatory mechanisms [Linden, 1994; Salvatore et al., 2000; Tilley et al., 2000]. However, controversy remains regarding protective signaling coupled to A_3-AdoR agonism. For this reason, the potential mechanisms of action were considered in the context of the varied protective responses, including early and delayed preconditioning and vascular protection [for review see Headrick and Peart, 2005].

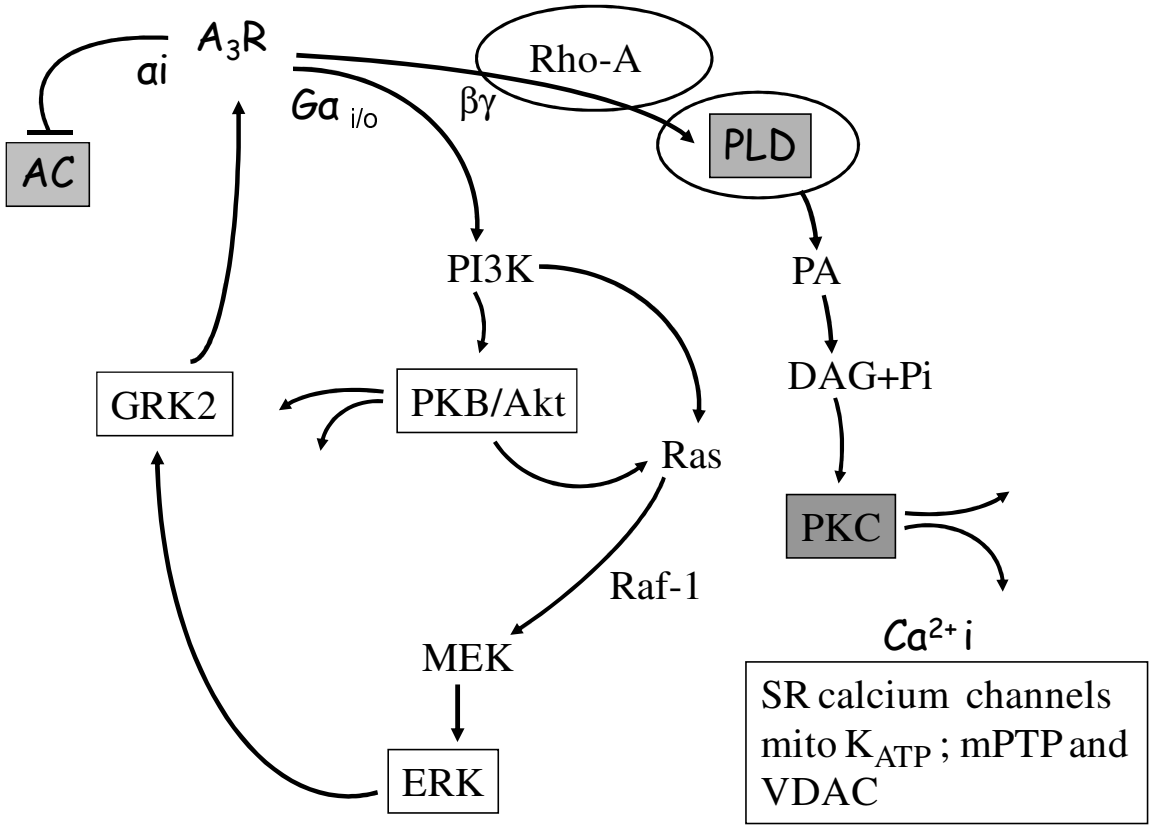


Fig. 1.9: Summary of the signaling pathways leading from the A_3-AdoR to the positive or negative modulation of PKB/Akt and ERK p44/p42 MAPK activation (Fredholm, 1995; Schulte and Fredholm, 2000; Graham et al., 2001; Trincavelli et al., 2002)

A_3-AdoRs in cardioprotection

Initial investigations of the A_3-AdoRs indicated that acute treatment with agonists produced cardioprotection characterized by reduced infarct size [Auchampach et al., 1997; Maddock et al., 2002] and apoptotic cell death [Maddock et al., 2002] associated with enhanced contractile function [Maddock et al., 2003; Gardner NM et al., 2004]. Some reports suggested that the A_3-AdoRs mediated protection occurs post-ischaemia [Jordan et al., 1999; Maddock et al., 2002], while others indicate that pre-ischaemic agonism is required for cardioprotection [Thourani et al., 1999; Flood et

al., 2003]. However, it was also suggested that the anti-infarct response of A_3-AdoR agonism was evident with either pre- or post–ischaemic treatment [Auchampach et al., 2003].

It was illustrated that selective A_3-AdoR agonists could trigger a potent protective response in isolated cardiomyocytes [Armstrong and Genote, 1994; Lee et al., 2001; Chaudary et al., 2004; Germack et al., 2004]. The fact that these receptors could trigger a potent cardioprotective response, despite its low levels shows that this receptor must be efficiently coupled to powerful cell signaling pathways to generate different protected phenotypes observed after or during continuous or transient A_3 receptor agonism [Headrick and Peart, 2005]. Liang et al. (1997) indicated in the avian heart that the A_1-AdoRs and A_3-AdoRs utilize different pathways to activate PKC. While both receptor subtypes induce an accumulation of diacylglycerol (DAG), this effect is more sustained after adenosine A_3 receptor stimulation.

Myocardial A_3-AdoR overexpression generates a protected state similar to that observed following acute treatment with A_3-AdoR agonists [Black et al., 2002]. From these studies, it has emerged that this receptor subtype enhance ischaemic tolerance and specifically overexpression of this receptor type significantly limits ATP depletion during ischaemia [Cross et al., 2002], which is consistent with adenosine-dependent enhancement of myocardial energy state [Fralix et al., 1993]. On the other hand, it has also been found that high levels of adenosine A_3 receptors generate nodal dysfunction and brady-cardiomyopathy [Black et al., 2002] but still confers protection even in such hearts.

A_1-AdoR mediated cardioprotection appears to be dependent upon Gi-dependent phospholipase C (PLC) activation, whereas the A_3-AdoR mediated protection involves preferentially phosphatidylethanolamine which converts phosphatidic acid into DAG [Lee et al., 2001; Parsons et al., 2000]. Thus, the A_3-AdoR response is attributed to selective RhoA-dependent activation of phospholipase D (PLD) [Liang et al., 1997; Lee et al., 2001; Parsons et al., 2000]. However, this has only been confirmed in avian cells and the distal end-effectors have yet to be identified. Differential coupling may explain the different temporal profiles of the adenosine A_1 and A_3 receptor mediated protection. The A_3-AdoR coupled phopholipase D / RhoA pathway mediates a less pronounced but a more sustained activation of downstream kinases relative to the A_1-AdoR coupled phospholipase C

pathway [Lee et al., 2001; Parsons et al., 2000]. A_3-AdoR mediated signaling cascades are summarized in Fig. 1.9.

The A_3-AdoRs can trigger ERK p44/p42 and PKB/Akt activation. However, as with the A_1-AdoRs, the data supports the requirement of ERK p44/p42 but not PKB/Akt in its protective actions during hypoxia-reoxygenation [Germack et al., 2004; Germack and Dickenson, 2005]. The A_3-AdoRs is also involved in the protection via grape-derived resveratrol [Das et al., 2005]. This response was shown to be associated with phosphorylation of PKB/Akt and cAMP response element binding protein (CREB). Inhibition of PI3-Kinase or MEK only partially limited CREB activation and protection, whereas simultaneous inhibition of these kinases and MEK completely blocked CREB activation and protection [Das et al., 2004]. In both adenosine A_1 and A_3 receptors mediated responses, there is evidence of convergence on common mediators / end-effectors such as the mitochondrial K_{ATP} channel [Tracy et al., 1998; Thourani et al., 1999a].

A_3-AdoR mediated preconditioning differs from ischaemic preconditioning which appears to selectively limit necrosis but not stunning [Auchampach et al., 1997; De Jonge et al., 2002]. Although both adenosine A_1 and A_3 receptor activation protect against ischaemic injury, agonists of the A_3-AdoR may be more promising as cardioprotective agents in the clinical setting due to lack of hemodynamic and anti-inflammatory effects, and a more sustained duration of protection than with A_1-AdoR agonists [Liang and Jocobson, 1998; De Jonge et al., 2002]. The molecular basis for early / acute preconditioning in response to A_3-AdoR activation is not well delineated.

However, early or classic preconditioning is thought to involve protein kinases converging on putative end-effectors such as the mitochondrial K_{ATP} channel, mitochondrial permeability transition pore (mPTP), voltage-dependent anion channel (a component of the mPTP), and apoptotic regulatory proteins Bcl-2 and Bad [Murphy, 2004; Cohen et al., 2000].

In addition to early preconditioning effects, the A_3-AdoR mediates delayed preconditioning similar to the A_1-AdoR [Tracey et al 1997; Takano et al., 2001]. It was also indicated that A_3-AdoR agonism in mice 24 hours prior to ischaemia triggers delayed PKC-dependent protection which is reflected in reduced contractile dysfunction and necrosis [Zhao and Kukreja, 2003].

Delayed preconditioning in response to receptor-dependent stimuli is thought to involve kinase-dependent activation of transcription factors which, in turn, induces a range of protein mediators of protection including iNOS, cyclooxygenase-2, aldose reductase, superoxide dismutase and heat shock proteins [Bolli, 2000; Stein et al., 2004]. However, as with early preconditioning, pathways may vary for different stimuli, e.g. aldose reductase inhibition in early preconditioning generates cardioprotection which is thought to be additive to adenosine A_3 receptor agonism [Tracey et al., 2000].

The A_3-AdoRs may also trigger a vasoprotective response. For example, Giannella et al. (1997) illustrated that A_3 receptor agonists reduced the effects of hypoxic coronary hyperemia in guinea pig hearts. The powerful vasoprotective actions of the A_3-AdoR was also illustrated in the ischaemic-reperfused mouse hearts [Flood and Headrick, 2002]. Studies of vascular protection with preconditioning and adenosine support the signaling common to myocardial protection such as the active role of PKC and the mitoK_{ATP} channel [Maczewski and Beresewicz, 1998]. However, the exact mechanism(s) of this vasoprotection remains to be elucidated.

1.17.2.5 Effect of species related differences and experimental models on the reactivity of AdoRs

The participation of adenosine in the cardioprotection of preconditioning was first demonstrated in rabbit hearts by Liu and coworkers in 1991: two chemically different non-selective adenosine receptor antagonists, 8-(p-sulfophenyl) theophylline (SPT) and PD115,199, infused shortly before the preconditioning cycle, successfully blocked IPC protection. Conversely adenosine or a selective A1 agonist, rPIA, followed by washout prior to sustained ischaemia caused a significant reduction in infarct size in these animals. These observations in the rabbit were confirmed by many others (see for example Thornton et al 1992). Further studies focused on the involvement of specific adenosine receptors and subsequent studies by Liu (1994) and Auchampach and their respective coworkers (1997) suggested that both A1 and A3 receptors which couple to the Gi proteins could trigger preconditioning in rabbit hearts.

These data in the rabbit heart were confirmed by others in various species using different approaches. For example, in dogs both PD115,199, a non-selective adenosine antagonist, and

DPCPX, a highly selective A1 receptor antagonist, administered prior to an IPC stimulus, prevented any reduction in infarct size (Auchampach et al., 1993).

However in a later study Auchampach et al (2004) failed to abolish preconditioning in dog hearts using three different A1 receptor antagonists. As was found in rabbit hearts, these observations could implicate involvement of other adenosine receptor subtypes. Involvement of adenosine in preconditioning of pig hearts has been shown by several workers (Schulz et al., 1995; Van Winkle et al., 1994). In addition, protection in both human atrial trabeculae and ventricular myocytes can be blocked with SPT or mimicked with either adenosine or PIA (Walker et al., 1995).

Initial studies could not demonstrate a role for adenosine in preconditioning of the rat heart because adenosine receptor blockade failed to block protection (Liu & Downey, 1992). Also in our own laboratory A1 receptor blockade could not abolish the protection elicited by 1x5 min ischaemia (Moolman & Lochner, unpublished data). The study by Cave et al (1993) cast further doubt on the significance of adenosine in the preconditioning of the rat heart since they could not demonstrate a role for adenosine in the functional recovery of such hearts. This observation has been confirmed in subsequent studies using more selective A1 antagonists, multiple cycle IPC protocols and by using infarct size as endpoint (for reviews see Cohen and Downey, 2008; Ganote & Armstrong, 2000). Despite intensive study the inability of adenosine to mimic preconditioning in the rat heart is still incompletely explained. A possible explanation may be the fact that adenosine activates MAPKAPK2 in the rat heart (Haq et al., 1998) and activation of p38MAPK during ischaemia/reperfusion has been shown to be detrimental in rat hearts (Moolman et al., 2006). It is possible however that adenosine may contribute to a limited extent to IPC in rats since a study by Headrick (1996) showed that an adenosine antagonist could blunt cardioprotection. Interestingly the differences between rat and rabbit hearts with respect to the role of adenosine in preconditioning, were also observed in isolated cardiomyocytes: neither a A1 agonist nor adenosine protected rat cardiomyocytes in vitro, while in contrast, rabbit cardiomyocytes could be preconditioned by adenosine receptor activation (Kin et al., 2005; Kis et al., 2003). However, in a rabbit myocyte model, the A_1-AdoR selective antagonist, DPCPX was unable to fully suppress the protective effect of IPC and had to be associated with an A_3-AdoR antagonist (Liu GS et al., 1994) suggesting that the A_1-AdoR subtype, as well as the A_3-AdoRs are involved in IPC mediated cardioprotection.

Genetic manipulation of mice has permitted further evaluation of the role of adenosine in preconditioning. In A1 knockout mice Lankford and coworkers (2006) observed abrogation of any protective effect of IPC whereas Guo et al (2001) could precondition A3 knockout mice.

However in a carefully conducted subsequent study Eckle and coworkers (2007) studied the ability of mice with deletion of either A1, A2A, A2B or A3 receptors to be preconditioned. In contrast to Lankford, they. were able to protect A1, A2A as well as A3 knockout mice. In fact only in A2B knockout mice IPC protection was abolished. It is suggested that failure to block protection of preconditioning in the A1, A2A, and A3 knockout mice is the result of receptors of multiple agonists released during an IPC protocol which mask the effects of knockout of a particular gene. It is of course also possible that adenosine does not play a role in preconditioning the mouse heart.

Reasons for differing outcomes with adenosine receptor blockade in varied models of IPC are not clear. Evidence has been presented for substantial species differences in adenosine handling and receptor activation (Headrick, 1996), which may determine differing roles for adenosine and contribute to the differing abilities of antagonists to limit these responses. Moreover the affinity and selectivity of adenosine receptor ligands vary across species. The differences between species may also be attributed to other agonists because of differences in their production during the preconditioning protocol. For example in the rat the opioid (or adrenergic?) receptor may be the principal one involved in IPC (Schultz et al., 1995) while in pigs bradykinin plays a major role (Schulz et al., 1995).

In summary, discordant results of adenosine receptor subtype activation in cardioprotection and particularly in the phenomenon of IPC, can largely be attributed to the multiplicity of receptor subtypes in a given cell or tissue, species differences and experimental models [for review see Mubagwa and Flameng, 2001].

1.18 Reactive oxygen species (ROS)

Biological systems have substantial ability to tolerate ROS under normal conditions. However, in the setting of ischaemia and reperfusion, this well-balanced system becomes disturbed. Ischaemia reduces the cellular antioxidant defenses and new danger exists as elevated H_2O_2 becomes increasingly capable of generating the destructive hydroxyl radical ($^{\cdot}OH$) [Hess and Manson, 1984]. Hydroxyl radicals are extremely reactive and may cause lipid peroxidation and damage to membrane proteins and sulfhydryl bonds [Tappel et al., 1973; Ferrari et al., 1982]. Metal ions, particularly iron, may play a role in generating hydroxyl radicals, hence the rationale for metal chelation during oxidative stress [Hess and Manson, 1984; Hiraishi et al., 1994]. Additional oxygen-related free radicals (nitric oxide, peroxinitrite, etc.) can also be produced causing important biological destructive or protective effects.

A vast amount of experimental literature supports the concept of oxidative stress mediated reperfusion injury which occurs when oxygen is reintroduced to ischaemic tissue [Hess and Manson, 1984; Park and Lucchesi 1999; Kilgore et a1., 1999; Zughaib et al., 1993; Opie, 1991; Ambrosio and Tritto, 1999; Weisfeldt et al., 1988]. This type of injury has been reported in the heart, kidney, liver, lung and intestine. Most of the data are derived from the indirect measurements of ROS and from the observations that free radical scavengers improve some aspects of reperfusion injury. For example, DMSO (free radical scavenger) added to the reperfusate of ischaemic rat hearts, decreased CPK release, diminished contraction band formation and preserved cellular morphology [Ganote, Simms and Safavi, 1982]. Superoxide dismutase and catalase administered during reperfusion in an occluded coronary artery canine model produced a reduction in infarct size (Jolly et al., 1984).

At the isolated cellular level it was shown that although cell injury occurs during ischaemia, most of the loss of cell viability occurs during reperfusion [Bolli et al., 1989; Becker et al., 1999; Vanden Hoek et al., 1997; 1998 and 2000]. Consequently, the application of antioxidants at the time of reperfusion may significantly improve the cell survival. However, it cannot be ignored that oxygen radical scavengers might have other complimentary, pharmacological or haemodynamic effects, apart from reducing oxygen toxicity. Furthermore, it is not clear if their positive effects are permanent or merely a delay in the development of necrosis [Zweier et al., 1987].

Despite the fact that ROS are produced primarily upon the reintroduction of oxygen following ischaemia, ROS generation during ischemia per se was also observed [Hess and Manson, 1984; Nohl and Jordan 1986; Becker et al., 1999; Kevin et al., 2003] and studies in cardiomyocytes showed the mitochondria to be the major source [Becker et al., 1999]. This concept is of major importance because these ischaemia-generated ROS appear to play an important signaling role [Carmody and Cotter, 2001] and to trigger the cardioprotective phenomenon of IPC [for review see Vanden Hoek et al., 1998].

1.18.1 Free radicals and oxidants also have protective effects

As discussed above, living organisms have not only adapted to protect against ROS, they have developed mechanisms for the beneficial use of free radicals [Bai and Cederbaum, 2001; Droge, 2002].

Much of the insight of this concept stems from the work done in ischaemic preconditioning. The significance of free radical generation in cardioprotection was demonstrated by the finding that antioxidants abolished the protection of preconditioning [Vanden Hoek et al., 1998, 2000], illustrating a definitive role for these radicals in phenomenon. The signaling pathways that connect the triggering ROS to the induction of preconditioning protection have also been intensely investigated and a central role has been established for the opening of the mitochondrial ATP-sensitive K^+ channel during ischaemia and reperfusion [Tang et sl., 2002]. Following opening of the mitochondrial ATP-sensitive K channel, both NO and ROS appear to be generated in isolated cardiomyocytes which lead to the cardioprotected state [Garlid et al., 2003].

In contrast to the role of ROS in triggering the cardioprotection of ischaemic preconditioning, it should be kept in mind that direct exposure to ROS may lead to contractile dysfunction [Persad et al., 1998; Zeitz et al., 2002], due to a reduction in SERCA activity, Ca^{2+} uptake and sarcoplasmic Ca^{2+} overload [Xu et al., 1997]. Increased intracellular ROS can also increase sodium calcium exchanger (NCX) activity [Goldhaber, 1996] and when this increase is coupled with increased cytosolic sodium (Na^+), it can lead to an increase in intracellular Ca^{2+} overloading.

1.19 Nitric oxide (NO)

Nitric oxide (NO) is a lipophilic, highly reactive, free radical gas with diverse bio-messenger functions. The measured half-life of NO is only 5 seconds in a physiological milieu [Fujimoto et al., 1998; Liu et al., 1998]. NO reacts with a wide range of substrates at a rapid rate and the most preferred chemical targets of NO include reactive oxygen species, other radicals, transition metals, thiols and molecular oxygen. The products of these reactions, for example, peroxy-nitrite (ONOO-), metal-nitrosyl adducts (M-NO), S-nitroso products and other derived species, contribute to the biological activity of arginine-derived NO. Under physiological conditions, peroxy-nitrite is sufficiently stable to diffuse some distance before reacting with target molecules such as membrane lipids, protein sulfhydryl groups [Gerschman et al., 1954; Chen et al., 2000], DNA [Zhang and Snyder, 1995] and cause cellular damage [Neuman et al., 2006].

Evidence also reveals the involvement of NO in a remarkable array of key physiological processes, including regulation of vascular tone [Ignarro et al., 1987; Palmer et al., 1988; Furchgott, 1988], platelet aggregation [Azuma et al., 1986], host defense [Hibbs et al., 1987], inflammation [Tiao et al., 1994; Harbrecht 1995], neurotransmission [Bredt et al., 1990], cell differentiation [Peunova and Enikolopov, 1995; Morbidelli 1996] and apoptosis [Sarih et al., 1993; Mannick et al., 1994].

1.19.1 Nitric oxide synthase (NOS) isoforms and NO synthesis

Mammalian nitric oxide synthases have been characterized and found to comprise three distinct isoforms that are 50 – 60% homologous, and distinguished by their histological expression, susceptibility to arginine-based inhibitors, intracellular localization, NO output and mode of regulation. These isoforms are products of distinct genes [Marsden et al., 1993; Xu et al., 1994] and are functionally categorized by whether their expression is constitutive or inducible.

Type 1 (NOS I / nNOS) was first identified in central and peripheral neuronal tissue, although it is also found in other tissue [Papapetropoulos et al., 1999]. It is constitutively expressed, and its activation depends on elevated intracellular Ca^{2+}, which promotes binding of calmodulin [Lincoln et al., 1997].

Type 2 (NOS 2 / iNOS) is an inducible form of the enzyme, which acts independently of intracellular Ca^{2+} levels [Lincoln et al., 1997; Stuehr et al., 1991]. It still requires calmodulin for its activation, but it binds even in the presence of low Ca^{2+} levels. It is found in vascular smooth muscle cells (VSMCs) [Beasley et al., 1991], macrophages [Lincoln et al., 1997], and to a small extent in platelets [Mehta et al., 1995] and usually requires cytokines or lipopolysaccharide for its activation [Papapetropoulos et al., 1999].

Type 3 (NOS 3 / eNOS), like NOS 1, is constitutively expressed and its activation is dependent upon the binding of calmodulin via increased Ca^{2+} [Forstermann et al., 1991]. However, NOS 3 can also be activated independently of Ca^{2+} elevation, by phosphorylation of various serine residues by a number of protein kinases [Dimmeler et al., 1999; Butt et al., 2000]. This isoform was first identified and cloned from vascular endothelial cells (Lamas et al., 1992; Pollock et al., 1991) and is also found in cardiac myocytes [Balligand et al., 1993] and platelets [Sase and Michel, 1995].

Cardiac myocytes express all three isoforms of NO synthase. NOS 1 and 3 are constitutively expressed and produce low amounts of NO, while NOS 2 is not usually expressed in cardiac myocytes but its expression is induced during the inflammatory response, common in heart failure patients [Ziolo et al., 2001]. All three NOS isoforms catalyze a five-electron oxidation of one of the equivalent guanido nitrogens of L-arginine to yield 1 mol each of nitric oxide and L-citrulline, at the expense of 1.5 mols nicotinamide adenine dinucleotide phosphate (NADPH) and 2 mols dioxygen [Lane and Gross, 1999]. The reaction involves two successive mono-oxygenation reactions, with N^{w}-hy-droxy-L-arginine produced as an intermediate. All NOS isoforms contain four prosthetic groups: flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), iron protoporphyrin IX (heme) and tetrahydrobiopterin (BH_4). The flavins are involved in electron storage and delivery, accepting two electrons from NADPH and then delivering single electrons to the heme group within the active site.

1.19.2 The involvement of NO in preconditioning-induced cardioprotection

NO has beneficial as well as detrimental actions on the heart. For example, in a study of ischaemia / reperfusion damage, it was observed that peroxynitrite ($ONOO^-$) and the hydroxyl radical ($^{\cdot}OH$) were formed as result of NO interaction with superoxide ($^{\cdot}O^-_2$) during early reperfusion and that

inhibition of this pathway leads to improved recovery of myocardial function [Naseem et al., 1995; Wang and Zweier, 1996]. It was found that NOS is activated by ischaemia and this activation was rapid during the whole ischaemic episode (particularly in the cytosolic fraction) and decreased significantly / disappears during reperfusion [Depre et al., 1996]. On the other hand, it was shown that if xanthine oxidoreductase (XOR) is presented with inorganic nitrite (NO^-_2) as an alternative substrate, the generation of NO from NO^-_2 protects the myocardium from ischaemia / reperfusion damage [Webb et al., 2004]. In addition, it was illustrated that NO donors decreased myocardial necrosis and decreased the reperfusion-induced endothelial dysfunction [Siegfried et al., 1992].

Pharmacological mimicking with NO donors can bring about the same cardioprotection as elicited by IPC. This can be abolished by administration of L-NAME during the IPC protocol [Lochner et al., 2000]. It also appears that endogenous NO production during an IPC protocol is sufficient to elicit protection [Prendes et al., 2007; Cohen, Yang and Downey, 2006]. Generation of NO also occurs in the cAMP / PKA signaling pathway: β_2-AR stimulation is associated with increased NOS 3 serine phosphorylation levels in endothelial cells [Ferro et al., 1999; Yao et al., 2003]. However, a PKA-independent component of β_2-AR-mediated NOS stimulation also brings about cardioprotection via activation of the ERK p44/p42 MAPK [Daaka et al., 1997; Shizukuda and Buttrick, 2002] as well as the PI3-K-Akt pathway [Chesley et al., 2000; Zhu et al., 2001].

β_3-AR stimulated activation of NO was shown to be accompanied by decreased contractility in humans [Gauthier et al., 2000] and various other experimental models [Cheng et al., 2001; Tavernier G et al., 2003; Barbier J et al., 2007]. The negative inotropic effects β_3-AR stimulation oppose those of the β_1-AR or β_2-AR, at high catecholamine concentrations, serving as a safety mechanism against increased sympathetic drive [Moens et al., 2010]. This negative inotropic effect was shown to be inhibited by non-selective NOS inhibitors. Immunohistochemical staining of ventricular biopsies showed the expression of NOS 3 but not NOS 2, suggesting an interaction between NOS 3 and the β_3-AR [Barouch et al., 2002].

1.20 The involvement of the K_{ATP} channel in cardioprotection

The **sarcolemmal K_{ATP} channel** was first described in cardiac ventricular myocytes [Noma, 1983]. This channel is a complex of two different proteins [Inagaki et al., 1995; 1996]. One subunit is an inwardly-rectifying potassium channel (Kir) subunit and it is thought that four of these subunits combine to form the channel pore. The sulfonylurea (SUR) subunit is the protein which confers a regulatory role as well as sensitivity of the channel to pharmacological agents and ATP [Ashcroft, 1996]. SUR 1 is highly expressed in pancreatic β-cells, while SUR 2 is highly expressed in cardiac and skeletal muscle cells. It is unknown in how many ways these different Kirs and SURs can interact but data suggest different combinations in different tissue types. Currently it is thought that Kir6.2 and SUR 2 form the cardiac sarcoK_{ATP} channel [Inagaki et al., 1996].

K_{ATP} channels are of intermediate conductance and are inhibited by physiological concentrations of ATP. They were originally termed ATP-dependent potassium channels because ATP was the first modulator studied. However, other endogenous modulators have since been identified and are now generally referred to as the ATP-sensitive channel. K_{ATP} channels (**sarcK_{ATP} and mitoK_{ATP} channel**) have also been found to be modulated by pH, fatty acids, NO, SH-redox state, various nucleotides, G-proteins and ligands (adenosine, acetylcholine, benzopyrans, cyanoguanidines, and more) [Edwards and Weston, 1993; Ming, Parent and Lavallee, 1997]. These channels are expressed in numerous tissue types including skeletal muscle, brain, kidney, heart, pancreatic β-cells and smooth cells [Noma, 1983; Edwards and Weston, 1993; Spruce et al., 1985; Treherne and Ashford, 1991], where they serve as metabolic energy sensors.

1.20.1 Properties of the mitochondrial K_{ATP} channel (mitoK_{ATP})

The mitoK_{ATP} channel was first identified in 1991 from single-channel recordings of the inner mitochondrial membrane [Inoue et al., 1991]. Two components of the channel have been identified, a 55kD channel protein and a 63 kD sulfonylurea receptor (SUR), based on its labelling with bodipy-glyburide [Grover and Garlid et al., 2000]. It appears that the mitoK_{ATP} channel has a heteromultimeric structure similar to that of the sarcolemmal K_{ATP} channel. Neither subunit has yet been cloned.

The main function of the mitochondrial potassium cycle is to regulate matrix volume [Garlid, 1998]. The mitochondrial potassium cycle consists of eletrophoretic K^+ uptake and electroneutral K^+ efflux across the inner membrane. The K^+ efflux is mediated by the K^+ / H^+ antiporter [Mitchell, 1961, 1966; Garlid, 1980], while the K^+ influx is mediated by the mitochondrial the K_{ATP} channel and by an inward K^+ leak due to diffusion caused by the high electrochemical gradient favouring inward flux [Garlid, 1980, 1998]. The K^+ / H^+ antiporter cannot sense changes in either of its substrates and it is regulated indirectly by the matrix Mg^{2+} and H^+ to sense changes in matrix volume and consequently, volume must change before the K^+ / H^+ antiporter adjusts to equal the rate of K^+ influx. This will cause transient swelling and results in a higher steady-state volume for as long mitoK_{ATP} channel remains open.

Despite extensive pharmacological evidence that mitoK_{ATP} channels are crucial for IPC [Auchampach et al., 1992; Fryer et al., 2000; Hide and Thiemermann, 1996] the question still remains as to how the opening of the mitoK_{ATP} channels might protect myocytes against ischaemic damage. It was proposed that inner membrane depolarization produced by the increased K^+ conductance may reduce mitochondrial Ca^{2+} entry through the calcium uniport, which in turns blunts mitochondrial Ca^{2+} overload.

K_{ATP} channel openers reverse inhibition of mitoK_{ATP} by ATP, ADP and palmitoyl CoA with $K_{1/2}$ values that are well within the ranges observed for plasma membrane K_{ATP} channels from various tissues [Cook and Quast, 1990]. Thus, the $K_{1/2}$ values are 1 μM and 0.4 μM for cromakalim and diazoxide, respectively [Garlid et al., 1996]. A new understanding of the pharmacology of mitoK_{ATP} inhibitors revealed that glyburide and 5-HD are ineffective in intact, respiring mitochondria [Beavis, Lu and Garlid, 1993] and it was suggested that this was probably dependent on the experimental model and conditions [Grover and Garlid, 2000].

1.20.2 The role of K_{ATP} in ischaemic preconditioning

While all investigators agree on the profound protection of IPC, there is less agreement on the molecular mechanism of protection. Initially it has been proposed that the K_{ATP} channel is the end-effector [Garlid et al., 1997]. The role of the K_{ATP} channels in cardioprotection has been complicated by the presence of these channels in both the cell membrane and the mitochondria.

Studies showing K_{ATP} openers to mimic IPC, are consistent with the notion that these channels are crucial to IPC, but do not prove it. Further proof required the use of pharmacological blockers of this channel. This was first illustrated in a canine model when the sarcolemmal K_{ATP} channel blocker, glyburide (glybenclamide) abolished IPC [Gross and Auchampach, 1992]. This blocker also abolished IPC in man [Tomai et al., 1994]. It is now generally accepted that the mitoK_{ATP} channel plays an important role in triggering IPC. These channels are not the end effectors of protection, but rather their opening before ischemia generates ROS that trigger entrance into a preconditioning state and activation of PKC [Pain et al., 2000].

It is evident that some degree of selectivity exists for K_{ATP} channel blockers. The mitoK_{ATP} blocker 5-HD efficiently abolishes the cardioprotective effects of all K_{ATP} channel openers tested, but has little effect on cardiac **sarcolemmal K_{ATP} channel** [Garlid et al., 1997; McCullough et al., 1991]. It is also of interest that 5-HD is incapable of blocking the vasodilator effects of K_{ATP} channel openers.

Interestingly, blockers of A_1-AdoR and K_{ATP} channel completely abolished IPC in similar animal models when administered alone. In neonatal rat cardiac myocytes it was found that A_1-AdoR activation also activated the K_{ATP} channel through a Gi coupled pathway [Kirsch et al., 1990]. It was also found that the A_1-AdoR agonist, R-PIA reduced infarct size in dogs and this effect was completely abolished by glyburide, suggesting adenosine receptor activation to be upstream of K_{ATP} channel activation [Grover, Sleph and Dzwonczyk, 1992; Van Winkle et al., 1994]. This pathway was also shown to be operative in man [Cleveland et al., 1997].

If it is assumed that adenosine receptor activation is upstream of K_{ATP} channel activation during IPC, then it becomes important to determine the signaling pathway linking the two systems. Adenosine A_1 receptor activation inhibits PKA and activates PKC, both of which are known to be involved with the K_{ATP} channel. PKC has been shown to be involved with activation of sarcolemmal K_{ATP} channel in patch clamp studies and its activation protects the myocardium in a glyburide-reversible manner [Hu et al., 1996; Speechly-Dick, Grover and Yellon, 1995]. Some studies also proved that IPC is associated with action potential duration shortening [Yao & Gross, 1994].

Convincing evidence also exists for a role of the mitoK_{ATP} channels as mentioned above. However, in the intact rabbit heart opening of mitoK_{ATP} channel failed while opening of sarcolemmal K_{ATP}

channel reduced infarct size [Haruna et al., 1998]. Diazoxide, which had been believed to be a mitoK_{ATP}-specific channel opener, failed to protect the heart in *kir6.2–/–* mice, which lacked surface K_{ATP} but preserved mitoK_{ATP} channels [Suzuki et al., 2003]. It could be possible that both the **sarcolemmal K_{ATP}** and **mito K_{ATP} channels** are necessary for IPC. Their protective functions could be complementary, but their importance may vary in different animal species or different experimental conditions.

1.21 Motivation and aims of study

As described previously, transient β-AR activation with ligands such as isoproterenol and dobutamine can mimick ischaemic preconditioning and elicit protection against a subsequent period of sustained ischaemia [Asimakis et al., 1994; Miyawaki and Ashraf, 1997; Nasa and Takeo, 1997; Lochner et al., 1999; Frances et al., 2003; Robinet, Hoizey and Millart, 2005]. As in the case of IPC, the exact mechanisms whereby activation of the β-AR signal transduction pathway and generation of cAMP during the triggering phase [Lochner et al., 1999], lead to cardioprotection, still need to be elucidated. In addition, it is not clear which of the three currently known β-AR receptors (β_1-, β_2- or β_3-AR) present in heart muscle, is involved in β-AR preconditioning.

A possible mechanism whereby transient β-AR activation elicits a cardioprotective response is via a process of demand ischaemia with associated raised adenosine levels. The role of adenosine in the process of IPC has been thoroughly investigated and the possibility exists that β-PC elicits protection in a similar manner as IPC.

In addition, β-adrenergic stimulation increases both NO [Balligand et al., 1999] and reactive oxygen species generation [Opie et al., 1979], which should be considered as possible mechanisms whereby β-PC mediates a cardioprotective response. As previously outlined, the main source of ROS appears to be the mitochondria where the mitoK_{ATP} channels play an essential role [Costa et al., 2005; Costa and Garlid, 2008] and it is possible that opening of the mitoK_{ATP} channel may act as a signal transduction element also in β-PC [Pain et al., 2000]. With the exception of p38 MAPK [Marais et al., 2001], relatively little is known about the role of the MAPK family in β-PC. In this study attention was focused on the role of ERK p44/p42 MAPK in association with PKB/Akt activation

(RISK pathway) during reperfusion. In addition, whether β-AR is associated with activation of the RISK pathway, as in IPC, still needs to be determined. Although the contribution of JNK to IPC has been investigated [Fryer et al., 2001; Ping P et al., 1999], no information is at present available regarding its role in β-PC and is a subject for future studies.

In view of this, the aims of this study were therefore to unravel the mechanisms involved in the cardioprotective response of β-PC and the following aspects were investigated:

1. The role of β1-, β2- and β3-adrenergic receptor subtypes in β-PC, using appropriate agonists and antagonists as well as the contribution of Gi protein and PKA to β-adrenergic preconditioning,

2. The role of the prosurvival kinases, PKB/Akt and ERK 44/p42 MAPKinase in β-drenergic preconditioning

3. Adenosine release and the relative contributions of the A_1-, A_2-, A_3-adenosine receptor subtypes and associated PI3-K / PKB /Akt and ERK activation during the triggering and mediatory phases of β-PC with isoproterenol

4. Involvement of the mitoK_{ATP} channel in the cardioprotection of β-PC, using the specific inhibitor 5-HD

5. The role of ROS in β-PC using the ROS scavenger N-acetyl-cysteine

6. The contribution of NOS activation and NO release to β-PC, using the NOS inhibitors, L-NAME and L-NNA

Chapter 2

Materials and Methods

2.1 Animals

Male Wistar rats weighing 250 to 350 g were used in this study. Rats were housed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) facility and their handling of laboratory animals was in accordance to institutional ethical guidelines. The rats had free access to food and water prior to anesthesia (30 mg pentobarbital / rat) by intraperitoneal injection.

2.2 Perfusion Technique (Fig. 2.1)

The hearts were rapidly excised and arrested in ice cold Krebs-Henseleit buffer, containing in mM/L: NaCl 119; $NaHCO_3$ 24.9; KCl 4.74; KH_2PO_4 1.19; $MgSO_4$ 0.6; $NaSO_4$ 0.59; $CaCl_2$ 1.25; glucose 10. The buffer was gassed with 95 % O_2 and 5 % CO_2 prior to and during the perfusion protocol.

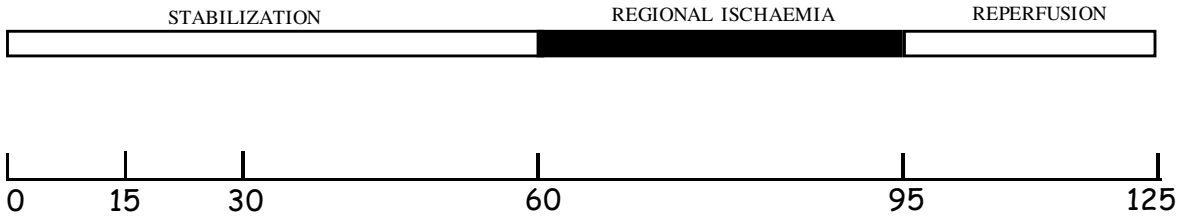
The hearts were mounted on the aortic cannula of the Neely-Morgan perfusion system and retrogradely perfused at 100 cm H_2O for 15 minutes during which time the left atrium was cannulated to allow atrial perfusion at a preload of 15 cm H_2O. Subsequent to the stabilization period, the mode of perfusion was changed to working heart mode for 15 minutes, the left ventricle ejecting against a hydrostatic pressure of 100 cm H_2O (afterload). This was followed by a retrograde perfusion episode of 30 minutes, prior to 35 minutes regional ischemia and a 30 minutes reperfusion period. The myocardial temperature was monitored by insertion of a temperature probe in the left atrium and controlled throughout the experiment. Drugs were applied via a side-arm into the aortic cannula, while the hearts were retrogradely perfused at a pressure of 100 cm H_2O.

2.3 Regional ischaemia

Regional ischaemia was applied by inserting a silk suture underneath and around the proximal LAD. Tightening of the suture resulted in occlusion of the coronary artery, cessation of regional myocardial perfusion and a 33 % reduction in total coronary flow.

Regional cyanosis of the area of the occluded vessel was also used as an indicator of effective occlusion of the vessel. Coronary artery occlusion was maintained for 35 minutes at 36.5 ^{o}C.

Fig. 2.1: Basic perfusion Protocol



2.4 End - points of ischaemic damage

2.4.1 Myocardial Function

Coronary (Qe) and aortic (Qa) flow rates in ml/minute were measured manually. The aortic pressure (mm Hg) was obtained through a side branch of the aortic cannula which was connected to a Viggo-spectramed pressure transducer. The peak systolic pressure (PSP) and heart rate (HR) were obtained from the recordings made. The following parameters were calculated:

- Cardiacoutput (CO) (ml/min) = (Qa + Qe)
- Stroke volume (SV) (ml/min) = (CO / HR)

The mean external power produced by the left ventricle (TW) in mWatts according to Kannengieser and co-workers, 1979:

$$TW = 0.002222(PAO-11.25)(CO)$$

Where

PAO = aortic pressure and CO = cardiac output

Measurements were made before and after ischaemia, during reperfusion. Functional recovery of hearts was determined by expressing post-ischaemic aortic output as a percentage of pre-ischaemic aortic output.

2.4.2 Determination of infarct size

Comprehensive studies on the effect of reperfusion time on infarct size were done on three different occasions and it was found in all series that infarct sizes (expressed as a percentage of the area at risk) were similar in hearts reperfused for 30 and 120 min after 35 min regional ischaemia (2h reperfusion : NPC:36.8±2.21 n=6; PC: 15.94±1.83 n=6; 30 min reperfusion: NPC: 37.82±1.54 n=15; PC: 16.80±2.08 n=13). Thus in this study hearts were routinely reperfused for 30 min before determination of infarct size.

At the completion of regional ischaemia and reperfusion, the silk suture around the LAD was permanently tied and a 0.25% Evan's blue solution infused into the heart to outline viable tissues. Hearts were removed, frozen, cut into 2 mm thick transverse tissue segments and incubated in 1 % triphenyl tetrazolium chloride (TTC) in phosphate buffer, pH 7.4 for 10 minutes. Damaged tissues take on a deep red coloration. Infarcted tissue areas are not stained and have a white colour. The reaction with TTC was stopped by placing the tissue segments in 10 % formalin. Tissue segments were placed between two glass plates and traced to outline the infarcted as well as the area at risk in each ventricular section. The left ventricle area at risk (R) and the area of infarct (I) tissue were determined using computerised planimetry (UTHSCSA Image Tool program, developed at the University of Texas Health Science Center at San Antonio, Texas). The infarct size was expressed as a percentage of the risk zone (I/R%).

2.4.3 Western Immunoblot analysis

Hearts were snap-frozen at the time intervals indicated in the results section. Immunoblotting and detection of total – and phospho - ERK p44 / p42 MAPKinase and PKB / Akt were performed using appropriate antibodies from Cell Signalling Technology. Immunoreactive bands obtained were analysed using densitometry.

2.4.3.1 Preparation of lysates

The lysis or protein extraction buffer contained the following: 20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1 mM β-glycerolphosphate, 1 mM $NaVO_3$, 50 µg/ml PMSF, 10 µg/ml Leupeptin, 10 µg/ml Aprotinin, 1 % Triton. $NaVO_3$ was prepared weekly; PMSF (phenylmethylsulfonyl fluoride) was added last to the buffer. Approximately 30 mg tissue was homogenized in 900 µl lysis buffer using a polytron homogenizer (2x4 seconds). After incubation on ice for 20 min, samples were centrifuged at 14500 rpm for 10 min to obtain the cytosolic fraction. The protein content of each sample was determined using the Bradford method (Bradford, 1975]. The protein concentration of all samples were adjusted and equalized to 20 µg / 9 µl with sample and lysis buffer after which samples were boiled for 5 min and stored at – 20^{o}C.

2.4.3.2 Western Immunoblot analysis

Samples were subjected to electrophoresis on a 12 % polyacrylamide gel (SDS –PAGE) using the standard BIO-RAD Mini Protean III system. The separated proteins were transferred to a Immobilon membrane (Millipore). Proper protein transfer and equal loading were routinely assessed using Ponceau-s staining and corrections were made if necessary. Non-specific binding sites on the membrane were blocked with 5% fat free milk in TBST (Tris-buffered saline + 0.1 % Tween 20) for 1-2 hours at room temperature and incubated overnight in the primary antibodies (Cell Signaling Technology, Massachusettes, USA) that recognize total or phosphorylated proteins: total ERK p44/p42 and phospho-ERK p44/p42 (Tyr-204 / Thr-202); total PKB / Akt and phospho-PKB / Akt (Ser-473). The membranes were washed with TBST (5x5 min) and then incubated with a diluted horseradish peroxidase-labelled secondary antibody (Amersham Life Science, Buckinghamshire, UK).

After thorough washing with TBST, membranes were covered with ECL detective reagents and briefly exposed to a autoradiography film (Amersham Hyperfilm ECL) to detect light emission via a non-radioactive method. Films were analysed using densitometry (UN-SCAN-IT, Silk Scientific Inc, USA).

2.5 Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). For multiple comparisons one-way analysis of variance (ANOVA) was utilised (GraphPad software. PrizmPlus Version 4.0). Post-hoc testing for differences between selected groups was done using Bonferroni's method. In view of the fact that Bonferroni is a strict post-hoc test which carries the risk of type II error, results were also analyzed using the Tukey or Newman-Keuls tests. Similar results were obtained. A minimum of 6 animals were used per experimental group and not more than 4 groups were included in each comparison. A p-value of <0.05 was considered significant.

Chapter 3

Role of β-adrenergic receptors in β-adrenergic preconditioning (β-PC)

The protection against ischaemia elicited by ischaemic preconditioning is associated with a reduction in infarct size and decreased reperfusion arrhythmias, diminished apoptosis and improved functional recovery upon reperfusion [Cohen et al., 2000; Downey and Cohen, 2006]. The protective effects of this phenomenon have been demonstrated to be mediated by G-protein coupled receptors and their associated signal transduction pathways [Das and Das, 2008; Gross and Gross, 2008]. Amongst others, the beta-adrenergic receptors (β-ARs) have been shown to be involved in ischaemic preconditioning [Asimakis et al., 1994; Tong et al., 2005]. In fact, transient β-AR activation with ligands such as isoproterenol and dobutamine mimicked ischaemic preconditioning and elicited protection against a subsequent period of sustained ischaemia – the so-called phenomenon of β-adrenergic preconditioning (β-PC) [Asimakis et al., 1994; Lochner et al., 1999; Miyawaki and Ashraf, 1997]. However, as in the case of ischaemic preconditioning, the exact mechanisms whereby activation of the β-adrenergic signal transduction pathway and generation of cAMP during the triggering phase [Lochner et al., 1999], lead to cardioprotection, still need to be elucidated.

It is also not clear which of the three β-AR receptors (β_1-, β_2- or β_3-AR) present in heart muscle, is involved in β-AR preconditioning. The β_1-AR predominates in heart muscle, the β_1/β_2 ratio being ~80:20 [Bristow et al., 1986], while the expression of β_3-AR is very low in the rat heart [Rozec and Gauthier, 2006]. The β_1-subtype couples primarily to the Gs protein, while the β_2- and β_3- subtypes couple to the Gi protein [Rozec and Gauthier, 2006]. Coupling to Gs may exert a proapoptotic effect, while Gi coupling is anti-apoptotic [Rozec and Gauthier, 2006; Zheng, Hau and Xiao, 2004]. It has also been shown that selective β_3-AR agonists exert negative inotropic effects in human ventricular muscle [Gauthier et al., 1996] and caused activation of the NO pathway and an increase in cGMP [Gauthier et al., 1998].

A number of studies point to a role for β_1-AR activation as trigger in β-AR preconditioning: (i) propranolol (a non-selective β-blocker) and atenolol (a more selective β_1-blocker) abolished

isoproterenol-induced protection, while the selective β_2-blocker IC1-118551 was without effect [Frances et al., 2003];

(ii) the specific β_1-adrenergic agonist xamoterol could elicit protection against ischaemia, which was abolished by atenolol and PKA inhibition [Robinet, Hoizey and Millart, 2005]; (iii) hypoxic preconditioning was attenuated by a β_1-selective blocker, metoprolol [Mallet et al., 2006]; (iv) desflurane and sevoflurane preconditioning was shown to be dependent on β_1-AR activation, since it could be blocked by esmolol and H89, a PKA inhibitor [Lange et al., 2006]. However, a major role for β_1-AR activation as trigger in β-PC cardioprotection may be questioned, in view of its well-established effects on necrosis and apoptosis. The fact that most membrane receptors coupled to the Gi protein are able to elicit cardioprotection, suggests that the β_2-AR may also be a strong candidate for triggering β-PC. Indeed, Tong and coworkers, 2005 found that preconditioning could not be elicited by isoproterenol in transgenic β_2-AR knock out mice. Furthermore, it was found that the Gi inhibitor pertussis toxin blocked isoproterenol-induced improvement in postischaemic function and reduction in infarct size [Tong et al., 2005]. As far as we know no information is available regarding a role for the β_3-AR in β-AR preconditioning.

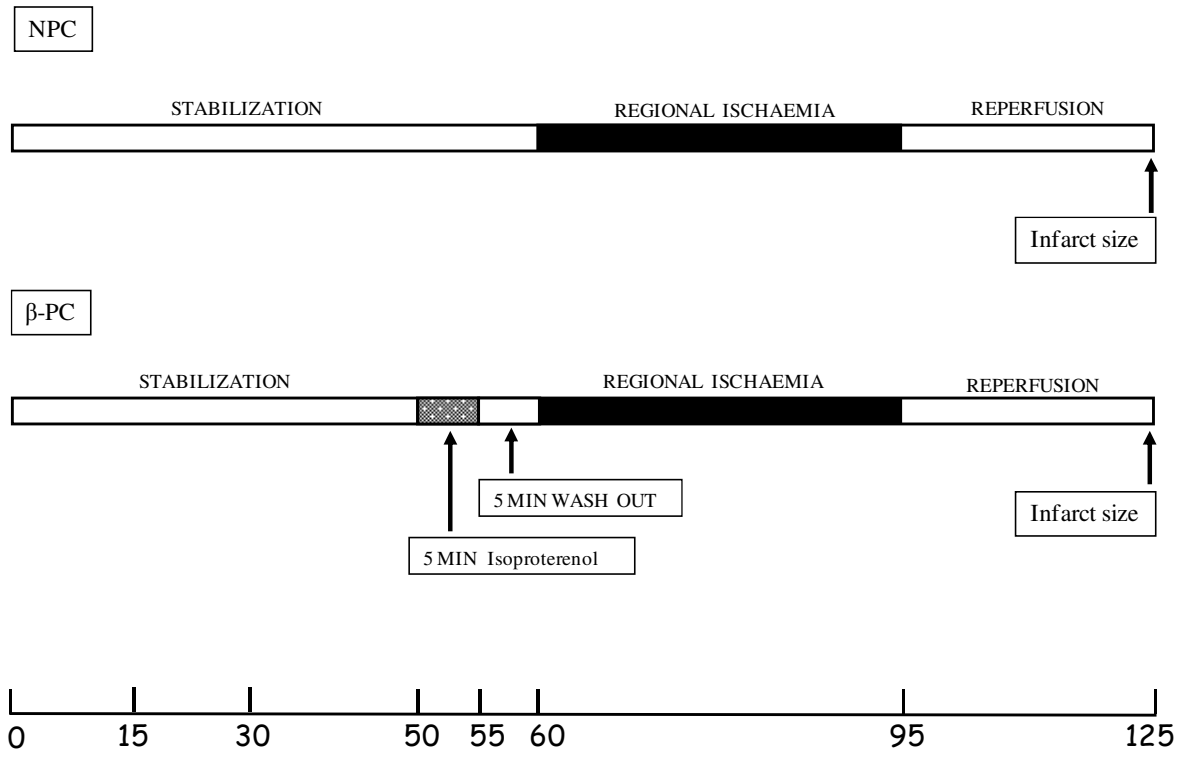
In view of the above, the aim of the present study was to evaluate the respective roles of the β_1-, β_2- and β_3-AR receptors in β-AR preconditioning by using selective agonists and antagonists. Infarct size and functional recovery during reperfusion were used as endpoints. In addition, the contribution of the Gi protein and PKA to the mechanism of isoproterenol-induced cardioprotection was investigated.

3.1 Methods

3.1.1 Investigating the effect of β-adrenergic preconditioning on haemodynamic parameters and myocardial infarct size (Fig. 3.1)

Hearts were subjected to the β-adrenergic preconditioning protocol in the following manner: hearts were subjected to a stabilization period of 15 minute retrograde perfusion which was followed by 15 minutes perfusion in the work heart mode at the end of which haemodynamic parameters were recorded. This was followed by a 20 minute retrograde perfusion, exposure to an appropriate β-AR agonist for 5 minutes and a 5 minute washout episode prior to 35 minutes regional ischaemia and 30 minutes reperfusion at the end of which haemodynamic parameters were recorded. Non preconditioned hearts were subjected to exactly the same perfusion protocol except that the β-AR agonist was not administered prior to regional ischaemia.

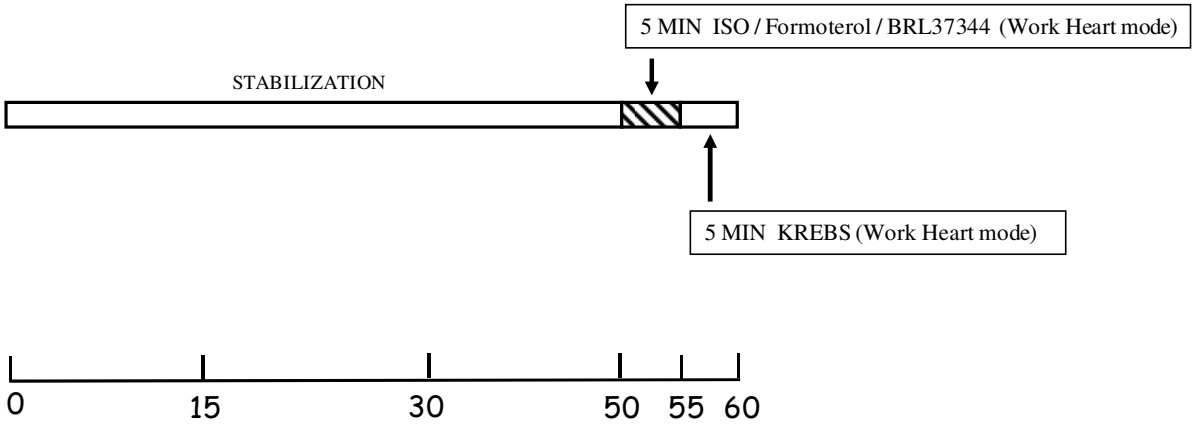
Experimental protocol: (Fig. 3.1)



3.1.2 Investigating the effectiveness of the 5 minutes washout episode after β-AR stimulation (Fig. 3.2)

The effectiveness of the 5 minute washout period applied after transient β-AR stimulation with the β_1/β_2-AR agonist, isoproterenol (ISO) (0.1 μM) / β_2-AR agonist, formoterol (1 nM) / β_3-AR agonist, BRL 37344 (1 μM) was investigated, to ensure that beta-adrenergic stimulation was effectively stopped and that all haemodynamic parameters returned to baseline prior to regional ischaemia. Subsequent to the 15 minutes stabilization period, the mode of perfusion was changed to working heart mode for 15 minutes (haemodynamic parameters recorded). This was followed by a retrograde perfusion episode of 20 minutes. β-adrenergic stimulation with isoproterenol / formoterol / BRL 37344 was applied when the heart was perfused in work heart mode for 5 minutes and haemodynamic parameters recorded. This was followed by a 5 minute washout episode, at the end of which haemodynamic parameters were again recorded.

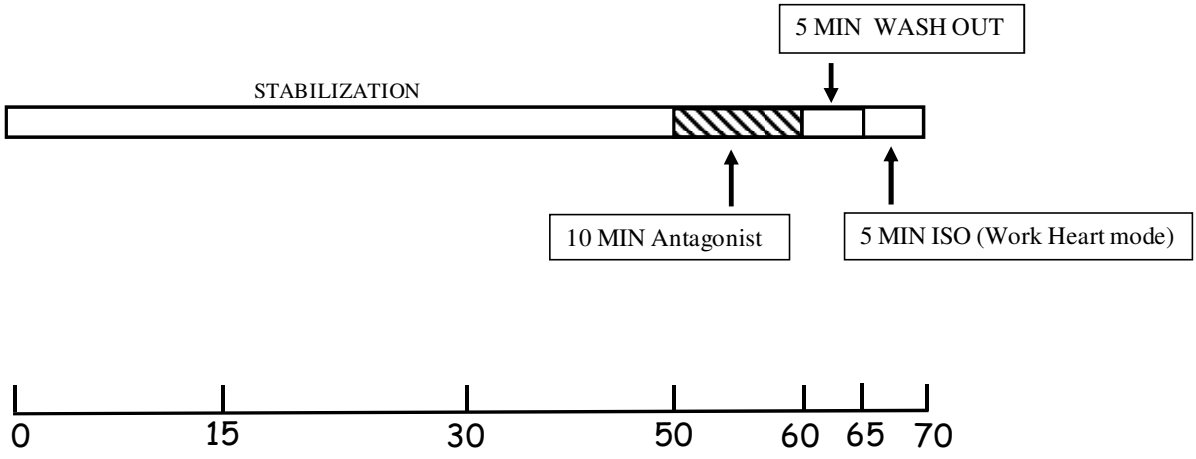
Experimental protocol: (Fig. 3.2)



3.1.3 To test the effectiveness of the 5 minute washout episode after the application of β-adrenergic antagonists on haemodynamic parameters (Fig. 3.3)

It was important to test the effectiveness of the 5 minute washout period applied after the application of various β-AR antagonists, to ensure complete removal and that all haemodynamic parameters returned to baseline prior to regional ischaemia. The β-AR antagonists were dissolved in DMSO and added to the Krebs-Henseleit buffer (final DMSO concentration 0.00023 % v/v). Subsequent to the 15 minutes stabilization period, the mode of perfusion was changed to working heart mode for 15 minutes and the haemodynamic parameters recorded. This was followed by a retrograde perfusion episode of 20 minutes. The β-AR antagonist was then applied for 10 minutes (CGP-20712A; β_1-ARs antagonist, 300 nM; ICI 118,551; β_2-ARs antagonist, 50 nM; SR 59230A; β_3-ARs antagonist, 100 nM). This was followed by a 5 minute washout period in retrograde mode, followed by a 5 minute episode of β-AR stimulation with isoproterenol (working heart) and recording of the haemodynamic parameters.

Experimental protocol: (Fig. 3.3)

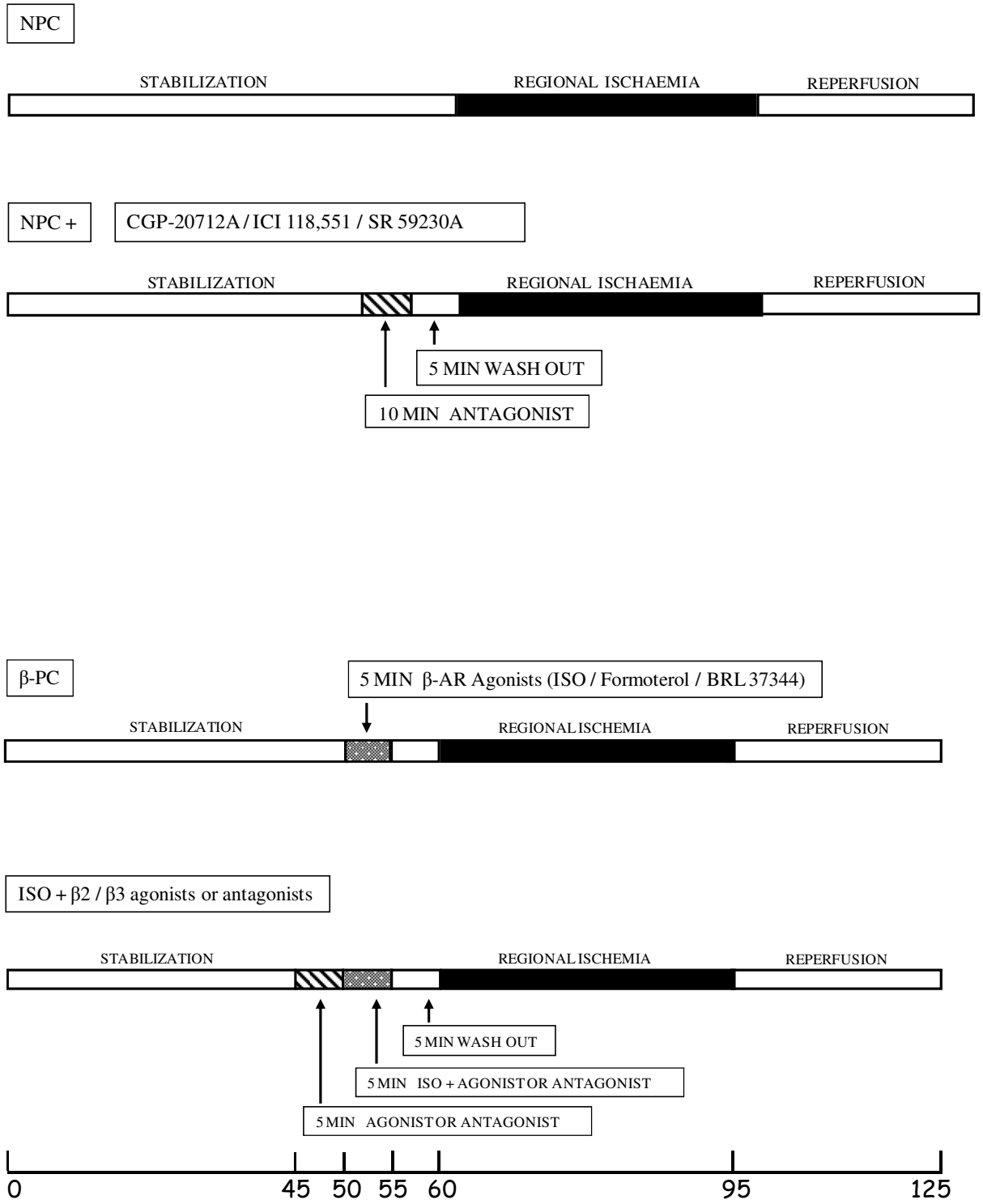


3.1.4 Exploring the β-adrenergic receptor subtype involved in β-adrenergic preconditioning (β-PC) (Fig. 3.4)

Non-preconditioned hearts (NPC) were subjected to a 15 minutes stabilization period after which the mode of perfusion was changed to working heart mode for 15 minutes (haemodynamic parameters recorded). This was followed by a 30 minute retrograde perfusion, 35 minutes regional ischaemia and 30 minutes reperfusion. In addition, non- preconditioned hearts were also exposed to the following β-adrenergic antagonists for 10 minutes followed by a 5 minute washout episode prior to regional ischaemia and reperfusion:
β_1-ARs antagonist (CGP-20712A) (300 nM), β_2- ARs antagonist (ICI 118,551) (50 nM) and β_3-ARs antagonist (SR 59230A) (100 nM). Haemodynamic parameters were recorded at the end of the 15 minute working heart mode prior to regional ischaemia and compared with haemodynamic parameters and infarct size at the end of reperfusion.

β-PC hearts were subjected to a stabilization period of 15 minute retrograde perfusion which was followed by 15 minutes perfusion in the work heart mode at the end of which haemodynamic parameters were recorded. This was followed by a 15 minute retrograde perfusion, isoproterenol 0.1 μM (β1/β2-PC) / formoterol 1 nM (β2-PC) / BRL 37344 1 μM (β3-PC) for 5 minutes and a 5 minute washout episode prior to 35 minutes regional ischaemia and 30 minutes reperfusion. The following β-adrenergic antagonists / agonists were applied prior to isoproterenol for 5 minutes as well as during isoproterenol administration: β_1-AR antagonist (CGP-20712A) (300 nM), β_2-AR antagonist (ICI 118,551) (50 nM), β_2- AR agonist (formoterol hemifumarate) (1 nM), β_3-AR antagonist (SR 59230A) (100 nM), β_3-AR agonists (BRL 37344) (1μM). After a 5 min washout episode, hearts were subjected to 35 min regional ischaemia during which time the temperature was carefully controlled and maintained at 36.5^{o}C. Hearts were retrogadely reperfused for 10 minutes, followed by 20 minutes perfusion in the work heart mode. The haemodynamic parameters recorded at the end of the 15 minute working heart perfusion prior to regional ischaemia were compared with those obtained during reperfusion. Infarct size was measured at the end of reperfusion following regional ischaemia.

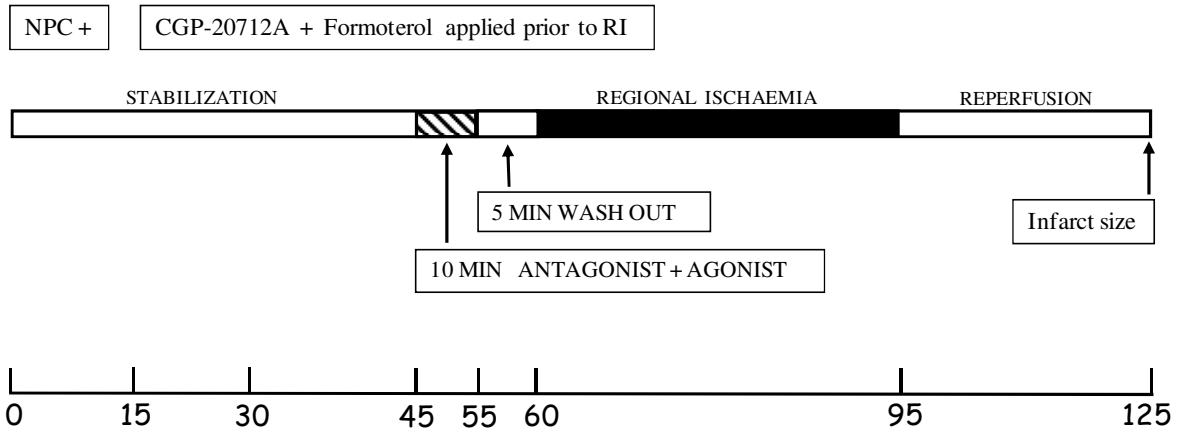
Experimental protocol: (Fig. 3.4)



3.1.5 Investigating the specificity of the β_1-AR antagonist (CGP-20712A) and its effects on β_2-AR stimulation with formoterol (Fig. 3.5)

To investigate the relevance of β_1-ARs in β_2- AR mediated cardioprotection, β_1-AR inhibition was applied in combination with the β_2- AR agonist. Hearts were subjected to a 15 minute retrograde perfusion which was followed by 15 minutes work heart mode. This was followed by another 15 minute retrograde perfusion after which the β_1-ARs antagonist, CGP-20712A (300 nM) and β_2- ARs agonist, formoterol (1nM) were applied for 10 minutes. This was followed by a 5 minute washout episode, 35 minutes regional ischaemia and 30 minutes reperfusion. Haemodynamic parameters were recorded at the end of the 15 minute working heart mode prior to regional ischaemia and compared with those obtained at the end of reperfusion following regional ischaemia. Infarct size was measured at the end of reperfusion.

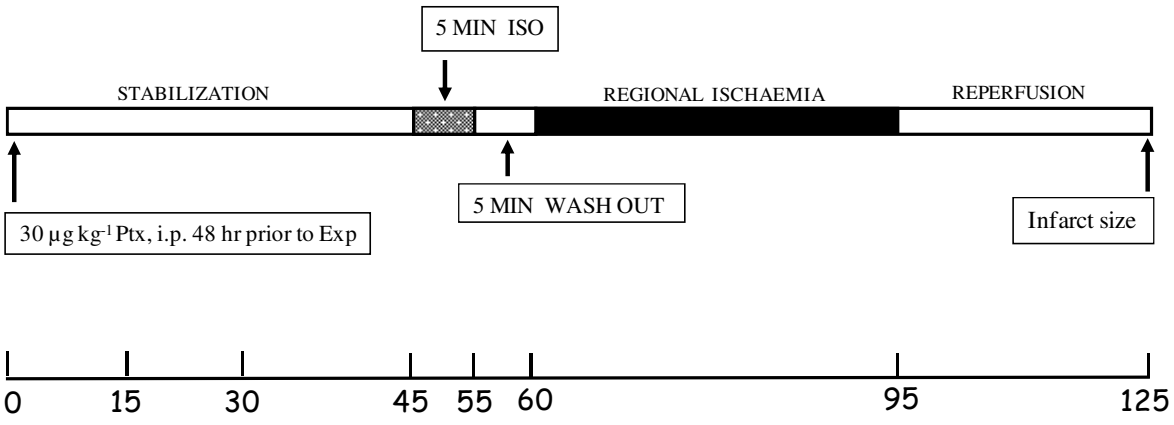
Experimental protocol: (Fig. 3.5)



3.1.6 Investigating the involvement of guanine nucleotide regulatory proteins (Gαi/o) in β-adrenergic preconditioning (Fig. 3.6)

Bordetella pertussis toxin (PTX), which catalyzes ADP-ribosylation of guanine nucleotide regulatory proteins and specifically, Gαi/o, thus functionally uncoupling this binding protein from its associated receptors, was examined to determine its involvement in the protective effect of β-adrenergic preconditioning. Pertussis toxin (Sigma-Aldrich) was diluted in normal saline and injected intra-peritoneally at a concentration of 30 µg/kg BW 48 hours before experimentation. Control animals were injected with an equivalent volume of the vehicle (saline solution). Hearts were preconditioned with ISO, 0.1 µM for 5 minutes followed by a 5 min washout episode, 35 min regional ischemia, 30 minutes reperfusion and the determination of infarct size at the end of reperfusion. Haemodynamic parameters were recorded at the end of the 15 minute working heart perfusion prior to regional ischaemia and compared with those obtained at the end of reperfusion.

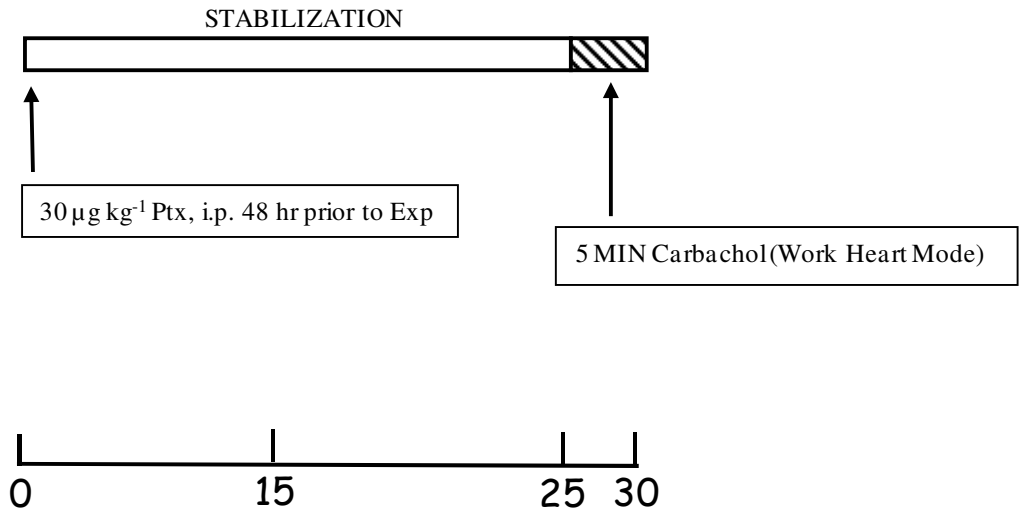
Experimental protocol: (Fig. 3.6)



3.1.7 Investigating the effectiveness of Gαi/o inhibition with carbachol (Fig 3.7)

Parasympathetic stimulation of the heart acts through muscarinic receptors, which mediates its signaling actions via pertussis toxin-sensitive Gαi/o proteins and affects the chronotropic and inotropic status of the heart. Effectiveness of Gαi/o inhibition with PTX was assessed by determining its ability to block the bradycardia response associated with carbachol, a muscarinic / cholinergic receptor agonist. After treatment with PTX (as described in 3.1.6), hearts were subjected to a 15 minute retrograde perfusion which was followed by 10 minutes perfusion in the working mode at the end of which the haemodynamic parameters were recorded. Carbachol (1 μM) was administered for 5 minutes and the haemodynamic parameters recorded at the end of this period.

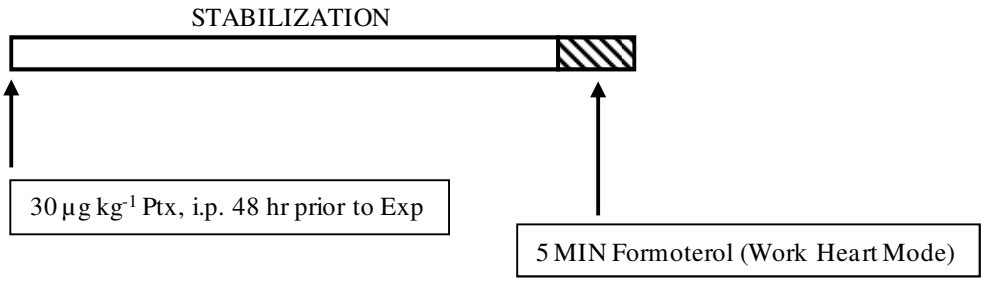
Experimental protocol: (Fig. 3.7)



3.1.8 Investigating the involvement of Gαi/o protein in β_2 –adrenergic receptor stimulation with formoterol (Fig. 3.8)

The β_2-AR couple to Gαs- and Gαi/o-protein, and evidence has accumulated that β_2-AR agonists can differentially activate either Gαs or Gαi/o-protein. The involvement of the Gαi/o regulatory protein in the specific actions of the selective β_2-ARs agonist, formoterol hemifumarate (1 nM) was assessed after treatment with PTX (30 µg/kg BW, ip.) as described in (3.1.6). Hearts were subjected to a 15 minute retrograde perfusion which was followed by 10 minutes perfusion in the working mode at the end of which haemodynamic parameters were recorded. Formoterol was administered for 5 minutes in work heart mode and haemodynamic parameters recorded at the end of this period.

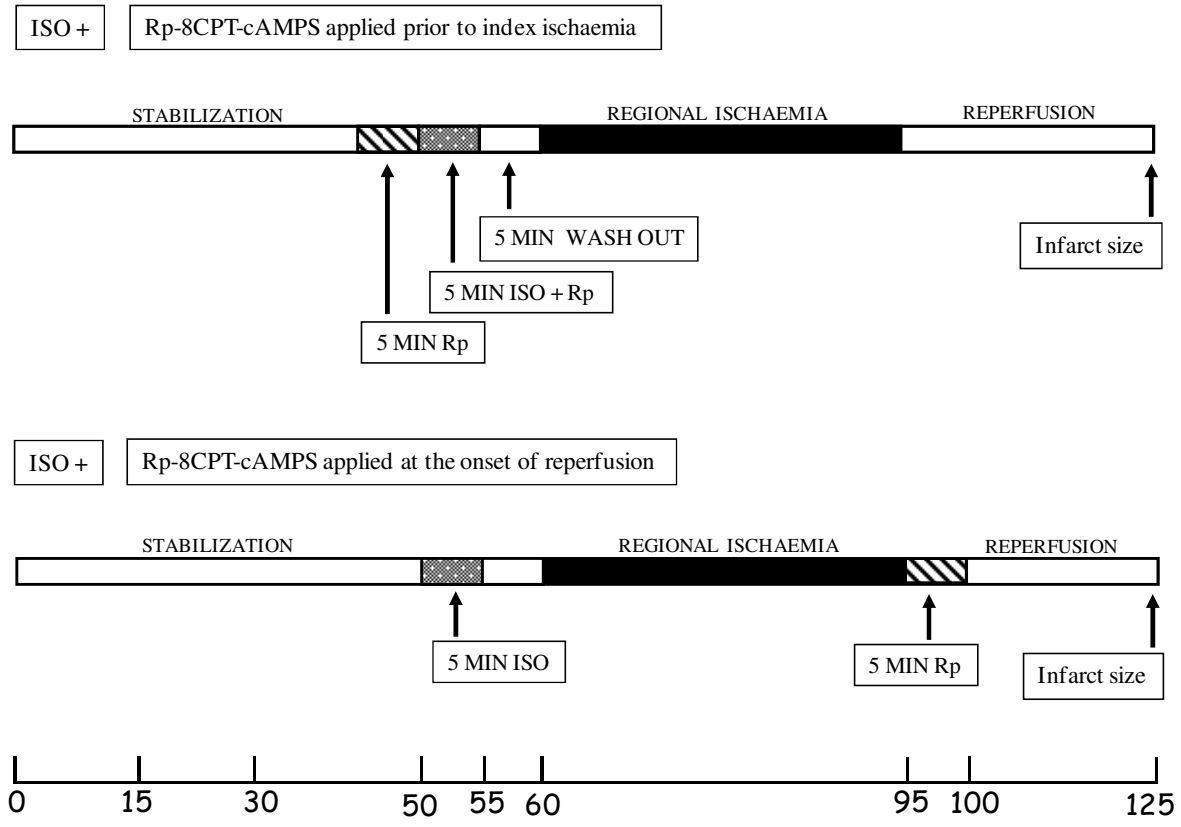
Experimental protocol: (Fig. 3.8)



3.1.9 Investigating the involvement of PKA in β-PC (Fig. 3.9)

The specific PKA inhibitor (Rp-8-CPT-cAMP) was dissolved in distilled water and added to the Krebs-Henseleit buffer (final concentration 16 μM) for 5 minutes prior to and during the 5 minute isoproterenol (0.1 μM) administration, followed by a 5 minute washout episode. Hearts were subsequently subjected to 35 minutes regional ischaemia and 30 minutes reperfusion, followed by the determination of infarct size at the end of reperfusion. In a separate group of experiments the PKA inhibitor (Rp-8-CPT-cAMP) was applied at the onset of reperfusion following regional ischemia. Haemodynamic parameters were recorded at the end of the 15 minute working heart perfusion prior to regional ischaemia and compared with those obtained at the end of reperfusion. Infarct size was determined at the end of reperfusion.

Experimental protocol: (Fig. 3.9)



3.2 Results

3.2.1 The effectiveness of the 5 minute washout episode after β- ARs stimulation (Table 3.1)

Before determining the efficacy of the β-AR agonists used to elicit cardioprotection, it was necessary to determine whether the 5 minute washout period was sufficient to eliminate all traces of the β-AR agonists studied.

During administration of isoproterenol, haemodynamic parameters such CF, AO and total work significantly increased after 1 minute. CF remained elevated, while the AO and total work were significantly reduced after 5 minutes. All haemodynamic parameters returned to baseline values after 5 minutes washout (Table 3.1).

Compared to isoproterenol, the application of the β_2-AR agonist formoterol had no effect any of the haemodynamic parameters during the 5 minute period of administration nor did any of the parameters differ significantly from baseline after a 5 minute washout episode (Table 3.2).

Administration of the β_3-AR agonist BRL 37344 for a period of 5 minutes had no effect on CF while AO and total work were significantly reduced throughout the treatment period. These parameters were shown to return to baseline values after 5 minutes washout (Table 3.3).

In summary, the results obtained with all three β-AR agonists showed that a washout period of 5 minutes was sufficient to remove all traces of the drugs before the onset of regional ischaemia.

Table 3.1: The haemodynamic parameters of isolated rat hearts before, and after 1, 3 and 5 min β-AR stimulation with isoproterenol as well as after 5 min washout

β-AR agonist: Isoproterenol (0.1 µM) (n=6)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
15 min WH	16.67±0.42	40.00±0.42	57.00±2.33	260.00±15.2	97.33±1.49	12.33±0.71
1 min WH+ ISO	23.67±1.66 *	53.50±3.69¥	77.17±4.91¥	303.00±7.33	101.20±2.01	17.55±1.41 *
3 min WH+ ISO	22.17±0.90 *	34.00±3.01 φ	56.17±3.63	311±11.00	95.83±1.49	11.91±0.89 δ
5 min WH+ ISO	20.00± 0.85 ¥	24.00±2.30 *φ	43.00± 2.76	299.00±9.15	93.50±1.47	8.42±0.67 φ
5 min Washout	17.00±0.44	39.67±2.49	56.33±3.07	244.00±3.41	95.50±1.72	12.06±0.79

¥ $p<0.05$ vs 15 min WH * $p<0.01$ vs 15 min WH δ $p<0.01$ vs 1min WH+ISO

φ $p<0.001$ vs 1min WH+ISO

Table 3.2: The haemodynamic parameters of isolated rat hearts before and after 1, 3 and 5 min β_2-AR stimulation with formoterol as well as after 5 min washout

β_2-AR agonist: formoterol (1 nM) (n=6)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
15 min WH	16.67± 0.42	42.83±2.10	59.90±2.39	231.0±0.42	98.33±1.97	13.08±0.70
1 min WH+ Formoterol	17.67±1.20	44.50±3.38	62.50±4.30	232.00±5.00	98.83±1.85	13.77±1.17
3 min WH+ Formoterol	19.00±1.23	45.67±2.94	64.33±3.87	245.00±5.00	98.67±2.15	14.15±1.08
5 minWH+ Formoterol	19.00±1.25	44.50±2.87	63.50±3.66	240.00±6.00	98.00±1.63	13.89±0.96
5 min washout	17.33±0.42	41.67±1.58	59.00±1.95	231.00±12.47	96.00±2.42	12.77±0.68

Table 3.3: The haemodynamic parameters of isolated rat hearts before and after 1, 3 and 5 min β_3-AR stimulation with BRL 37344

β_3-AR agonist: BRL 37344 (1 µM) (n=6)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
15 min WH	16.00±0.0	36.33±1.33	53.33±1.33	221.00±1.76	92.00±1.73	10.72±0.19
1 min WH+ BRL 37344	16.00±0.0	22.00±3.05 *	38.00±3.05 *	262.00±3.51 *	89.33±2.84	7.54±0.54 *
3 min WH+ BRL 37344	15.17±0.83	18.67±3.52 *	34.67±3.52 *	260.00±6.48 *	90.00±2.88	7.16±0.68 ¥
5 minWH+ BRL 37344	15.17±0.83	18.00±3.42 *	33.17±3.56 ¥	266.00±7.89 *	89.33±0.88	6.47±0.55 ¥
5 min washout	16.00±0.0	32.00±1.02	47.33±1.76	212.00±4.35	87.00±0.0	9.22±0.34

*p<0.05vs15 min WH

¥p<0.01vs15 min WH

3.2.2 a The effect of β-adrenergic preconditioning with isoproterenol, formoterol or BRL 37344 on mechanical recovery during reperfusion following regional ischaemia (Table 3.4)

The basal coronary flow (CF), aortic output (AO), cardiac output (CO), heart rate (HR), peak systolic pressure (PSP), and total work during stabilisation prior to regional ischaemia were similar in NPC as well as β-PC groups. The values were pooled for comparison purposes. Similarly, the aortic output, cardiac output and total work measured during reperfusion after RI were significantly reduced in all groups when compared to the baseline values. Hearts preconditioned with either isoproterenol (β1/β2-PC) or the β_2-AR agonist formoterol (β2-PC) exhibited significant increases in AO, CO and total work during reperfusion after RI, when compared with NPC hearts. However, hearts preconditioned with combination of isoproterenol and formoterol (β1/β2-PC + β_2-AR agonist) showed no significant change of haemodynamic parameters measured after RI, illustrating no cumulative effects.

In contrast to isoproterenol and formoterol, the β_3-AR agonist BRL 37344 (β3-PC) did not improve post-ischaemic functional recovery and the values obtained were similar to those of untreated NPC hearts. Interestingly, preconditioning with a combination of isoproterenol and BRL 37344 (β1/β2-PC + β_3-AR agonist) resulted in functional recovery similar to β1/β2-PC alone.

In summary, preconditioning with isoproterenol or the specific β_2-AR agonist, formoterol, resulted in a significant improvement in functional recovery, while the β_3-AR agonist BRL 37344 was without effect.

Table 3.4: Effect of β-adrenergic receptor stimulation on mechanical recovery during reperfusion after 35 min coronary artery ligation

(A) **β-AR agonist: isoproterenol** (0.1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.56±0.34	41.50±1.06	56.84±1.18	254±5.22	100.7±1.70	12.70±0.33
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI (n=18)	15.85±022	40.24±1.02	56.24±1.06	263±5.68	101.2±1.87	12.90±0.42
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

# P< 0.05 vs β-PC After RI

(B) β_2-AR agonist: formoterol hemifumarate (formoterol) (1nM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+β2 agonist After RI (n=6)	15.58±0.40 ✶	29.00±3.09 ✶	45.00±3.23 ✶	251±7.40	92.17±2.07	9.637±0.52 ✶
βPC+β2 agonist After RI (n=6)	16.00±0.0 ✶	21.38±3.85 ✶	37.38±3.85 ✶	268.3±15.41	92.75±3.01	7.60±1.01 ✶

✶p<0.001 vs NPC After RI

(C) **β_3-AR agonist: BRL 37344** (1 μM)

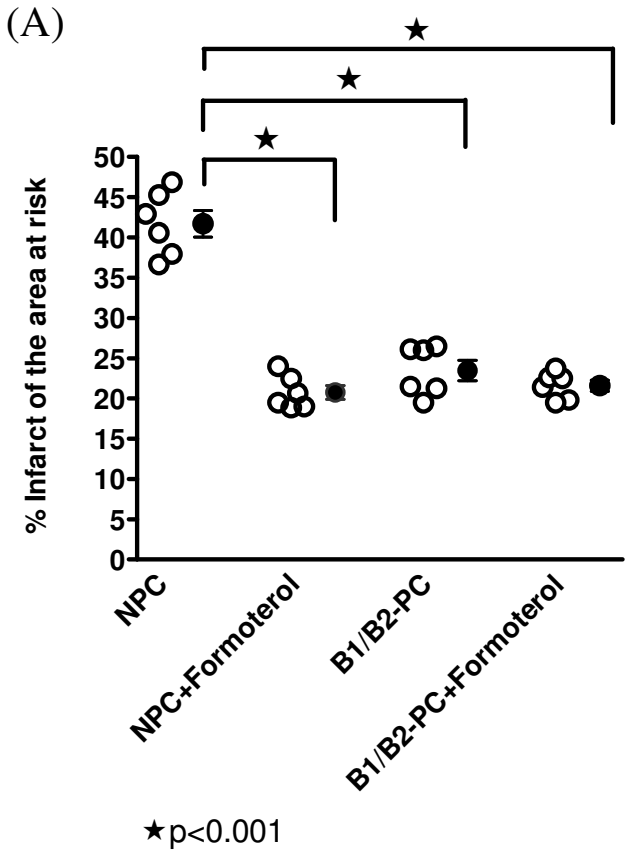
	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+β3 agonist After RI (n=6)	9.5±2.56	13.60±4.66	23.00±7.09	189±47.44	72.19±18.07	4.84±1.38
βPC+β3 agonist After RI (n=6)	12.30±0.73	21.20±1.20 ✶	33.50±1.25 ✶	265±8.58	92.88±1.97	6.74±0.39 ✶

✶p<0.001 vs NPC After RI

3.2.2 b The effect of β-AR preconditioning with isoproterenol, formoterol or BRL 37344 on infarct size (Fig. 3.10 A and B)

The area at risk zone (54.42±0.65%), expressed as a percentage of the left ventricular volume was similar in NPC and β-PC groups, as well as in all other experimental groups in which various pharmaceutical agents were applied. This implied that all results obtained were comparable.

The cardioprotective effect of β1/β2-PC with isoproterenol was clearly illustrated when comparing the reduced IS of β1/β2-PC, 22.01±0.65% to the large IS of untreated NPC hearts, 41.72±1.65%, $p<0.001$. Similarly, NPC hearts treated with formoterol significantly reduced IS (20.74±1.43%, $p<0.001$ vs NPC). The β_2-AR agonist, formoterol applied prior to and during β1/β2-PC, did not add to the cardioprotective effect of β1/β2-PC, since the application of this agonist did not further reduce the IS of β1/β2-PC. The β_3-AR agonist, BRL 37344, applied in the same setting, had no effect on the IS of NPC hearts but significantly increased the IS of hearts exposed to β1/β2-PC, 35.68±1.61%, $p<0.001$ vs β1/β2-PC.



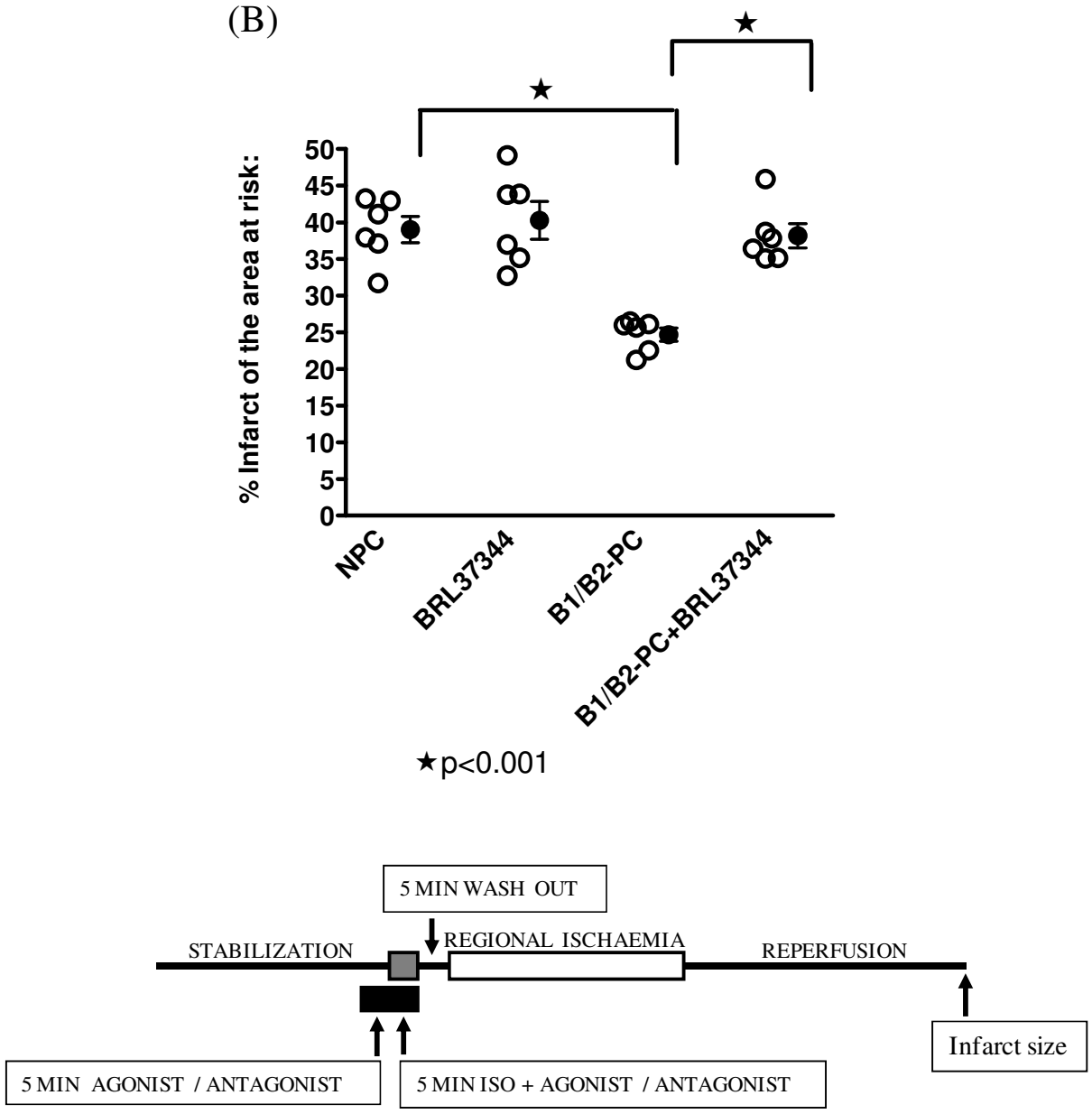


Fig. 3.10: The effect of preconditioning with the β_1/β_2-AR agonist (isoproterenol) (A), the β_2-AR agonist (formoterol) (A) or β_3-AR agonist (BRL 37344) (B) on infarct size

3.2.3 The effect of the 5 minute washout episode after application of β-adrenergic antagonists on haemodynamic parameters (Table 3.5 A, B and C)

Before the effects of β-AR antagonists on the response of the heart to β-adrenergic preconditioning could be evaluated, it was essential to establish (i) whether the antagonist could counteract the effects of isoproterenol and (ii) whether a 5 minute washout period was sufficient to remove both isoproterenol and its antagonists before the onset of regional ischaemia.

The effects of β-AR stimulation with ISO after β_1-AR, β_2-AR or β_3-AR blockade with CGP-20712A, ICI 118,551 or SR 59230A respectively, abolished the significant increase in CF, AO, CO and total work seen with isoproterenol after 1 minute (Table 3.1). These haemodynamic parameters returned to baseline values after 5 washout.

Table 3.5 A: The haemodynamic parameters of isolated rat hearts before and after 5 min β_1-AR inhibition followed by β-AR stimulation with isoproterenol (0.1 µM)

β_1-AR antagonist: CGP-20712A (300 nM) (n=4)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
15 min WH before β1antagonist	16.00± 0.00	37.00±1.00	53.00±1.00	256.00±11.92	94.50±1.19	11.26±0.43
1 min WH+ISO after β1antagonist	18.00±0.81	37.50±2.75	56.00±3.16	273.50 ±21.57	98.00±4.14	12.33±1.27
3 min WH+ISO after β1antagonist	17.50±1.25	33.00±3.41	51.00±4.65	283.00±17.55	95.50±3.37	10.91±1.33
5 min WH+ISO after β1antagonist	17.50±1.23	27.00±2.51	44.50±2.51	286.00±17.38	93.00±1.47	9.27±0.91
5 min Washout	16.00±0.00	36.25±1.49	51.75±1.18	291.00±12.22	93.25±1.28	10.89±0.45

Table 3.5 B: The haemodynamic parameters of isolated rat hearts before and after β_2-AR inhibition followed by β-AR stimulation with isoproterenol (0.1 µM)

β_2-AR antagonist: ICI 118,551 (50 nM) (n=4)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
15 min WH before β2antagonist	16.67±0.66	36.00±2.30	53.33±1.33	276.00±29.05	94.00±3.51	11.17±0.35
1 min WH+ISO after β2antagonist	20.67±0.66	40.00±0.00	60.67±0.66	312.00±6.43	101.30±2.18	13.30±0.27
3 min WH+ISO after β2antagonist	21.33±0.66 *	28.00±2.30 ¥	49.33±1.76 δ	354.00±39.01	93.00±1.52	10.11±0.66 ¥
5 min WH+ISO after β2antagonist	22.00±15 *	21.33±2.66 € φ	42.00±3.05 *δ	322.00±44.51	91.33±2.33	8.56±0.80 φ
5 min Washout	17.33±0.66	36.00±2.30	53.33±2.90	300.00±7.21	91.33±0.66	10.87±0.59

* $p<0.05$ vs 15 min WH before β2 antagonist

€ $p<0.01$ vs 15 min WH before β2 antagonist

¥ $p<0.05$ vs 1 min WH+ISO after β2 antagonist

φ $p<0.01$ vs 1 min WH+ISO after β2 antagonist

δ $p<0.001$ vs 1 min WH+ISO after β2 antagonist

Table 3.5 C: The haemodynamic parameters of isolated rat hearts before and after β_3-AR inhibition followed by β-AR stimulation with isoproterenol (0.1 µM)

β_3-AR antagonist: SR 59230A (100 nM) (n=4)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
15 min WH before β3antagonist	16.67± 0.66	36.00±2.00	53.33±1.33	270.00±27.47	93.67±1.20	11.14±0.28 €
1 min WH+ISO after β3antagonist	18.67±0.66	40.00±2.30	58.67±2.40	313.00±16.80	100.00±1.55	13.04±0.49
3 min WH+ISO after β3antagonist	18.00±1.55	28.00±0.00 φ *	46.67±0.66 φ	317.00±33.50	94.00±1.00	9.74±0.24 ¥
5 min WH+ISO after β3antagonist	18.00±15	25.00±0.00¥ *	41.33 ±0.66 ¥ δ	372.00±20.30	90.00±0.57	8.26±0.10 ¥ δ
5 min Washout	16.67±0.66	31.33±3.17	48.00±4.83	317.00±22.08	91.67±0.88	10.06±0.73

* p<0.05 vs 15 min WH before β3 antagonist

δ p<0.01 vs 15 min WH before β3 antagonist

€ p<0.05 vs 1 min WH + ISO after β3 antagonist

φ p<0.01 vs 1 min WH + ISO after β3 antagonist

¥ p<0.001 vs 1 min WH + ISO after β3 antagonist

3.2.4 a The effect of β_1-AR (CGP-20712A), β_2-AR (ICI 118,551) or β_3-AR antagonists (SR 59230A) on mechanical recovery during reperfusion following regional ischaemia (Table 3.6)

The aortic output, cardiac output, and total work after regional ischaemia (RI) were significantly lowered in the β1/β2-PC+β1 antagonist group ($p<0.05$) when compared with the β1/β2-PC group. The coronary flow, heart rate and peak systolic pressure remained unaffected. Pretreatment of β1/β2-PC hearts with the β_2-AR antagonist ICI 118,551 caused a significant reduction in the aortic output in comparison with the β1/β2-PC group after RI, while the coronary flow, cardiac output, heart rate and peak systolic pressure and total work unchanged. Interestingly, treatment of NPC hearts with the β_3-AR antagonist resulted in a significant increase in aortic output during reperfusion compared to the NPC hearts. Furthermore, β_3-AR blockade combined with β1/β2-PC, caused a significant increase in AO, compared with β1/β2-PC alone (25.33±1.33 vs 18.00±2.78, $p<0.05$). In fact, administration of the β_3-AR antagonist elicits cardioprotection, similar to β1/β2-PC alone.

Table 3.6: Effect of β-adrenergic receptor antagonists on mechanical recovery during reperfusion of β-adrenergic receptor preconditioned hearts

(A) β-AR agonist: isoproterenol (0.1 µM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.37±0.29	40.74±0.55	55.97±0.57	264±6.34	100.70±1.40	12.78±0.19
NPC After RI	8.95±1.17	8.13±1.61 #	17.08±2.04 #	199±5.95	89.71±2.83	3.63±0.59 #
β1/β2-PC Before RI (n=18)	16.09±0.14	40.70±0.71	56.96±0.81	264±5.27	104.00±1.43	13.14±0.27
β1/β2-PC After RI	13.27±0.68	19.43±1.73	32.70±2.20	237±11.7	82.43±2.63	6.67±0.40

# P< 0.05 vs β1/β2-PC After RI

(B) β_1-AR antagonist: CGP-20712A (300 nM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+β1 antagonist After RI (n=6)	7.30±3.10	5.60±3.42	12.90±5.28	138.±56.75	89.33±2.02	3.85±1.43
β1/β2-PC+β1 antagonist After RI (n=6)	7.20±2.93	7.00±3.02 ϕ	14.20±5.83 ϕ	161±66.0	55.40±22.83	3.024±1.29 ϕ

ϕP< 0.05 vs β1/β2-PC After RI

(C) β_2-AR antagonist: ICI 118,551 (50 nM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+β2 antagonist After RI (n=6)	6.37±3.69	3.12±2.02	9.62±5.71	148 ± 86	45.06± 52.04	1.88±1.12
β1/β2-PC +β2 antagonist After RI (n=6)	13.42±0.59	9.75±1.97 ⌖	23.42±1.86	255±11.04	88.43±1.85	5.122±0.24

⌖ P< 0.05 vs β1/β2-PC After RI

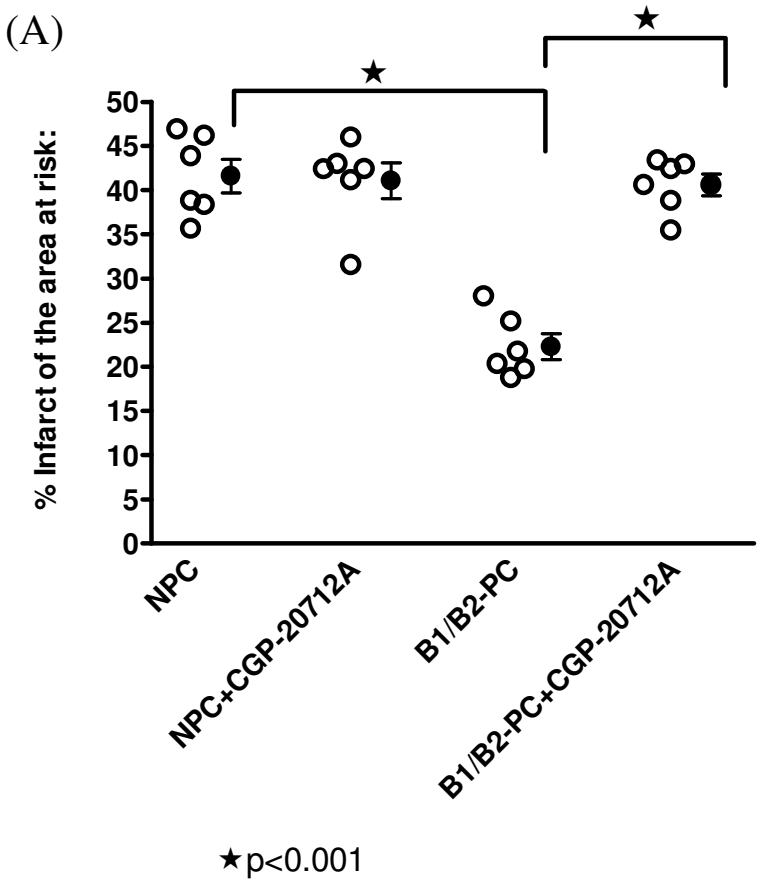
(D) β_3-AR antagonist: SR 59230A (100 nM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+β3 antagonist After RI (n=6)	11.38±1.32 £	18.50±2.75 £	29.88±4.06 £	247±17.64	88.87±3.19	6.32±1.23 £
β1/β2-PC +β3 antagonist After RI (n=6)	13.08±0.35	25.33±1.33	39.75±1.91	272±13.01	92.35±1.30	8.00±0.44

£ p<0.05 vs NPC After RI

3.2.4 b Effect of β_1-AR (CGP-20712A), β_2- AR (ICI 118,551) or β_3-AR antagonists (SR 59230A) on infarct size after β1/β2-AR preconditioning with isoproterenol (Fig. 3.11 A; B and C)

The IS of NPC hearts averaged at 41.60±1.89%, compared to the significantly reduced IS of hearts exposed to β1/β2-PC, 22.26±1.46%, p<0.001 vs NPC. Antagonists to β_1-ARs (CGP-20712A, 300nM) or β_2-ARs (ICI 118,551, 50 nM) applied prior to and during β-adrenergic preconditioning, followed by a washout episode prior to regional ischemia, significantly increased infarct size (41.60±1.89% and 44.25±1.28%, p<0.001 vs β1/β2-PC, respectively) and abolished the protective effect of β1/β2-PC. In contrast, the β_3-AR (SR59230A, 100 nM) antagonist applied in the same manner, had no effect on the reduction in infarct size elicited by β1/β2-PC.



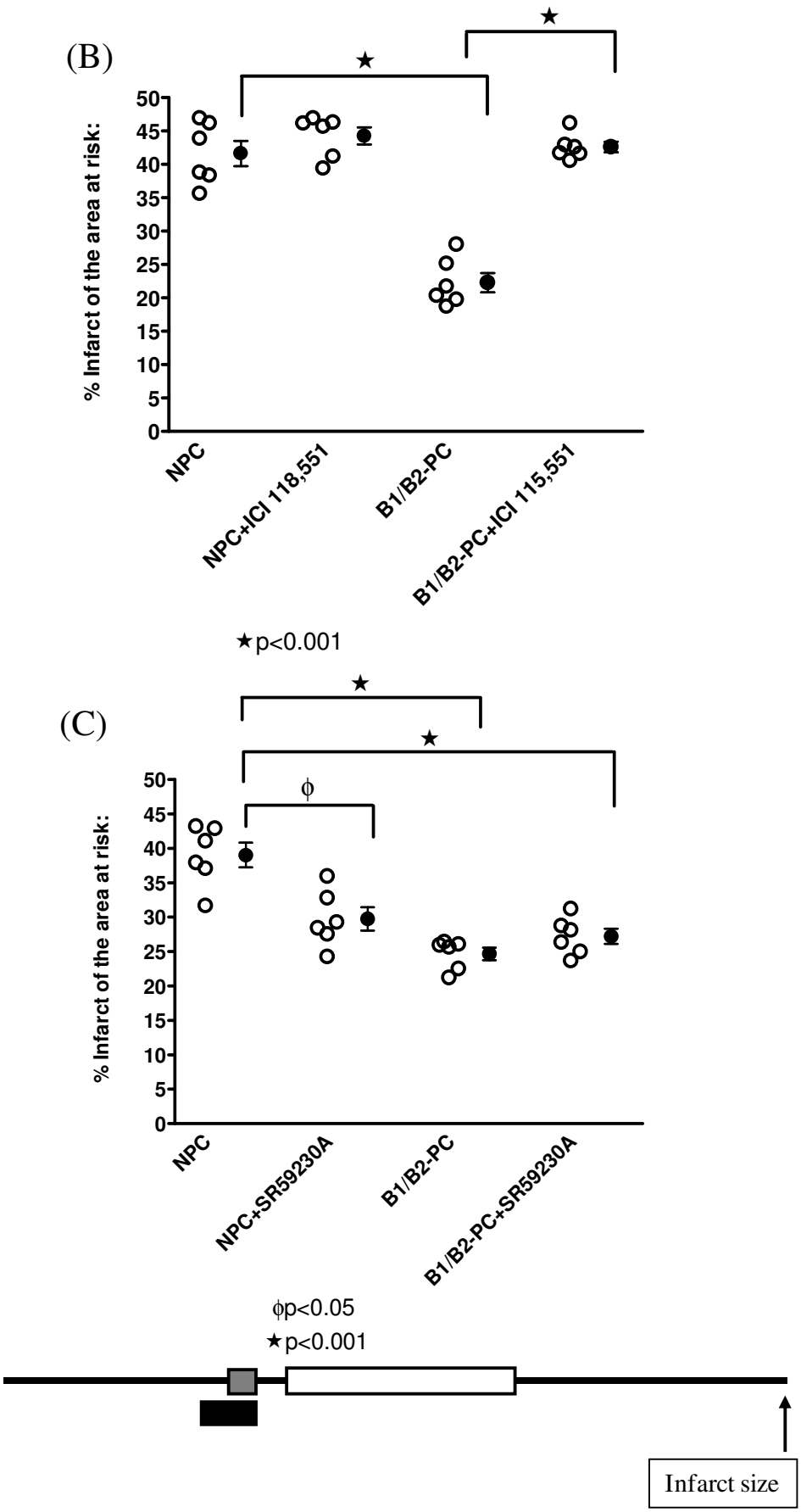


Fig. 3.11: Effect of β_1-AR (CGP-20712A) (A), β_2- AR (ICI 118,551) (B) or β_3-AR antagonists (SR 59230A) (C) on IS in β1/β2-PC

3.2.5 a The effect of the β_1-AR antagonist (CGP-20712A) on β_2-AR stimulation with formoterol on mechanical recovery during reperfusion after regional ischaemia (Table 3.7)

The β_2-specific agonist formoterol, caused a significant increase in CF, AO and CO during reperfusion, when compared with the functional performance of NPC hearts. However, the β_1-AR antagonist, CGP-20712A (300 nM) and β_2- ARs agonist, formoterol (1 nM) when applied in combination to hearts prior to regional ischaemia had no significant effect on any of the haemodynamic parameters during reperfusion, when compared with formoterol alone.

Table 3.7: Effect of β_1-AR inhibition (CGP-20712A) and β_2-AR stimulation (formoterol) on mechanical recovery during reperfusion after 35 min coronary artery ligation

β_1-AR antagonist: CGP-20712A + β_2-AR agonist: formoterol

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.56±0.23	43.78±0.87	59.90±0.91	266±6.28	97.24±1.30	12.88±0.23
NPC After RI	10.25±0.90 #	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #

# P< 0.001 vs NPC before RI

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+Formoterol After RI (n=6)	15.58±0.40 ✶	29.00±3.09 ✶	45.00±3.23 ✶	251±7.40	92.17±2.07	9.637±0.52 ✶
NPC+ CGP-20712A + formoterol After RI (n=6)	14.34±2.66	20.00±4.12	34.33±6.74	227±47.51	76.50±15.48	7.10±1.47

✶p<0.001 vs NPC After RI

3.2.5 b The effect of the β_1-AR antagonist (CGP-20712A) on infarct size after preconditioning with formoterol (Fig. 3.12)

Preconditioning with formoterol (β2-PC), significantly reduced the IS of NPC hearts (20.74±1.43%, p<0.001 vs NPC). The application of the β_1-ARs antagonist, CGP-20712A in combination with formoterol prior to RI, caused a slight but significant increase in IS, thus negatively affecting the β_2-AR mediated cardioprotective effects (32.55±0.92%, p<0.01 vs formoterol).

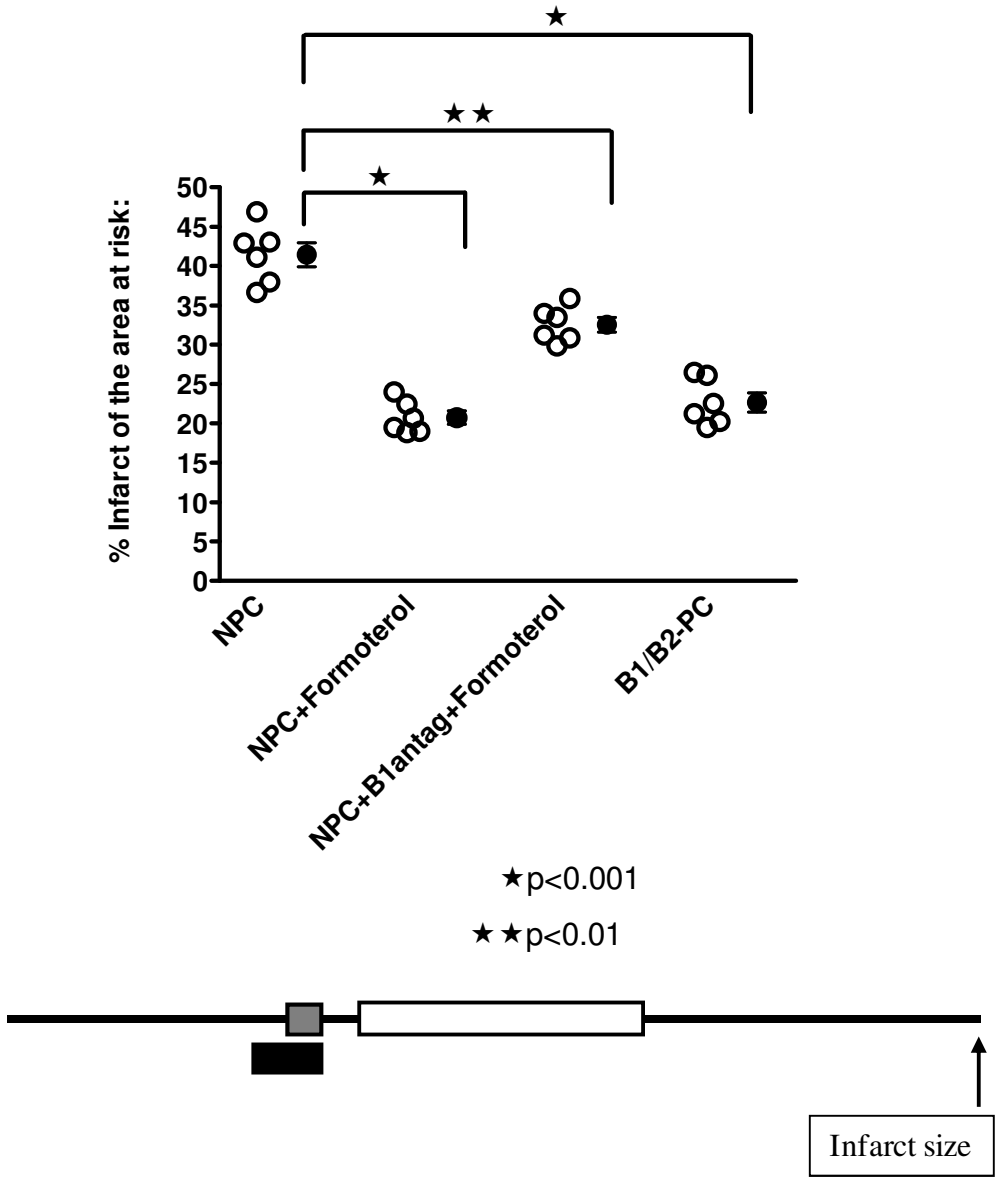


Fig. 3.12: The effect of the β_1-AR antagonist (CGP-20712A) and β_2-AR stimulation with formoterol on infarct size

3.2.6 The role of PTX sensitive Gαi/o proteins in β-adrenergic preconditioning

3.2.6 a The effectiveness of Gαi/o inhibition (Table 3.10 A and B)

Effectiveness of Gαi/o inhibition with PTX (30 µg kg^{-1}) was assessed by determining its ability to block the bradycardia response associated with carbachol, a muscarinic / cholinergic receptor agonist. Administration of carbachol (1 µM) for 5 min caused a 51-52 % reduction in CF, AO, CO and heart rate of untreated control hearts. PTX treatment significantly decreased the negative inotropic and chronotropic effects of carbachol (% change: AO 22; CO 19; heart rate 21), with no change in PSP and total work.

Table 3.8 A: The haemodynamic parameters before and 5 min after application of carbamylcholine chloride / carbachol to isolated rat hearts

Muscarinic / cholinergic receptor agonist: carbachol (1 µM) (n=3)

Total perfusion time (min)	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
15 min WH	17.33±0.66	40.67±4.05	58.00±4.61	232.00±22.85	98.33±2.96	12.63±1.12
20 min WH+ Carbachol	8.50±0.50 *	20.00±2.30¥	28.50±2.75 ¥	112.00±2.88 δ	114.3±2.33 δ	7.45±0.63 δ
	(51 %)	(51 %)	(51 %)	(52 %)		
25 min WH+ washout	16.67±0.66	40.00±3.46	56.67±4.05	203.00±12.74	100.00±3.00	12.69±1.23

* $p<0.001$ vs 15 min WH as well as 25 WH+washout

¥ $p<0.01$ vs 15 min WH as well as 25 WH+washout

δ $p<0.05$ vs 15 min WH as well as 25 WH+washout

Table 3.8 B: The hemodynamic parameters before and 5 min after the application of carbachol to isolated hearts taken from rats pretreated with PTX

Muscarinic / cholinergic receptor agonist: carbachol (n=4)

Total perfusion time (min)	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
PTX+15 min WH	16.50± 0.50	34.00±0.81	50.50±0.95	241.00±9.00	95.00±1.05	10.69±0.29
PTX+20 min WH+ Carbachol	14.88±0.65	26.50±0.95	40.88±1.32	190.00±10.91	97.00±1.78	9.06±0.32
		(22 %)	(19 %)	(21 %)		
PTX+25 min WH+ washout	16.00±0.00	24.00±4.89	40.00±4.89	222.00±20.00	94.50±3.06	8.79±0.91

3.2.6 b The involvement of the Gαi/o protein in β2-PC with formoterol (Table 3.9)

The involvement of the Gαi/o regulatory protein in the specific actions of the selective β_2-ARs agonist, formoterol was assessed 48 hours after PTX (30 µg kg^{-1}) pretreatment. Results show that haemodynamic parameters recorded after the application of formoterol for 5 minutes did not differ significantly from values recorded prior to, or after formoterol treatment (compare Table 3.2).

Table 3.9: The haemodynamic parameters of isolated hearts taken from rats pretreated with PTX (30 µg kg^{-1}) before and after 1, 3 and 5 min β_2-AR stimulation with formoterol

β_2-AR agonist: formoterol (n=4)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
PTX+5 min WH	16.67±0.66	37.33±1.33	54.00±2.00	261.00±13.80	95.33±1.45	11.43±0.30
PTX+1 min WH+ Formoterol	18.67±0.66	41.33±1.33	60.00±2.00	241.00±29.36	98.67±1.20	13.22±0.35
PTX+3 min WH+ Formoterol	20.67±1.76	42.67±1.33	63.33±2.90	264.00±11.67	99.00±1.15	13.96±0.59
PTX+5 min WH+ Formoterol	21.33±1.33	42.67±1.33	64.00±2.30	261.00±5.66	98.00±1.73	13.90±0.41
PTX+5 min WH+ Washout	17.33±0.67	36.00±0.00	53.33±0.66	263.00±17.04	94.33±1.85	11.03±0.10

3.2.7 a The role of PTX sensitive Gαi/o protein in β1/β2-PC and β2-PC (Table 3.10)

Experimental protocols were applied 48 hours after treatment with pertussis toxin.
Haemodynamic parameters were recorded at the end of the 15 minute working heart mode prior to regional ischaemia and compared with haemodynamic parameters and infarct size at the end of reperfusion. As shown previously, the AO, CO and total work of NPC hearts after regional ischaemia and 30 minutes reperfusion were significantly lower than those from hearts exposed to β1/β2-PC. PTX pretreatment had no affect on any of the haemodynamic parameters of NPC hearts or hearts exposed to β1/β2-PC. In contrast, PTX pretreatment significantly reduced AO, CO and total work of hearts exposed to β_2-AR stimulation with formoterol (β2-PC), suggesting a role for the Gαi/o protein in β_2-AR-induced cardioprotection.

Table 3.10: The effect of PTX sensitive Gαi/o protein inhibition on mechanical recovery of hearts exposed to β1/β2-PC (ISO) or β2-PC (formoterol)

β1/β2-PC: Isoproterenol

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.56±0.23	43.78±0.87	59.90±0.91	266±6.28	97.24±1.30	12.88±0.23
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI (n=18)	15.97±0.15	40.39±1.19	56.11±1.33	252±8.01	98.25±1.28	12.40±0.34
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

# P< 0.05 vs β1/β2-PC After RI

Table 3.10: (continued)

PTX (30 ug/kg, i.p., 48 hrs prior to isolation) + β1/β2-PC (Isoproterenol)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+PTX After RI (n=6)	11.33±2.46	11.25±3.06	23.25±5.23	224±47.57	69.90±13.9	4.70±1.17
β1/β2-PC+PTX After RI (n=6)	14.75±0.55	20.92±2.59	35.67±3.03	261±11.42	88.17±1.36	6.99±0.66

β2-PC: Formoterol

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
β2-PC After RI (n=6)	15.58±0.40 ✶	29.00±3.09 ✶	45.00±3.23 ✶	251±7.40	92.17±2.07	9.637±0.52 ✶

✶p<0.001 vs NPC After RI

PTX (30 ug/kg, i.p., 48 hrs prior to isolation) + β2-PC (Formoterol)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+PTX After RI (n=6)	11.33±2.46	11.25±3.06	23.25±5.23	224±47.57	69.90±13.9	4.70±1.17
NPC+PTX + β2-PC After RI (n=6)	13.83±0.83	15.00±2.13 ¥	28.93±2.48 ¥	250±3.76	88.35±2.02	6.30±0.45 ε

¥p<0.01 vs β2-PC After RI

ε p<0.001 vs β2-PC After RI

3.2.7 b The effect of PTX sensitive Gαi/o protein inhibition on infarct size of hearts exposed to β1/β2-PC and β2-PC (Fig. 3.13)

NPC hearts show the characteristic large IS (40.24±2.36%), whereas the IS of hearts exposed to β1/β2-PC was significantly reduced (23.20±1.29%, p<0.001 vs NPC). The IS of NPC hearts treated with the β_2-AR agonist, formoterol (β2-PC) was significantly reduced (20.65±0.82% p<0.001 vs NPC) compared to those of NPC hearts. PTX had no effect on the IS of NPC hearts (PTX+NPC group), NPC hearts treated with formoterol (PTX+NPC+Formoterol group) or β1/β2-PC hearts (PTX+ β1/β2-PC group).

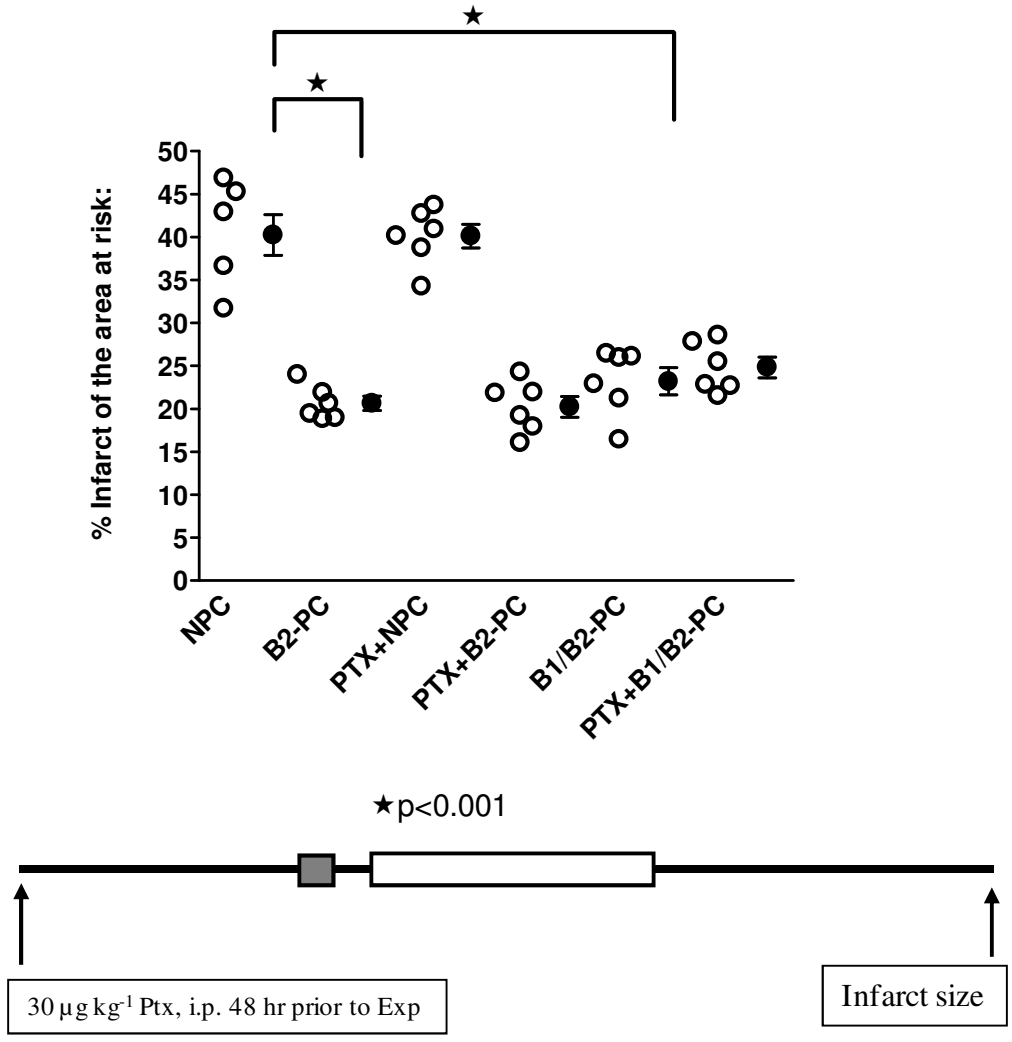


Fig. 3.13: The effect of PTX sensitive Gαi/o protein inhibition on infarct size of hearts exposed to β1/β2-PC and β2-PC

3.2.8 a The involvement of PKA in β-adrenergic preconditioning (Table 3.11)

The role of PKA activation in the cardioprotection elicited by β1/β2-PC with isoproterenol, was evaluated by using the specific PKA inhibitor, Rp-8-CPT-cAMP (16 μM). Rp-8-CPT-cAMP, when applied either prior to (trigger phase) or after regional ischemia / at the onset of reperfusion, had no effect on mechanical performance of NPC hearts, while it significantly reduced the AO, CO and total work of β1/β2-PC hearts.

Table 3.11: Effects of PKA inhibition prior to RI or during reperfusion on mechanical recovery of hearts exposed to β1/β2-PC

(A) β1/β2-PC: Isoproterenol (0.1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=12)	15.20±0.38	42.40±0.67	56.35±0.68	270±3.85	102.5±1.53	13.00±0.34
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI (n=12)	15.96±0.21	40.00±1.06	56.15±1.07	260±8.45	102.90±1.92	13.09±0.44
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

# P< 0.05 vs β1/β2-PC After RI

(B) PKA inhibitor: Rp-8-CPT-cAMPS (Trigger) (16 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+Rp-8CPT After RI (n=6)	7.75±2.52	10.13±3.58	17.75±6.02	177±56.67	61.75±19.42	3.84±1.35
β1/β2-PC + Rp-8CPT After RI (n=6)	7.25±2.33	6.16±2.85 Φ	14.51±5.03 Φ	209±68.04	61.00±19.67	2.89±1.18 Φ

Φ P< 0.05 vs β1/β2-PC After RI

Table 3.11: (continued)

(C) PKA inhibitor: Rp-8-CPT-cAMPS (Reperfusion) (16 µM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+Rp-8CPT After RI (n=6)	9.50±1.96	7.66±2.23	17.17±3.87	215±43.54	74.80±15.27	3.47±0.77
β1/β2-PC + Rp-8CPT After RI (n=6)	8.667±1.90	6.75±3.34 €	15.42±4.76 €	254±53.61	72.10±14.51	2.61±1.10 €

€ P< 0.05 vs β1/β2-PC After RI

3.2.8 b The effect of PKA inhibition on infarct size of hearts exposed to β1/β2-PC (Fig. 3.14)

β1/β2-PC significantly decreased IS (22.75±1.23%, p<0.001) compared to NPC hearts (42.04±1.63). The PKA inhibitor, RP-8-CPT-cAMP applied prior to regional ischaemia in the βPC+RP group (35.74±1.40%, p<0.001 vs β1/β2-PC) or at the onset of reperfusion, the β1/β2-PC + RP R10 group (32.55±2.92%, p<0.01 vs β1/β2-PC) significantly increased IS and consequently decreased the protective effect of β1/β2-PC.

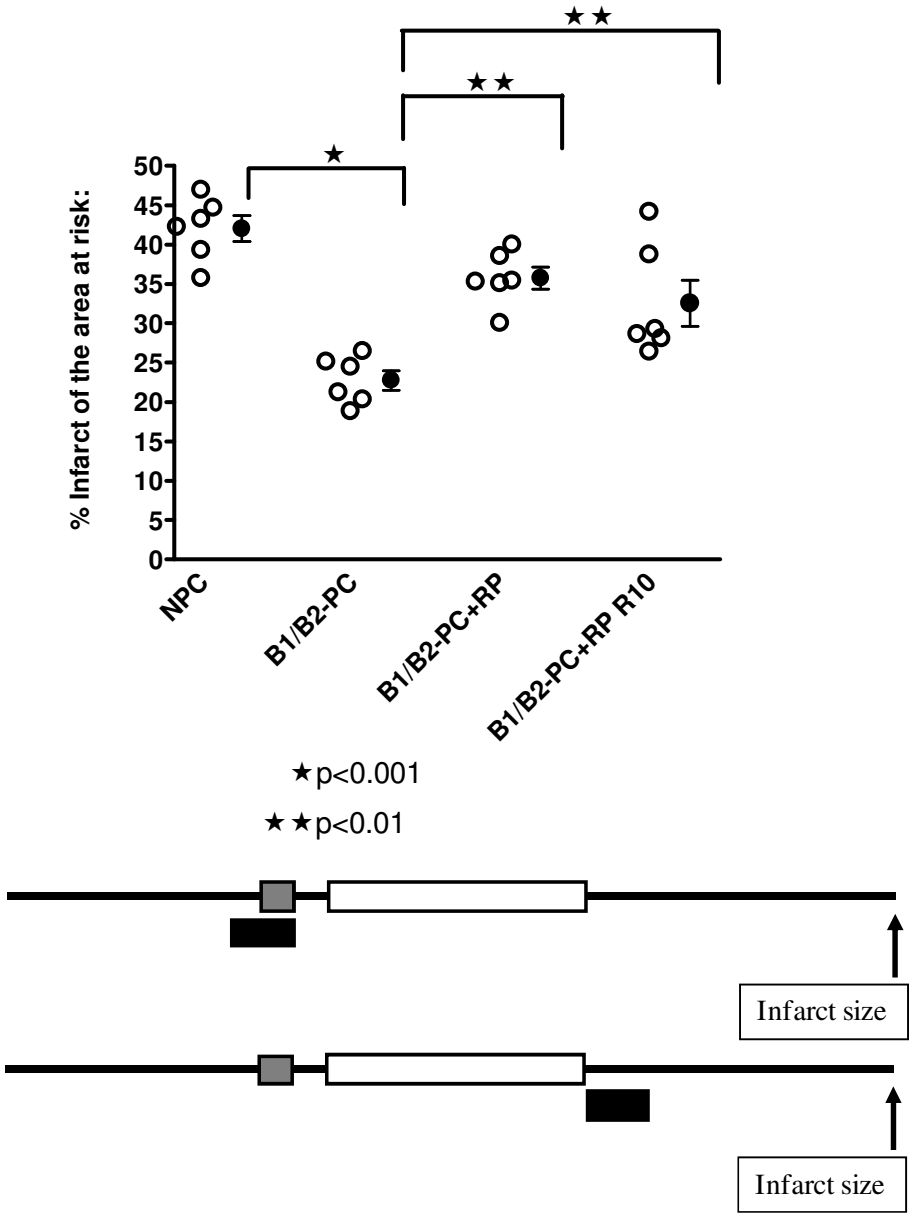


Fig. 3.14: The effect of the PKA inbibitor (RP-8-CPT-cAMP) on infarct size in β1/β2-PC

3.3 Discussion

The most significant observations made in this section were the following: (i) β-PC can be elicited via stimulation of either β_1- or β_2-, but not β_3-AR receptors; (ii) β-PC can be blocked by either β_1- or β_2-AR blockade; (iii) β_3-AR blockade appears to be cardioprotective, while a combination of β1/β2-PC and β_3-AR blockade augments protection and (iv) demand ischaemia is not involved in the reduction of necrosis, but in attenuating stuning.

3.3.1 The role of β-adrenergic receptors in the cardioprotective effects of β-adrenergic preconditioning (β-PC)

It is well established that in the heart, β-AR stimulation by catecholamines (norepinephrine and epinephrine) serves as a powerful regulatory mechanism to maintain excitation contraction coupling (ECC) in normal functioning hearts and to enhance cardiac performance in response to stress, injury or exercise [Lohse et al., 2003; Hata et al., 2004]. Stimulation of β_2-AR but not β_1-AR activates Gi proteins in adult rat cardiomyocytes [Zheng et al., 2004], while both β-AR subtypes are able to stimulate the classic / traditional Gs-AC-cAMP-PKA signaling pathway [Xiao et al., 1995; Daaka et al., 1997; Kilts et al., 2000]. β_1-AR activated stimulation of the Gs-adenylyl cyclase-cAMP-PKA pathway, can be disseminated throughout the cell, whereas β_2-AR-evoked cAMP signaling is spatially and functionally compartmentalized, due its attachment to Gi protein [Xiao and Lakatta, 1993; Kuschel et al., 1999; Xiao, 2001]. Thus, Gi coupling can qualitatively and quantitatively modify the outcome of Gs signaling.

While recognizing the significance of long-term or chronic β_1-AR and β_2-AR stimulation and the concomitant signaling pathways, in the development of various aetiologies of heart failure, it is reasonable to believe that the selective down-regulation of β_1-ARs and the up-regulation of β_2-AR-Gi signaling in the failing heart, elicit opposing effects on survival of cardiomyocytes and may represent cardiac adaptive mechanisms to protect myocytes against apoptosis and consequent contractile dysfunction. The results obtained in the present study demonstrated how transient activation of either β_1-AR or β_2-AR elicits cardioprotection against a long subsequent period of sustained ischaemia, similar to that induced by ischaemic preconditioning.

In an attempt to determine the trigger of cardioprotection elicited by β-AR stimulation, it was argued that isoproterenol, a potent β_1/β_2-AR agonist, causes demand or supply ischaemia and that its mechanism is, in fact, similar to that of ischaemic preconditioning. Within the first min of isoproterenol, CF, AO and HR were significantly increased, while stroke volume remained unchanged. During the remainder of administration period, a progressive decline in stoke volume occurred, despite unchanged coronary flow (Table 3.3). The significance of these events is supported by the fact that the combination of isoproterenol and the β_3-AR agonist, BRL 37244, which abolished the positive inotropic effect of isoproterenol alone, led to an increase in infarct size.

However, β_3-AR per se reduced AO and CO, without cardioprotection. In addition, transient stimulation of the β_2-AR with formoterol was equally effective in eliciting cardioprotection, while having no effect on the contractile behaviour of the heart during the 5 min period of administration (Table 3.2). It therefore appears unlikely that transient changes in contractile behaviour of the heart during administration of the β-adrenergic receptor agonist and possible development of ischaemia trigger the process. Unfortunately tissue high energy phosphates were not measured at the end of 5 minute isoproterenol administration. The significant activation of PKB/Akt and ERK p44/p42 MAPK seen at this stage as reported recently (Fig. 4.4 A and B) also confirm the absence of ischaemia, since it is well-established that activation of these kinases does not occur during an ischaemic episode.

Thus far, several studies indicated the involvement of the β_1-AR in cardioprotection: (i) propanolol (non-selective β-blocker) abolished protection, while the selective β_2-AR blocker ICI 118551 had no effect [Frances et al., 2003]; (ii) the specific β_1-adrenergic agonist xamoterol could elicit protection against ischaemia, which was abolished by atenolol [Robinet et al., 2005]; (iii) hypoxic preconditioning was attenuated by a selective β_1-selective blocker, metoprolol [Mallet et al., 2006]; (iv) pharmacological preconditioning with desflurane and sevoflurane was shown to be dependent on β_1-AR activation, since it could be blocked by esmolol and H-89, a blocker of PKA [Lange, 2006]. Although the role of β_1-AR in cardioprotection may be questioned, in view of its well-established effects on necrosis and apoptosis, the period of administration during a β-AR preconditioning protocol is probably too short to elicit such harmful effects.

β_1-AR induces hypertrophy and apoptosis via the time dependent β_1-AR signaling switch from PKA to Ca2+ / calmodulin-dependent kinase II (CAMK II) dominant pathway which is independent of

the classic cAMP-PKA pathway [Communal et al., 1999; Zaugg et al., 2000]. The CaMK II pathway may be clinically relevant, since inhibition thereof is a potential target to prevent adverse cardiac remodeling, particularly myocyte hypertrophy and apoptosis in the context of enhanced β_1-AR signaling. The results obtained in the present study suggest a role for PKA both during the triggering and mediating phases (Fig. 3.14), but the involvement of CAMK II still needs to be determined. On the other hand, the β_2-AR promotes cell survival via the PKA-dependent β_2-AR phosphorylation switch from Gs to Gi protein coupling [Daaka et al., 1997; Zou et al., 1999], which minimizes the receptor mediated inotropic response while enhancing the cardioprotective effects mediated by a signaling pathway involving Gi, G$\beta\gamma$, PI3K and PKB/Akt [Zhu et al., 2001].

The results obtained using either isoproterenol plus β_2-AR blockade or the specific β_2-AR agonist, formoterol hemifumarate suggest a definite role for the β_2-AR in β-AR cardioprotection, since the selective β_2-AR antagonist ICI-18851 completely abolished isoproterenol-induced cardioprotection (Fig. 3.11 B). This drug has a high degree of specificity and selectivity for the β_2-AR (Ki 2.0±0.4 nM, β2/1 selectivity ratio 123:1) [Bristow et al., 1986] and was used at a concentration of 50 nM in the present study, similar to that used by several others [Communal et al., 1999; Kaumann, et al., 1996].

The significance of the β_2-AR in β-AR preconditioning was confirmed by using the potent, selective and long-acting β_2-AR agonist formoterol hemifumarate which has a 330 fold selectivity for β_2- over β_1- receptors [Anderson, 1993; Naline, 1994]: transient administration of this drug elicited the same reduction in infarct size as isoproterenol, while significantly improving mechanical performance during reperfusion (AO during RI (ml/min) isoproterenol: 18.00±2.78; formoterol: 29.00±3.09) when compared to isoproterenol (Table 3.4 and Fig 3.10 A). A role for the β_2-AR in preconditioning was further confirmed by the finding that hearts of β_2-receptor knockout mice could not be preconditioned [Tong, 2005].

Interestingly, simultaneous administration of isoproterenol and formoterol hemifumarate did not have additive effects, suggesting a similar mode of action. Despite using a high-specificity β_2-AR agonist, formoterol hemifumarate, simultaneous administration of the β_1-AR antagonist CGP-20712A, partially attenuated the beneficial effects of formoterol on infarct size (Fig. 3.12), but not on functional recovery (Table 3.7).

This is probably due to the presence of endogenous catecholamines in our preparation and should be investigated further. Previous studies on the role of β_1- and β_2-AR subtypes in preconditioning were done on reserpinized animals or after treatment with β-hydroxydopamine to eliminate the effects of the endogenous catecholamines [Frances et al., 2003; Robinet et al., 2005].

3.3.2 Role of the Gi proteins in β-AR preconditioning

Contrary to expectations, our results showed that the cardioprotective actions of isoproterenol (β1/β2-PC) were not mediated via the PTX sensitive Gi regulatory protein (Table 3.10 and Fig. 3.13). It is well established that the β_1-AR exerts its effects via the Gs protein. In view of the preponderance of this receptor subtype compared to the β_2-AR, it is possible that stimulation of the β_1-AR by isoproterenol overrides the effects of Gi inhibition and still causing cardioprotection.

In the case of formoterol (β2-PC), pretreatment with PTX yielded interesting results namely that although the infarct size remained unchanged, the parameters of functional recovery were significantly reduced, suggesting that the Gi protein does contribute to β_2-AR preconditioning (Table 3.9 and Fig. 3.13). Another possibility is that the Gi protein is not involved in tissue necrosis, but does affect stunning during reperfusion. The dosage of PTX was proven to be sufficient, since it could significantly attenuate the effects of carbachol in our model (Table 3.8).

Results reported on the effects of the Gi proteins in cardioprotection are contradictory. Many studies support a role for this regulatory protein in cardioprotection, for example (i) it was shown in the isolated rat heart that pretreatment with PTX completely abolished the cardioprotective effects of ischaemic preconditioning [Schultz et al., 1998].
(ii) the Gi/o protein is a crucial component in the cardioprotection of hypoxic preconditioning in neonatal rat cardiomyocytes [Chen and Xia, 2000].
(iii) β_2-AR preconditioning, could be blocked by PTX pretreatment [Oldenberg et al., 2002; Tong et al., 2004]. It was also reported that PKA inhibition blocked the protection of preconditioning and isoproterenol, consistent with the role of PKA in the switching of β_2-AR coupling from Gs to Gi [Tong et al., 2005].

However, in contrast to the above, it has also been reported that ischaemic preconditioning in the rat heart does not to involve the PTX sensitive regulatory Gi protein [Downey et al., 1993; Piacentini et al., 1995]. Furthermore, Frances and coworkers (2003) could not show involvement of the β_2-AR in β-PC, since they found that β_2-AR blockade did not abolish isoproterenol-induced preconditioning. These controversial findings are probably due to differences in the protocols since they used a much higher ICI-18851 concentration than us (2 μM vs 50 nM) combined with a lower isoproterenol concentration (20 nM) in reserpinised animals. Clearly more work is required to solve these controversies.

It should be borne in mind that the type and duration of the β-AR stimulation ultimately determines the signaling cascade and cardioprotective response obtained, i.e. acute β-AR stimulation such as β-PC will differ markedly from chronic β-AR stimulation which eventually enhance and develop into CHF. In the scenario of β-AR preconditioning, stimulation of the receptor (β_1- or β_2-AR) is short lived and transient and the signaling patterns will differ from those of chronic β-AR stimulation.

Finally, although the results obtained are strongly suggestive of major involvement of the β_2-AR, subsequent studies using specific β_1-AR agonists, denopamine and xamoterol hemifumarate showed that specific activation of the latter receptor is equally effective in producing cardioprotection [Salie and Lochner, unpublished observations]. In view of these observations, it was decided to use isoproterenol as agonist in all subsequent studies in which the mechanism of protection was investigated. Studies evaluating the mechanism of β_2-AR stimulation are currently in progress.

3.3.3 What happens downstream of the β-AR ? A role for PKA

PKA has been implicated to be an important role player in agonist-induced receptor desensitization and down-regulation [Benovic et al., 1988]. PKA-dependent β_1-AR phosphorylation promotes receptor desensitization, whereas PKA-dependent β_2-AR phosphorylation switches the receptor G protein coupling from Gs to Gi [Daaka et al., 1997] which reduces the receptor-mediated intropic response while enhancing its cardioprotective effect during chronic β-AR stimulation.

Previous studies from our laboratory showed that ischaemic preconditioning as well as treatment with forskolin or isoproterenol rapidly and transiently increase tissue levels of cAMP and PKA activation prior to sustained ischemia [Lochner et al., 1999; Makaula and Lochner, 2005]. This in turn leads to desensitization of the β-AR and subsequent protection against ischaemic damage [Lochner et al., 1999]. In the present study, it was illustrated that the selective PKA blocker, Rp-8-CPT-cAMP administered prior to sustained ischemia or at the onset of reperfusion completely abolished the cardioprotective effect of β1/β2-PC, in both instances, suggesting that PKA is an essential component in β1/β2-PC induced cardioprotection (Table 3.11 and Fig. 3.14). Unfortunately the effect of PKA inhibition was not studied in the case of β2-PC, which does not allow for distinction of the role of this kinase in each receptor subtype.

It is also possible that the rapid activation of PKA which occurs during a preconditioning protocol, causes a Gs to Gi switch in the case of the β_2-agonist formoterol, explaining the lack of a positive inotropic response observed with this drug (Table 3.11).

The mechanism whereby PKA activation during a preconditioning protocol elicits protection as well as downstream events remains to be established. Possible mechanisms of PKA action include (i) calpain inhibition, resulting in reduced hydrolysis of structural proteins, reduced sarcolemmal fragility and less cell death [Inserte et al., 2004] (ii) increased HSP27 activation [Marais et al., 2005] (iii) phospholamban phosphorylation, causing increased SR Ca^{2+} uptake and a reduction in cytosolic Ca^{2+} [Sichelschmidt et al., 2003] (iv) inhibition of the small GTPase RhoA and its downstream Rho-kinase. The latter kinase is known to enhance cardiac damage in acute ischaemia [Sanada et al., 2004; Dong, Leung and Manser, 1998; Shimokawa, 2002] and Sanada and coworkers (2004) suggested that transient pre-ischaemic activation of PKA reduces infarct size through Rho-kinase inhibition. It is also possible that, both in the case of β_1-and β_2-AR preconditioning, PKA activation leads to switching their coupling from Gs to Gi and activation of ERKp42/p44, as has been demonstrated by Martin and coworkers [Martin et al., 2004].

Interestingly administration of the inhibitor during the first 10 min of reperfusion of β1/β2-AR preconditioned hearts also abolished the beneficial effects of prior preconditioning, while having no effect on the NPC hearts (Fig. 3.14). As far as we know, this is the first demonstration of the significance of PKA activation during reperfusion of β-AR preconditioned hearts, the mechanism of

which remains to be determined. It may occur via its cross-talk with PKB/Akt contributing to activation of the RISK pathway, which is also characteristic of β-AR preconditioning (see chapter 5). It is also possible that PKA activation during reperfusion elicits protection by inhibition of GSK-3beta which, in turn, limits the induction of mitochondrial permeability transition [Juhaszova et al., 2004]. The finding that short-term PKA inhibition during reperfusion has no effect on infarct size of NPC hearts is in contrast to the findings in rabbits using H89 [Lange et al., 2009].

3.3.4 Cardioprotection of β-PC does not involve β_3-AR

The role of the β_3-AR in the cardiovascular system remains controversial. However, increasing evidence suggest that it acts as a brake in sympathetic overstimulation [Moens et al., 2010]. Unlike the stimulation of β_1-AR and β_2-AR subtypes, the activation of β_3-AR leads to negative regulation of cardiac contractility via the Gi-coupled signaling [Gauthier et al., 1996], increased NO production and an increase in intracellular cGMP levels [Moens et al., 2010]. β_3-AR - induced NO production was initially linked to eNOS [Gauthier et al., 1998], but recent data indicate that these receptors can also modulate NO production via nNOS and iNOS [Maffei A et al., 2007].

To delineate the contribution of the β_3-AR to isoproterenol - induced cardioprotection, use was made of the β_3-AR antagonist SR 59230A and agonist BRL 37344, respectively. This study illustrated that the selective β_3-AR agonist, BRL 37344 significantly reduced haemodynamic parameters after 1 to 5 minutes application, which returned to baseline values after 5 minutes washout (Table 3.3), demonstrating the presence and negative inotropic nature of the β_3-AR in the rat myocardium. This agonist was capable of completely reversing the immediate positive inotropic and chronotropic effects of isoproterenol (Table 3.4 C) and explains why BRL 37344 abolished the cardioprotective effects of isoproterenol, as shown by the significant increased infarct size (Fig. 3.10 B).

As expected, the results showed that the selective β_3-AR antagonist, SR 59230A, applied prior to sustained ischaemia, had no effect on haemodynamic parameters or the infarct limiting capabilities of β1/β2-PC (Table 3.6 D and Fig. 3.11 C), suggesting that this receptor is not involved in isoproterenol – induced cardioprotection. Interestingly, NPC hearts exposed to SR 59230A per se displayed similar contractile recovery as β-PC hearts, as well as a significant reduction in infarct size (Table 3.6 D and Fig. 3.11 C). However, the exact mechanism whereby this is accomplished needs to be determined. It is known that the β_3-AR functions as a negative inotrope which is

activated only after extended periods of exposure to higher levels of catcholamines [Lafontan et al., 1994]. Activation of this receptor during transient stimulation of the β_1-AR and β_2-AR as occurs during a preconditioning protocol is therefore unlikely to occur. In summary, the results presented in this chapter indicate roles for the β_1- as well as the β_2-AR in cardioprotection, while β_3-AR is not involved.

3.3.5 The correlation between measured endpoints: infarct size and functional recovery

The results obtained stress the significance of the endpoint used in the interpretation of the data and suggest that use of one endpoint only could yield misleading interpretations. Reduction in infarct size is regarded by many researchers as the "gold standard" for evaluation of the effects of preconditioning, but this is not always associated by improvement in mechanical recovery during reperfusion due to concomitant stunning [Cohen et al., 1999; Lochner et al., 2003].

In the present study the reduction in infarct size β1/β2-AR preconditioned hearts was associated with a significant increase in functional recovery. This could be an indication of the efficacy of β-adrenergic preconditioning, demonstrating that the protection afforded by this intervention was sufficient to override the effects of stunning (Table 3.1; 3.2 and 3.3). However the opposite was also observed namely that a reduction in postischaemic function was not necessarily associated with an increase in infarct size (Tables 3.5 and 3.10). A possible explanation is that measurement of infarct size is perhaps not sensitive enough to pick up relatively small changes in infarct size or that the intervention used (e.g. pertussis toxin pretreatment) was without effect on necrosis, while attenuating the beneficial effects of the intervention on stunning, leading to a further reduction in functional recovery. Nevertheless, the data suggest that care should be exercised when final conclusions regarding the effects of interventions are based on results obtained from one endpoint only.

Chapter 4

Investigating the role of the prosurvival kinases, PKB/Akt and ERK p44/p42 MAPKinase in β-adrenergic preconditioning

However, it is well established that the (PI3K) PKB/Akt and ERK p44/p42 MAPK signaling cascades are activated in response to the stimulation of a wide range of receptors, including those for growth factors (RTK) and GPCRs [Widmann et al., 1999]. Activation of these signaling cascades prior to lethal ischaemia has been shown to be associated with ischaemic preconditioning induced cardioprotection [Tong et al., 2000; Fryer et al., 2001]. In addition, it has also been shown that ischaemic preconditioning protects the heart by phosphorylating the prosurvival kinases PKB/Akt and ERK p44/p42 MAPK, during early reperfusion [Hausenloy et al., 2004]. Pharmacological manipulation and up-regulation of these pro-survival kinases, which are also referred to as the Reperfusion Injury salvage Kinases (RISK), as an adjunct to reperfusion may protect the myocardium from reperfusion-induced cell death and provide a novel approach to salvage viable myocardium and limit infarct size [Hausenloy and Yellon, 2004].

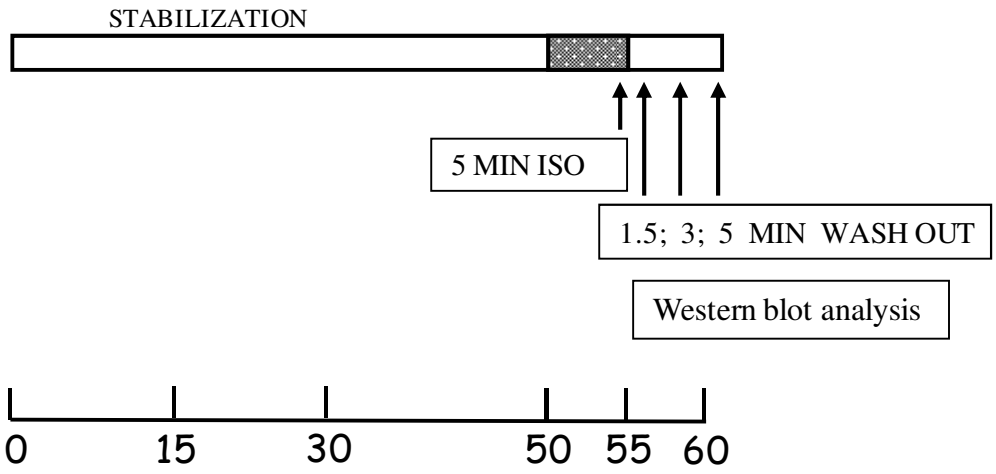
The involvement of these prosurvival kinases in β-adrenergic preconditioning has not yet been studied. Thus the aim of this study was to establish the involvement of these kinases prior to sustained ischaemia as well as at the onset of reperfusion in the context of β-adrenergic preconditioning. The PI3K inhibitor, wortmannin and the MEK inhibitor, PD 98,059 were used to achieve these objectives.

4.1 Methods

4.1.1 Investigation of the expression of total and phosphorylated PKB/Akt and ERK p44/p42 MAPKinase during β1/β2-PC (Fig. 4.1)

To establish the involvement of PKB/Akt and ERK p44/p42 MAPKinase during the preconditioning protocol prior to sustained ischaemia, β1/β2-PC hearts were freeze-clamped immediately after 5 min application of isoproterenol (0.1 μM). In another group, β1/β2-PC hearts were freeze-clamped after 1.5 min, 3 min and 5 min of washout prior to sustained ischaemia and analysed using Western blot analysis for total and phosphorylated PKB/Akt and ERK p44/p42 MAPKinase.

Experimental protocol: (Fig. 4.1)



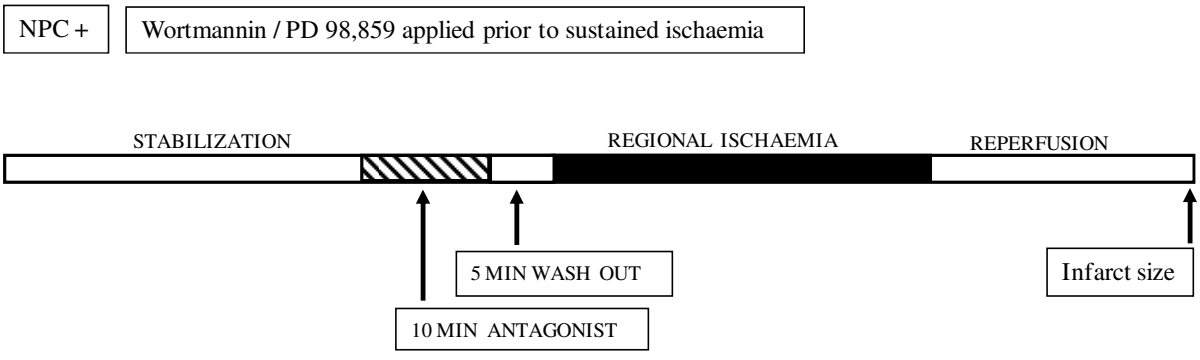
4.1.2 The effect of PI3-Kinase / PKB/Akt and ERK p44/p42 MAPKinase on functional recovery and infarct size in β1/β2-PC (Fig. 4.2 A and B)

Non-preconditioned hearts were subjected to a 30 minute stabilization period followed by a 30 minute perfusion in the working heart mode, 35 min regional ischaemia and reperfusion. After 50 min of stabilization, β1/β2-PC hearts were preconditioned with isoproterenol (0.1 μM) for 5 minutes followed by a washout episode of 5 min, 35 min regional ischaemia and reperfusion (as described in chapter 2).

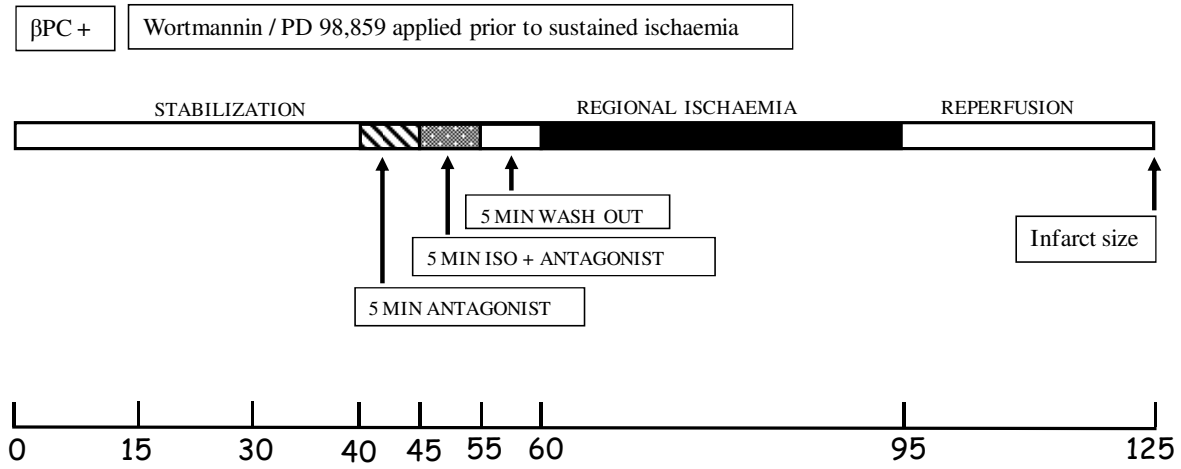
The PI3-Kinase inhibitor, wortmannin (final concentration 100 nM) or the MEK inhibitor, PD 98,059 (final concentration 10 μM) was dissolved in DMSO, the concentration of which was less than 0.00023 % vol/vol in the Krebs-Henseleit buffer. These inhibitors were applied in Krebs-Henseleit buffer to the NPC hearts for 10 minutes, followed by a 5 min washout episode prior to 35 min regional ischaemia and reperfusion (Fig. 4.2 A).

In the case of β1/β2-PC hearts, the inhibitors were administered for 10 min while isoproterenol (0.1 μM) was added to the perfusate for the last 5 min. This was followed by a 5 min washout episode, 35 min regional ischaemia and reperfusion (Fig. 4.2 A). In a separate group of experiments, wortmannin or PD 98,059 was applied at the onset of reperfusion for a period of 5 min. Haemodynamic parameters were recorded at the end of the 15 minute working heart mode prior to regional ischaemia and compared with haemodynamic parameters and infarct size at the end of reperfusion (Fig. 4.2 B).

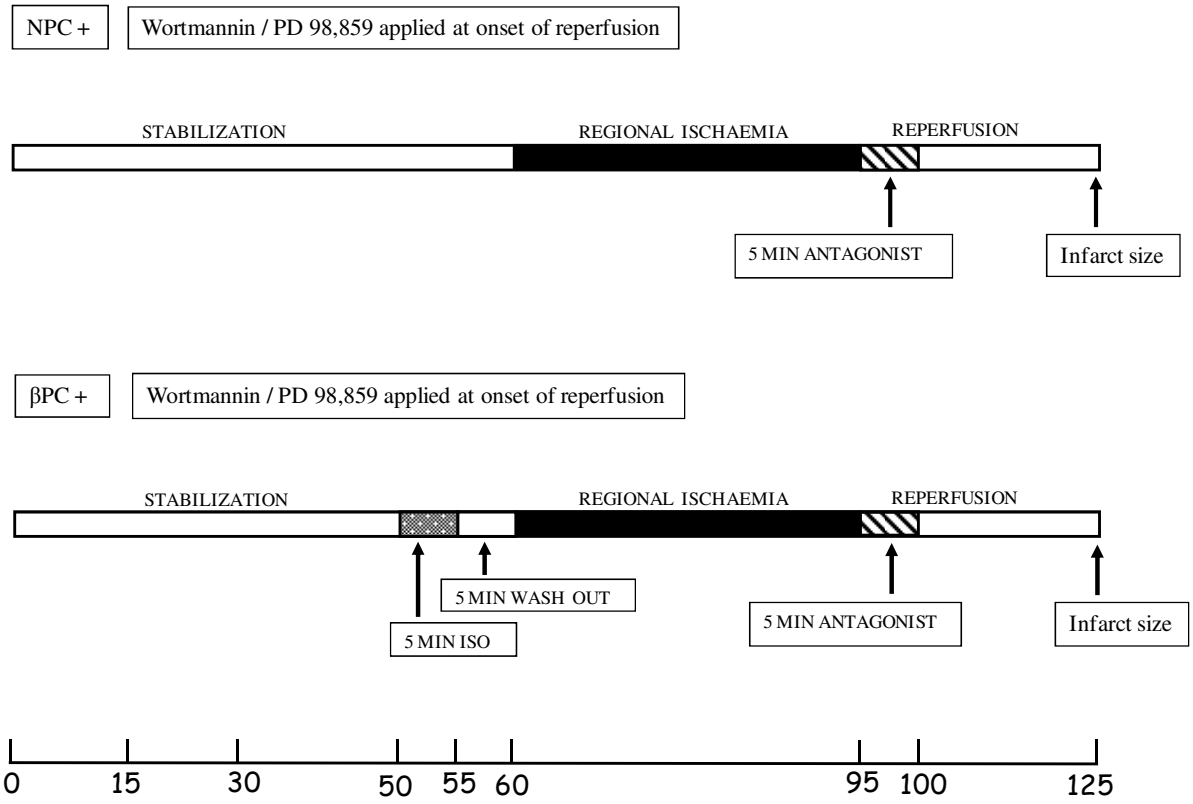
Experimental protocol: (Fig. 4.2 A)



Experimental protocol: (Fig. 4.2 A) (Continued)



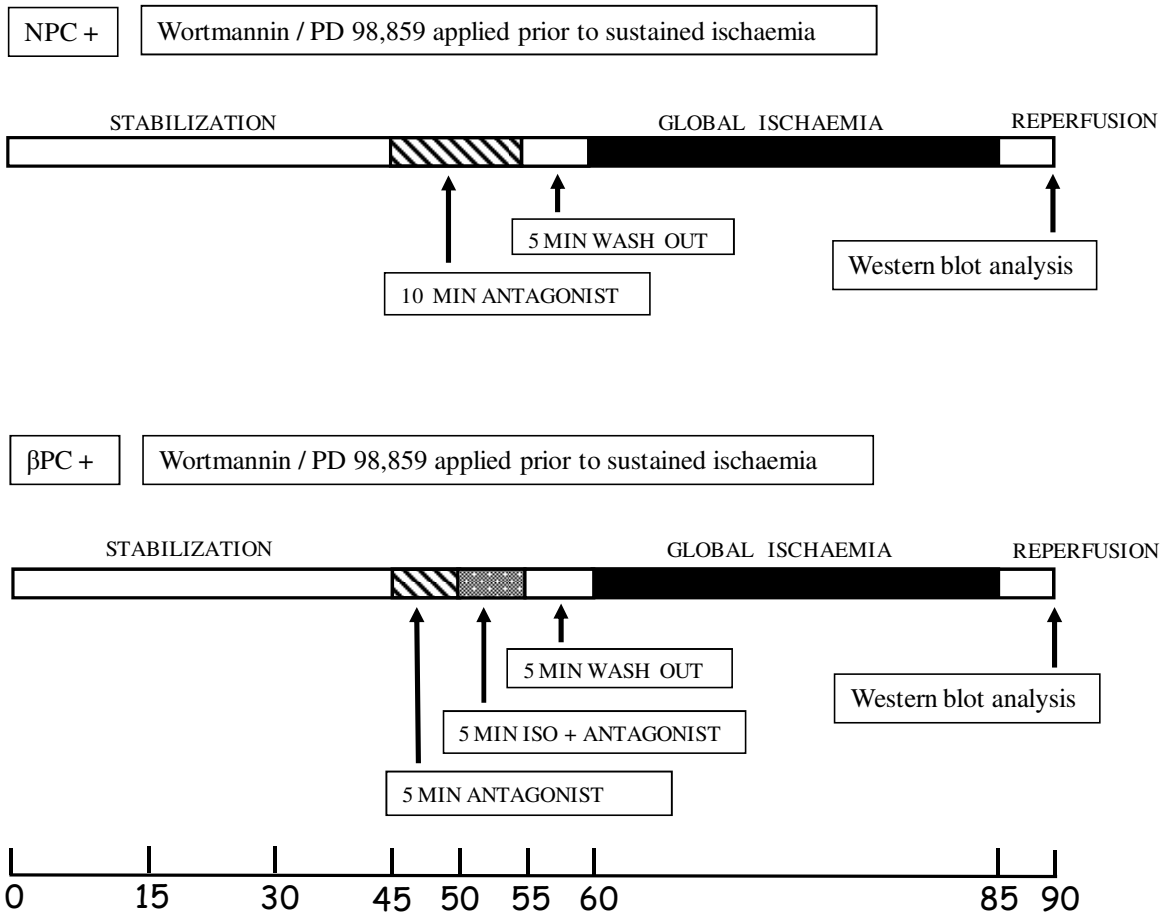
Experimental protocol: (Fig. 4.2 B)



4.1.3 Investigation of the expression of total and phophorylated PKB/Akt and ERK p44/p42 MAPKinase in β1/β2-PC during early reperfusion using Western blot analysis (Fig. 4.3)

Non-preconditioned hearts were subjected to a 30 minute stabilization period followed by a 30 minute retrograde perfusion, 25 minutes global ischaemia, followed by freeze- clamping after 5 minutes reperfusion. β1/β2-PC hearts were preconditioned with isoproterenol (0.1 μM) for 5 minutes followed by a washout episode of 5 min, 25 min global ischaemia and freeze-clamping after 5 min reperfusion. Freeze-clamped hearts were analysed using Western blot analysis for total and phosphorylated PKB/Akt and ERK p44/p42 MAPKinase. In addition, hearts were treated with wortmannin or PD 98,059, as outlined in 4.1.2, freeze-clamped after 5 min reperfusion and processed as described above.

Experimental protocol: (Fig. 4.3)



4.2 Results

4.2.1 Western blot analysis of total and phosphorylated PKB/Akt and ERK p44 / p42 MAPKinase after β1/β2-PC and during the washout episode (WO) (Fig. 4.4 A and B)

Expression of total PKB/Akt was similar at all time intervals studied. Western blot analysis for phosphorylated PKB/Akt of hearts subjected to β1/β2-PC illustrated a significant increase of this kinase at the end of 5 min administration of isoproterenol (0.1 μM) (fold increase: 3.47±1.12, $p<0.001$ vs negative control). The activation of PKB/Akt remained significantly elevated at 1.5 min (4.45±0.25), 3 min (3.94±0.15) as well as after 5 min (3.93±0.36) washout prior to global ischaemia (Fig. 4.4 A), i.e. until the onset of sustained ischaemia.

Similarly, Western blot analysis for phosphorylated ERK p44/p42 MAPKinase of hearts exposed to β1/β2-PC illustrated a significant increase in phosphorylated ERK p44 MAPKinase (2.46±0.17, $p<0.001$ vs negative control). Phosphorylated ERK p44 MAPKinase remained elevated at 1.5 min and 3 min washout, and increased even further after 5 min washout (fold increase: 3.50±0.20, $p< 0.001$ vs 1.5 min and 3 min washout, respectively) (Fig. 4.4 B).

Even though, ERK p42 MAPKinase was less activated compared to p44 MAPKinase, the p42 MAPKinase followed a similar trend after 5 min washout (fold increase: 2.77±0.08, $p<0.01$ vs 1.5 min washout, $p<0.05$ vs 3 min washout, respectively) (Fig. 4.4 B). Expression of total ERK p44/p42 MAPKinase after β1/β2-PC, at 1.5 min, after 3 min as well as after 5 min washout did not differ significantly from negative control values, respectively (Fig. 4.4 B).

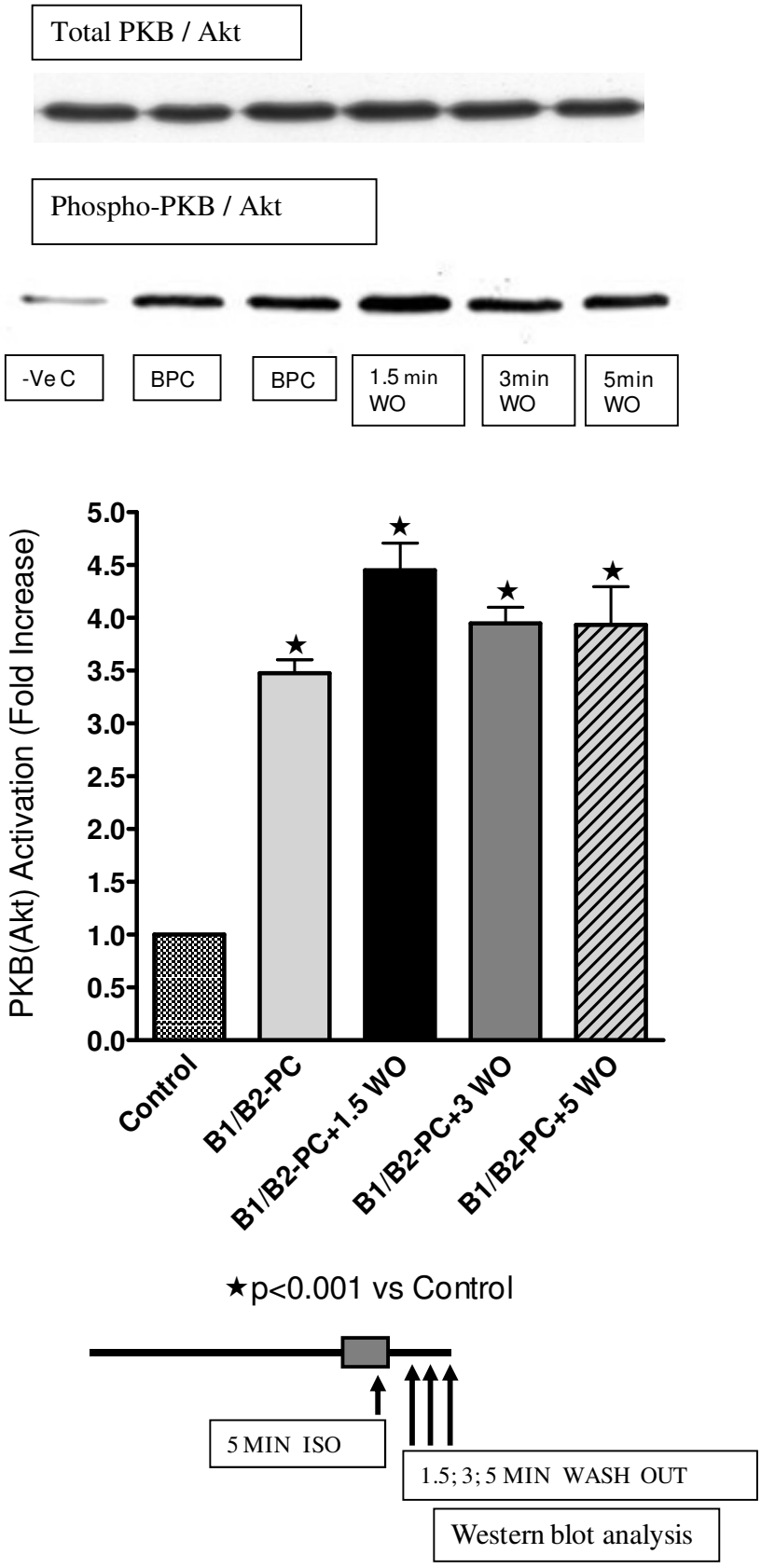


Fig. 4.4 A: PKB/Akt activation after β1/β2-PC, as well as after 1.5 min, 3 min and 5 min washout following β-adrenergic stimulation

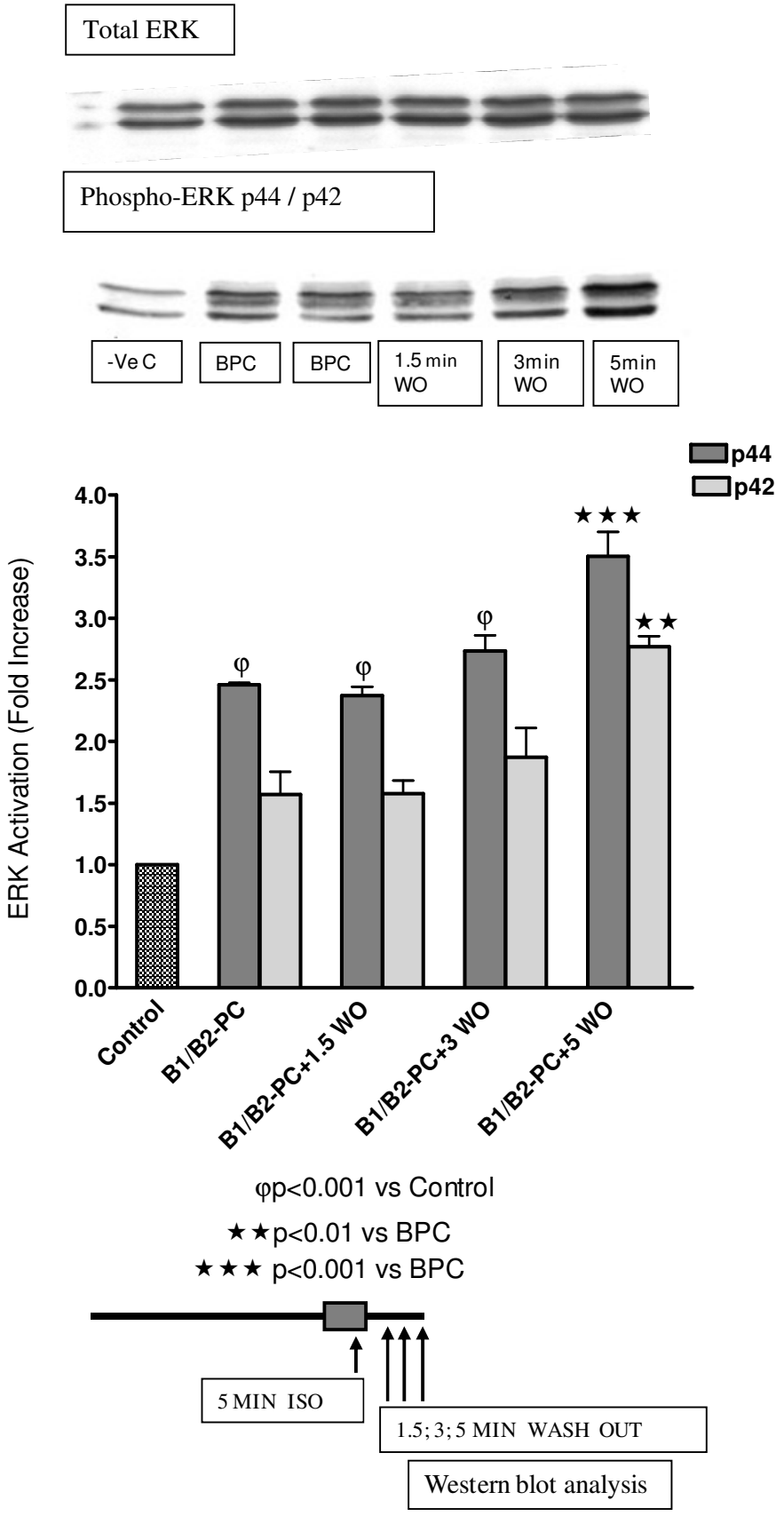


Fig. 4.4 B: ERK p44/p42 MAPKinase activation after β1/β2-PC, as well as after 1.5 min, 3 min and 5 min washout following β-adrenergic stimulation

4.2.2 The role of PKB/Akt and ERK p44 / p42 MAPKinase activation on functional recovery of hearts exposed to β1/β2-PC (Table 4.1 A and B)

As was demonstrated in Chapter 3, preconditioning of hearts with isoproterenol (0.1 μM), caused a significant increase in AO, CO and total work during reperfusion, when compared with those of NPC hearts. The PI3-Kinase inhibitor, wortmannin (100 nM) applied prior to regional ischaemia (trigger phase) or at the onset of reperfusion of hearts exposed to β1/β2-PC, significantly reduced haemodynamic parameters such as CF, AO, CO and total work, illustrating a definite role for PI3-Kinase-PKB/Akt in the cardioprotective effects of β1/β2-PC during both the preconditioning protocol as well as early reperfusion (Table 4.1 A). In the same experimental setting, the MEK (ERK p44/p42 MAPK) antagonist, PD 98,059 applied prior to regional ischaemia (trigger phase) or at the onset of reperfusion of hearts exposed to β1/β2-PC, had no significant effect on any of the parameters of functional recovery during reperfusion of β1/β2-PC hearts (Table 4.1 B).

Table 4.1 A: Effects of PI3-K - PKB/Akt inhibition with wortmannin on mechanical recovery during reperfusion of β1/β2-PC hearts

β-AR agonist: Isoproterenol (0.1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI	14.67±0.55	42.00±0.77	55.30±1.41	270±5.41	104.1±2.10	13.27±0.49
NPC After RI (n=6)	10.25±0.90 €	7.250±1.01 * #	19.01±1.02 * #	235±15.30 €	86.80±2.13 *	3.61±0.22 * #
β1/β2-PC Before RI	15.83±0.47	39.33±2.73	55.25±1.30	253±9.07	101.8±2.64	12.95±1.30
β1/β2-PC After RI (n=6)	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

€p< 0.05 vs NPC Before RI

*p< 0.001 vs NPC Before RI

# p< 0.05 vs β1/β2-PC After RI

Table 4.1 A: (continued)

PKB/Akt inhibitor: Wortmannin (Trigger) (100 nM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI	16.20±0.12	37.00±1.54	53.10±1.63	274.00±12.95	98.83±2.88	11.89±0.67
NPC After RI (n=6)	6.00±2.55 *	7.50±3.10 *	13.40± 5.63 *	144.00±60.69 *	62.60±21.54 *	3.62±1.12 *
β1/β2-PC Before RI	16.10±0.12	40.00±1.67	56.00±1.67	254.00±16.23	102.20±2.51	12.79±0.47
β1/β2-PC After RI (n=6)	6.12±2.51 #	4.40±2.07 €	10.40±4.28 ¥	129.00±54.64	37.07±19.09	2.51±1.04 ¥

*p< 0.001 vs NPC before RI

# p< 0.05 vs β1/β2-PC After RI

€ p< 0.001 vs β1/β2-PC After RI

¥ p< 0.01 vs β1/β2-PC After RI

PKB/Akt inhibitor: Wortmannin (Reperfusion) (100 nM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI	15.40±0.24	41.00±0.63	55.20±0.58	282.00±13.41	101.00±2.33	12.64±0.27
NPC After RI (n=6)	5.40±2.35 *	3.20±1.94 *	8.50± 3.71 *	154.00±65.90 *	48.19±19.79 *	1.52±0.67 *
β1/β2-PC Before RI	16.10±1.00	38.40±0.97	54.40±0.97	261.00±18.04	98.76±3.10	12.30±0.11
β1/β2-PC After RI (n=6)	6.30±2.70 #	4.30±2.26 €	10.60±4.87 ¥	149.00±61.60	50.56±20.71	2.02±0.95 ¥

*p< 0.001 vs NPC before RI

# p< 0.05 vs β1/β2-PC After RI

€ p< 0.001 vs β1/β2-PC After RI

¥ p< 0.01 vs β1/β2-PC After RI

Table 4.1 B: Effects of MEK (ERK p44/p42 MAPK) inhibition with PD 98,059 on mechanical recovery during reperfusion of β1/β2-PC hearts

β-AR agonist: Isoproterenol (0.1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI	14.67±0.55	42.00±0.77	55.30±1.41	270±5.41	104.1±2.10	13.27±0.49
NPC After RI (n=6)	10.25±0.90 €	7.250±1.01 * #	19.01±1.02 * #	235±15.30 €	86.80±2.13 *	3.61±0.22 * #
β1/β2-PC Before RI	15.83±0.47	39.33±2.73	55.25±1.30	253±9.07	101.8±2.64	12.95±1.30
β1/β2-PC After RI (n=6)	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

€p< 0.05 vs NPC Before RI *p< 0.001 vs NPC Before RI # p< 0.05 vs β1/β2-PC After RI

MEK (ERK p44/p42 MAPK) inhibitor: PD 98,059 (Trigger) (10 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=6)	16.80±0.20	38.60±2.08	54.50±2.09	268.00±14.05	101.40±6.07	12.35±1.13
NPC After RI	8.50±2.70 *	6.82±2.16 *	14.34±4.74 *	179.00±57.17 *	59.34±18.79 *	3.74±0.90 *
β1/β2-PC Before RI (n=6)	16.60±0.36	36.40±0.97	52.80±1.35	268.00±26.30	106.60±2.16	12.68±0.47
β1/β2-PC After RI	10.50±3.05	12.60±3.95	22.10±6.92	223.00±56.35	70.26±17.67	5.11±1.36

*p< 0.001 vs NPC Before RI

MEK (ERK p44/p42 MAPK) inhibitor: PD 98,059 (Reperfusion) (10 μM)

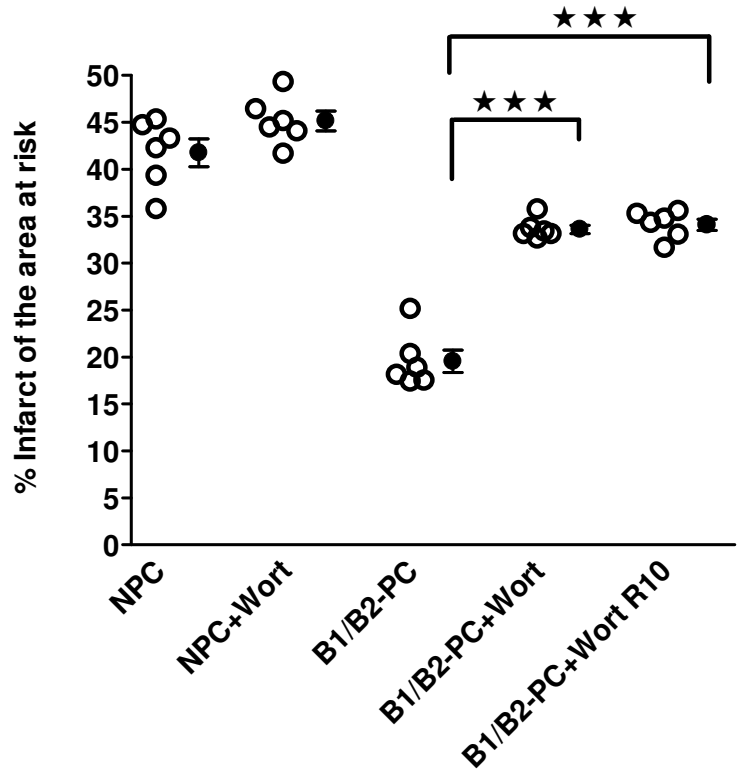
	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=6)	16.60±0.60	39.80±1.14	56.40±1.28	295.00±10.82	103.10±3.55	13.29±0.57
NPC After RI	8.70±2.29 *	7.90±3.11 *	16.50±5.14 *	205.00±52.46 *	88.47±1.61	3.30±1.03 *
β1/β2-PC Before RI (n=6)	16.00±0.72	43.60±2.85	60.10±3.13	282.00±12.07	101.10±5.52	13.35±1.12
β1/β2-PC After RI	12.30±0.48	15.90±3.42	28.20±3.81	226.00±9.14	78.39±1.85	5.97±0.77

*p< 0.001 vs NPC Before RI

4.2.3 The effect of PI3-Kinase - PKB/Akt and ERK p44 / p42 MAPKinase inhibition on infarct size (IS) in β1/β2-PC (Fig. 4.5 A and B)

As shown previously, β1/β2-PC with isoproterenol (0.1 μM) caused a significant reduction in infarct size, when compared with NPC hearts (41.76±1.48%, p<0.001 vs βPC). The PI3-Kinase inhibitor, wortmannin (100 nM) applied prior to regional ischaemia (trigger phase) or at the onset of reperfusion of hearts exposed to in β-adrenergic preconditioning, significantly increased IS and abolished the cardioprotective effects of β-preconditioning in both experimental settings (33.61±0.44% and 34.08±0.61%, p<0.001 vs 19.55±1.19 % of β1/β2-PC, respectively) (Fig. 4.5 A). Similarly, the MEK (ERK p44/p42 MAPK) antagonist, PD 98,059 applied prior to regional ischaemia (trigger phase) or at the onset of reperfusion of hearts exposed to β1/β2-PC, significantly increased IS (44.56±1.70% and 40.36±1.65%, p<0.001 vs β1/β2-PC, respectively) (Fig. 4.5 B).

(A)



★★★p<0.001 vs BPC

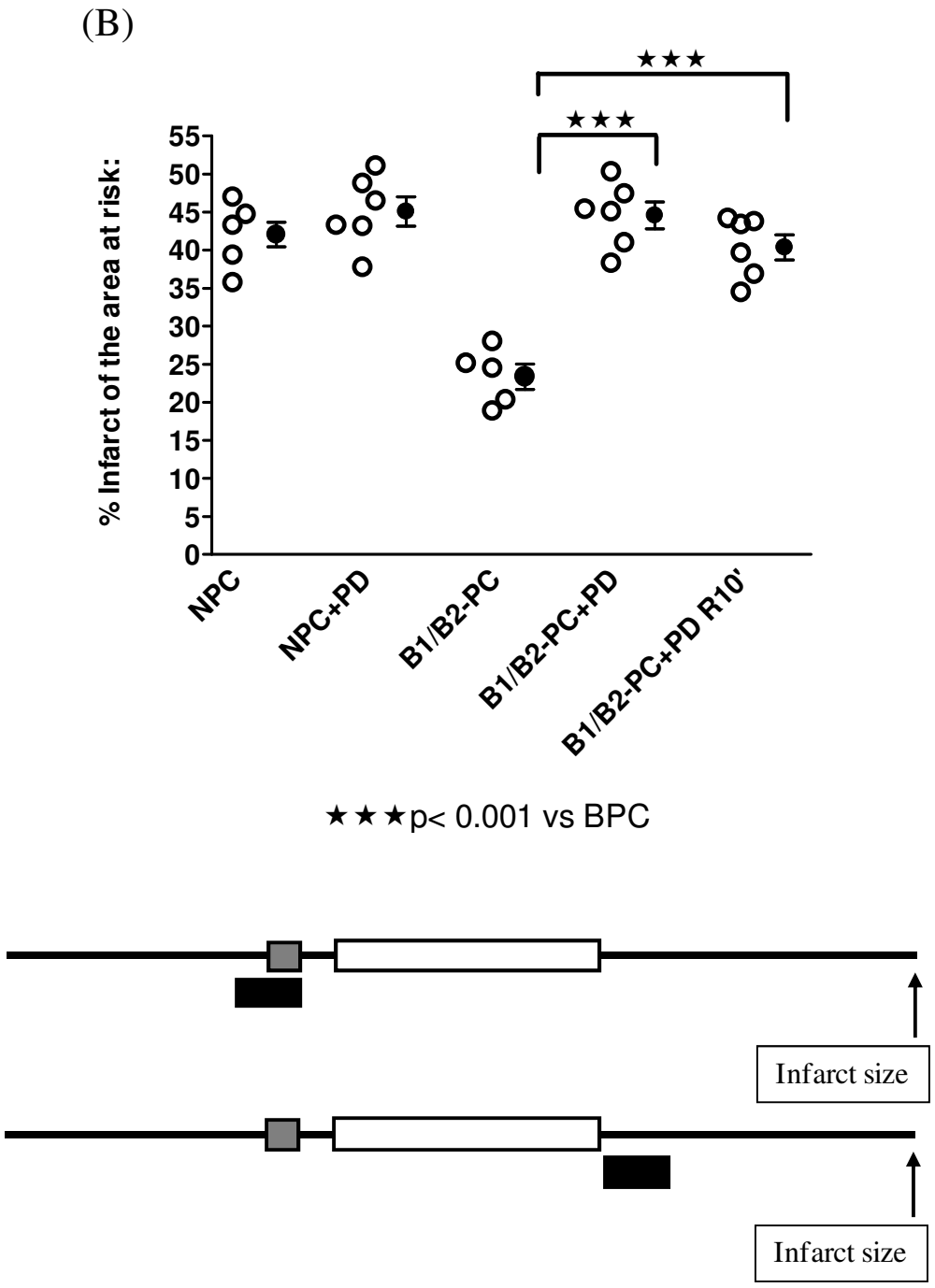


Fig. 4.5: The effect of PI3-Kinase - PKB/Akt inhibition (wortmannin) (A) and MEK- ERK p44 / p42 MAPKinase inhibition (PD 98,059) (B) on infarct size in β1/β2-PC

4.2.5 Western blot analysis of total and phosphorylated PKB/Akt and ERK p44 / p42 MAPKinase in β1/β2-PC at early reperfusion (Fig. 4.6 A, B, C and D)

Western blot analysis for phosphorylated PKB/Akt of hearts subjected to β1/β2-PC illustrated a significant fold increase of phosphorylated PKB/Akt (2.24±0.17, $p<0.05$ vs NPC) which was significantly suppressed with the application of wortmannin prior to global ischaemia in the β-PC+ group (1.18±0.15, $p<0.001$ vs β1/β2-PC) (Fig. 4.6 A).

Similarly, Western blot analysis for phosphorylated ERK p44/p42 MAPKinase of hearts subjected to β1/β2-PC illustrated a significant fold increase of ERK p44 (2.49±0.06, $p<0.001$ vs NPC) and ERK p42 (2.29±0.05, $p<0.01$ vs NPC). The application of wortmannin prior to global ischaemia (β-PC+) significantly suppressed the activation of ERK p44 (1.86±0.09, $p<0.01$ vs β1/β2-PC) as well as ERK p42 during reperfusion (1.35±0.13, $p<0.001$ vs β1/β2-PC) (Fig. 4.6 B).

The application of PD 98,059 (β-PC+) in the same experimental setting had no effect on the activation of PKB/Akt (Fig. 4.6 C). However, PD 98,059 significantly reduced the activation of ERK p44 (1.46±0.17, $p<0.001$ vs β1/β2-PC) as well as ERK p42 (1.23±1.33, $p<0.001$ vs β1/β2-PC) (Fig. 4.6 D). In all experimental conditions, the expression of total PKB/Akt, ERK p44 as well as ERK p42 MAPKinase was unaltered, compared to the negative controls.

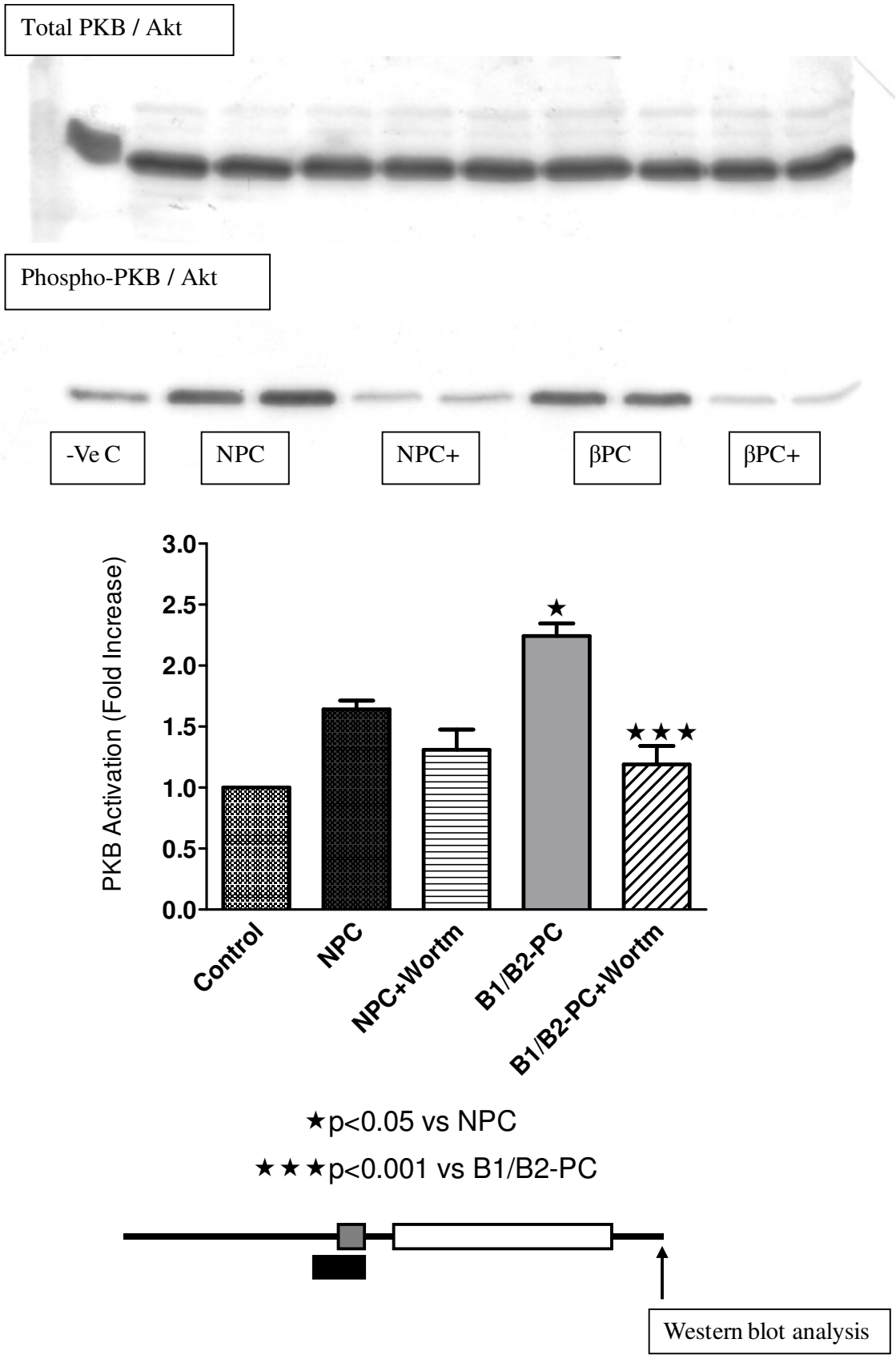


Fig. 4.6 A: The effect of PI3-K inhibition with wortmannin on PKB/Akt expression during early reperfusion

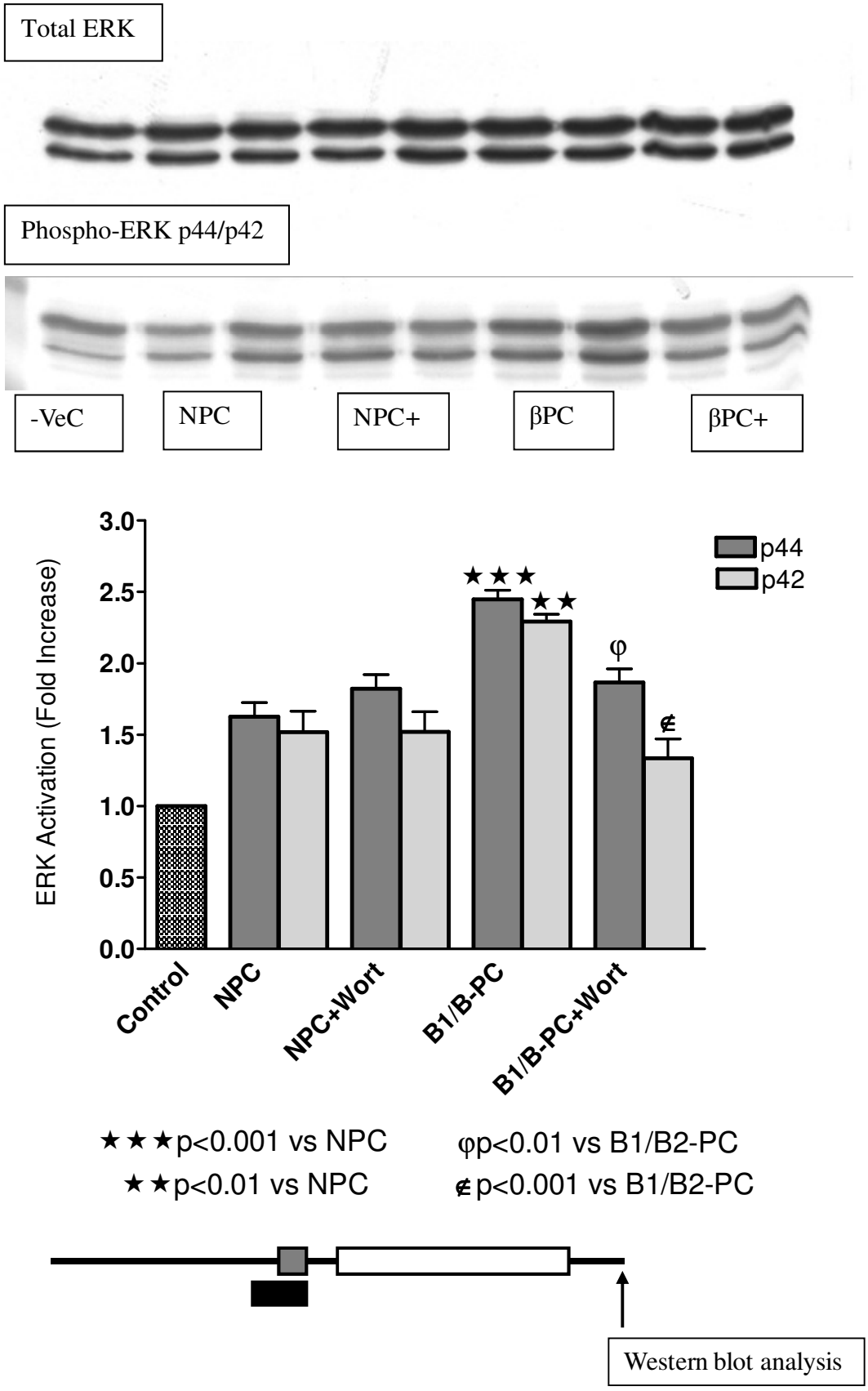


Fig. 4.6 B: The effect of PI3-K inhibition with wortmannin on ERK p44/p42 MAPKinase expression during early reperfusion

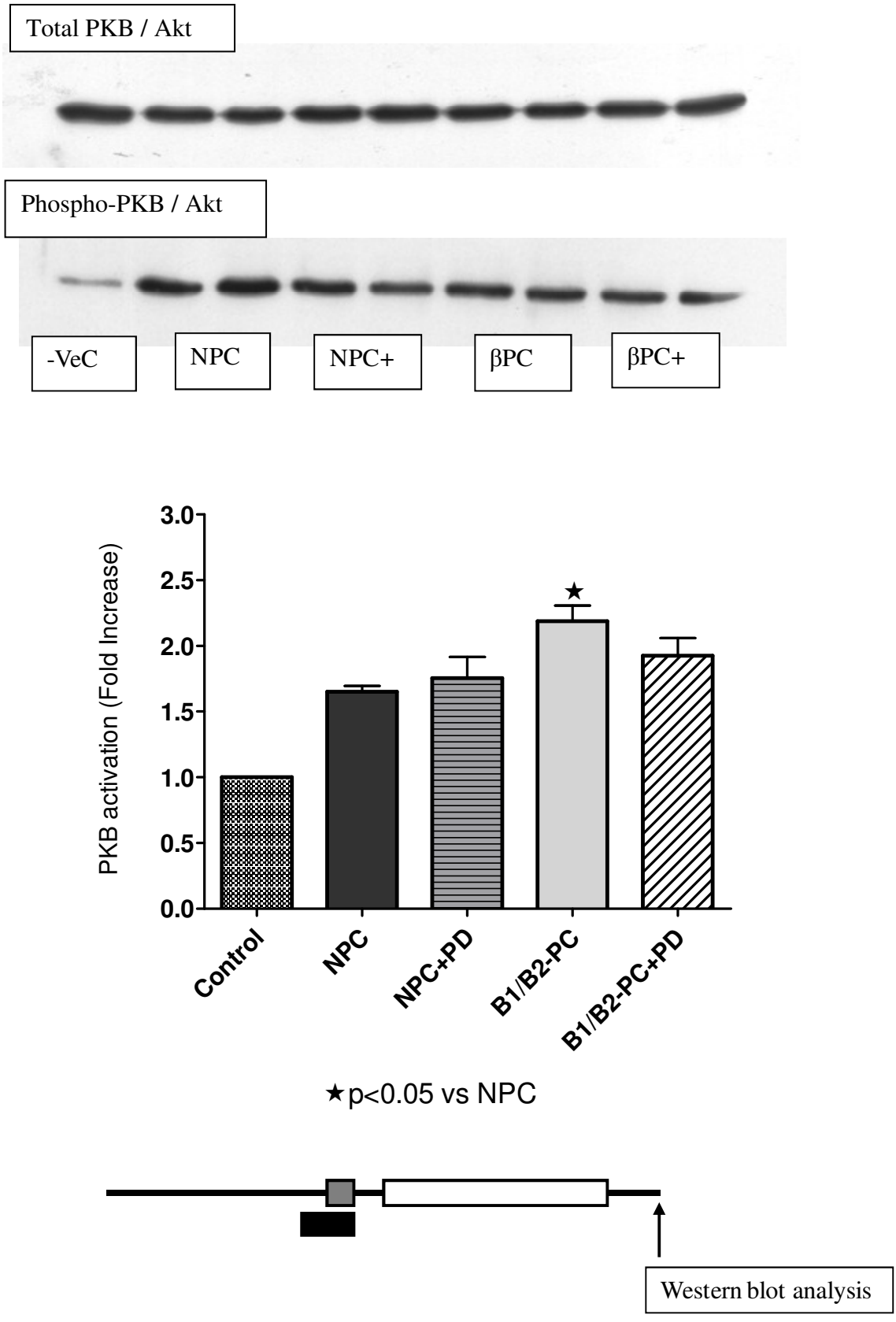


Fig 4.6 C: The effect of MEK (ERK p44/p42 MAPKinase) inhibition with PD 98,059 on PKB/Akt expression during early reperfusion

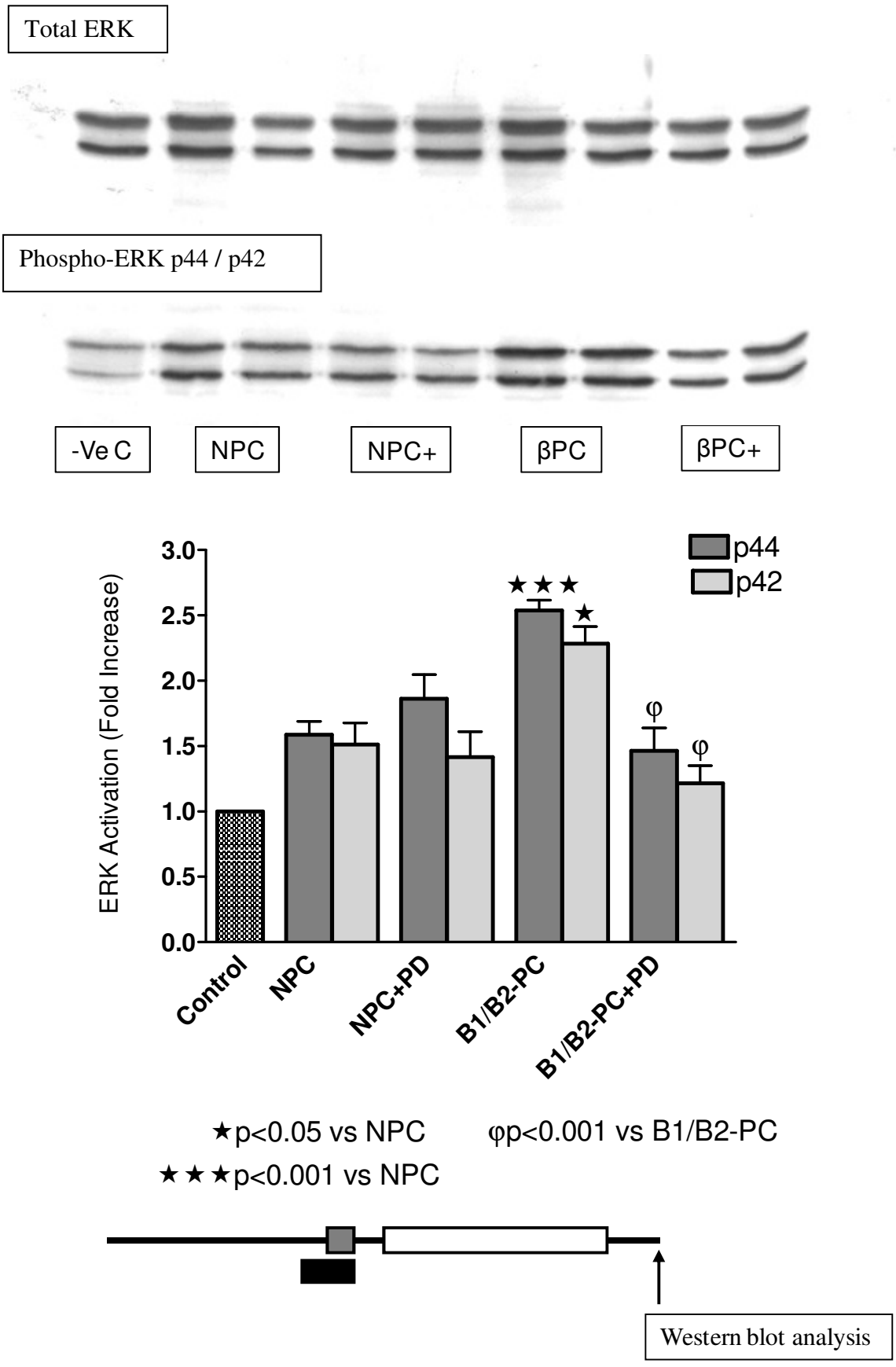


Fig. 4.6 D: The effect of MEK (ERK p44/p42 MAPKinase) inhibition with PD 98,059 on ERK p44/p42 MAPKinase expression during early reperfusion

4.3 Discussion

In this study, the cardioprotection of β1/β2-PC was shown to be dependent on the activation of PI3K-PKB/Akt and ERK p44/p42 MAPK during the administration of isoproterenol as well as during the washout episode prior to sustained ischaemia (Fig. 4.4 A and B), suggesting that these two kinases play an important role in triggering protection. This was substantiated by the fact that inhibition of these kinases during this phase, abolished protection, in this regard, it was shown in this study that inhibition of PI3K-PKB/Akt (with Wortmannin) or inhibition of ERK p44/p42 MAPK (with PD 098,059) prior to sustained ischaemia also significantly reduced their activation at reperfusion (Fig. 4.6 A and C). As far as we know, this is the first demonstration that activation of these two survival kinases is a prerequisite for eliciting β-AR cardioprotection. As expected, activation of these two kinases during early reperfusion, is also a required for the protective action of β1/β2-PC. The significantly increased infarct size and reduced cardioprotective effects (Fig. 4.5 A and B) associated with inhibition of these kinases, emphasize the significance of activation of the RISK pathway during reperfusion in cardioprotection.

Although both kinases are associated with cardioprotection, it was shown in this study that they have a interdependent relationship to achieve the cardioprotective response seen in β1/β2-PC, since inhibition of PI3K-PKB/Akt (with Wortmannin) during the triggering phase not only caused significant inhibition of PI3K-PKB/Akt but also of ERK p44/p42 MAPK activation (Fig. 4.6 A and B), whereas inhibition of ERK p44/p42 MAPK (with PD 098,059) significantly reduced ERK p44/p42 MAPK but had no significant effect PI3K-PKB/Akt activation (Fig. 4.6 C and D), indicating that ERK p44/p42 MAPK activation depends largely on PI3K-PKB/Akt activation and not vice versa. These observations suggest that ERK p44/p42 MAPK activation during early reperfusion is perhaps more significant in β1/β2-PC induced cardioprotection, since abolishment of cardioprotection could be obtained in the presence of PI3K-PKB/Akt activation, as was shown with the administration of PD 098,059 during the β1/β2-PC preconditioning protocol (see Fig. 4.6 C).

The results obtained in our study are in agreement with the activation of the RISK pathway observed in ischaemic preconditioning. These kinases are known to be activated prior to lethal

ischaemia [Tong et al., 2000; Fryer et al., 2001] as well as at the time of reperfusion [Hausenloy et al., 2004] and suggested to mediate ischaemic preconditioning induced cardioprotection.

Activation of the anti-apoptotic pro-survival kinase signaling cascades, (PI3K) PKB/Akt and ERK p44/p42 MAPK, has been implicated in cellular survival through their recruitment of anti-apoptotic pathways of protection [Gross et al., 2000]. These include the phosphorylation and inactivation of a diverse array of substrates, responsible for mediating cardioprotection, including GSK-3β [Michael et al., 2004], proapoptotic proteins such as BAD [Datta et al., 1997], the mitochondrial permeability transition pore [Shanmuganathan et al., 2005], BAX [Tsuruta et al., 2002; Weston et al., 2003], BIM [Weston et al., 2003], p53 and caspases [Cardone et al., 1998; Erhardt et al., 1999], GLUT4, transcription factors (IKK-α), P70S6K [Chung, et al., 1994; Lehman and Gomez-Cambronero, 2002], NOS [Dimmeler et al., 1999] and PKC [Le Good et al., 1998].

Chapter 5

The function of adenosine, its receptors (A_1, A_{2A}, A_{2B} and A_3) and downstream targets in the cardioprotective phenomemon of β-adrenergic preconditioning

Having established the involvement of the β-adrenergic receptor subtypes and subsequent signaling in triggering the cardioprotection of β-adrenergic preconditioning, the next aim was to further elucidate the mechanism of β-adrenergic receptor mediated cardioprotection.

β-adrenergic receptor stimulation per se causes an increased workload on the heart and could conceivably elicit demand ischaemia with concomitant adenosine production. Indeed, stimulation with isoproterenol has been shown to cause adenosine release, due to an imbalance between oxygen supply and demand [Duessen and Schrader, 1991]. The relative importance of adrenergic stimulation and demand ischaemia as important preconditioning stimuli remains unclarified, however it was shown that demand ischaemia can precondition the myocardium while increased demand alone without ischaemia had marginal preconditioning effects. This may be of clinical relevance to patients with severe stenosis exposed to stressful stimuli before the development of myocardial infarction [Sharaf et al., 2000].

In the heart, adenosine has potent electrophysiological effects [Drury, and Szent-Gyorgi, 1929; Belardinelli, Linden and Berne, 1989] and the transient, reversible slowing of the heart rate (negative chronotropic effects) and impairment of atrioventricular conduction (negative dromotropism), antagonize the effects of catecholamines (anti-adrenergic) [Schrader, Baumann and Gerlach, 1977]. The role of adenosine as a trigger of ischaemic preconditioning has been intensely studied and reviewed by several authors [Cohen and Downey, 2008; Headrick and Peart, 2005; Lasley et al., 2007]. Unlike bradykinin and opioids, the other two autacoids involved in this process, adenosine is not dependent on opening of the mitochondrial K_{ATP} channel or release of ROS, but activates phopholipase C and / or PKC directly [Cohen and Downey, 2008].

Adenosine exerts its effects almost exclusively via four currently defined G-protein-coupled receptors (GPCRs): A_1, A_{2A}, A_{2B} and A_3 [Fredholm et al., 2001]. However, the effect of adenosine itself on the ischaemic responses may be more complex than modulation of these receptors above [Ashton et al., 2007], since it has been shown that adenosine can modulate cardiac tolerance to ischaemia or hypoxia via non-receptor-mediated metabolic or substrate actions [Bolling et al., 1994; Peart and Headrick, 2003].

The A_1-AdoRs and A_3-AdoRs participate in the intracellular signaling that triggers ischaemic preconditioning [Dougherty et al., 1998; Miura and Tsuchida, 1999]. Conversely, participation of the A_{2A}-AdoRs and A_{2B}-AdoRs has been identified in the protective phenomenon of postconditioning [Zhao et al., 2003; Kin et al., 2005; Yang et al., 2005]. During reperfusion activation of PKC initiates the activation of the low sensitivity A_{2B} receptor, making it responsive to adenosine which accumulated during sustained ischaemia. This would limit its activation only to periods of excessive adenosine accumulation (Peart and Headrick, 2007). In addition, role of the A_{2B} receptor in reperfusion has been demonstrated by the fact that A_{2B} agonists infused at reperfusion mimics preconditioning [Eckle et al., 2007].

In this chapter the role of adenosine release and the relative contribution of the A_1, A_{2A}, A_{2B} and A_3 receptors during the triggering and mediatory phases of β1/β2-PC were investigated. In addition, the involvement of PI3-K and ERK signal transduction pathways in the mechanism of β-adrenergic receptor mediated cardioprotection were further elucidated.

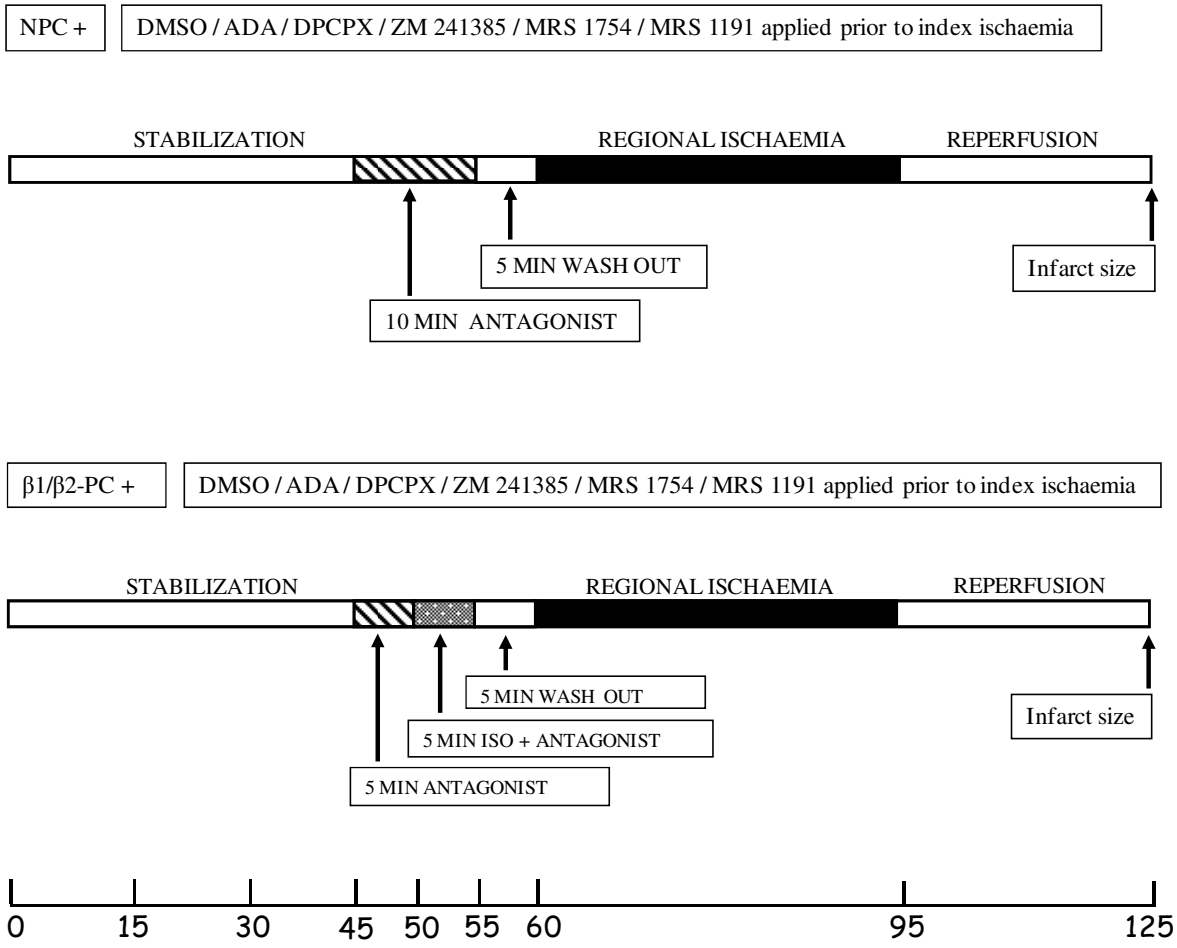
5.1 Methods

5.1.1 Investigating the role of adenosine and the adenosine A_1, A_{2A}, A_{2B} and A_3 receptors in β1/β2-PC (Fig. 5.1 A and B)

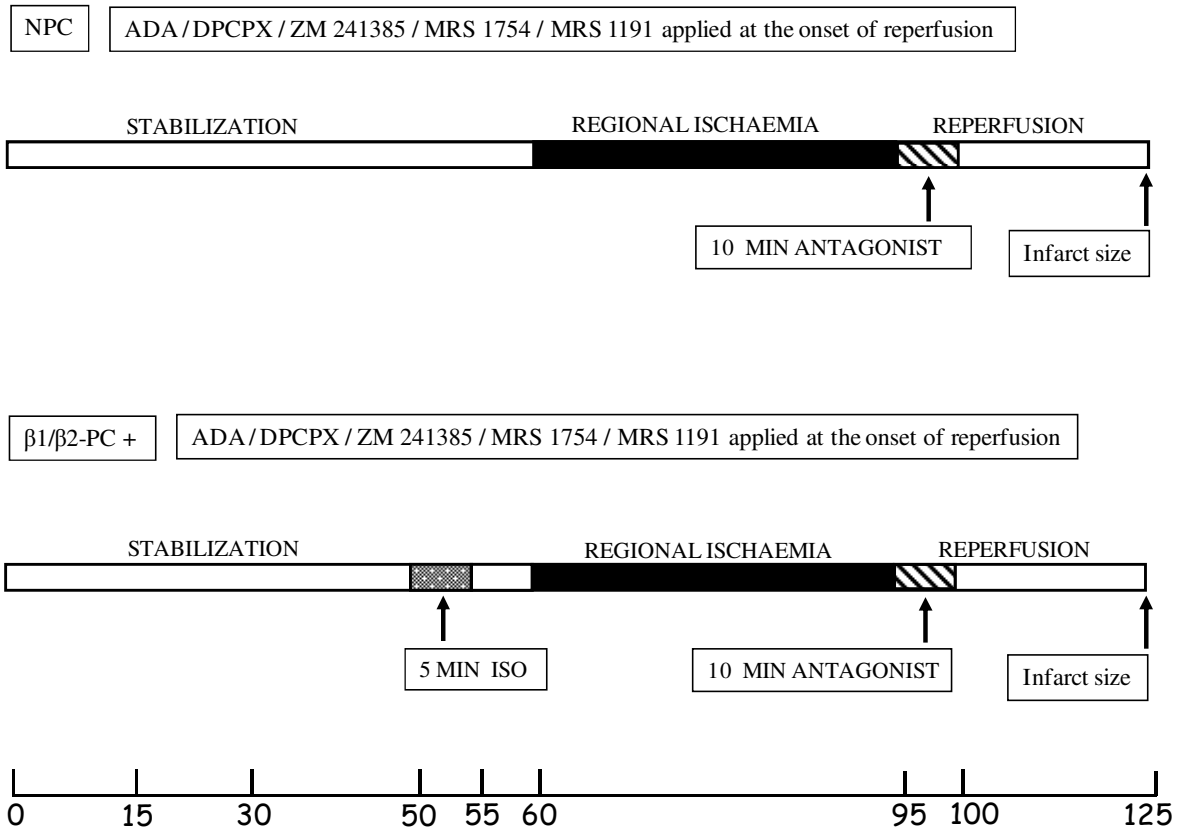
Non-preconditioned hearts as well as β1/β2-PC hearts were exposed to administration of the following drugs: adenosine deaminase (0.3 U/ml); A_1-AdoR antagonist (DPCPX) (1μM); A_3-AdoR antagonist (MRS 1191) (1μM); A_{2A}-AdoR antagonist (ZM241385) (1 μM); A_{2B}-AdoR antagonist (MRS1754) (1μM). In the NPC hearts, drugs were administered for 10 minutes followed by a 5 minute washout episode prior to 35 minutes regional ischaemia and reperfusion. In the case of the β1/β2-PC hearts, isoproterenol (0.1 μM) administration was initiated 5 min after the antagonists and co-administered for a further 5 min. The rest of the protocol was similar for the two groups.

In separate groups of experiments adenosine deaminase or antagonists of the adenosine A_1, A_{2A}, A_{2B} or A_3 receptor were applied during the first 10 min of reperfusion after regional ischemia. Haemodynamic parameters were recorded at the end of the 15 minute working heart mode prior to regional ischaemia and compared with those measured at the end of reperfusion following regional ischaemia. Adenosine deaminase was dissolved in distilled water whereas the adenosine antagonists were dissolved in DMSO and applied in Krebs-Henseleit buffer, respectively. The concentration of DMSO in the Krebs-Henseleit buffer was less than 0.00023 % vol/vol. To ensure that DMSO had no effect on the pharmacological agents used, DMSO (0.00023 %) alone was applied in the same experimental setting.

Experimental protocol: (Fig. 5.1 A)



Experimental protocol: (Fig. 5.1 B)

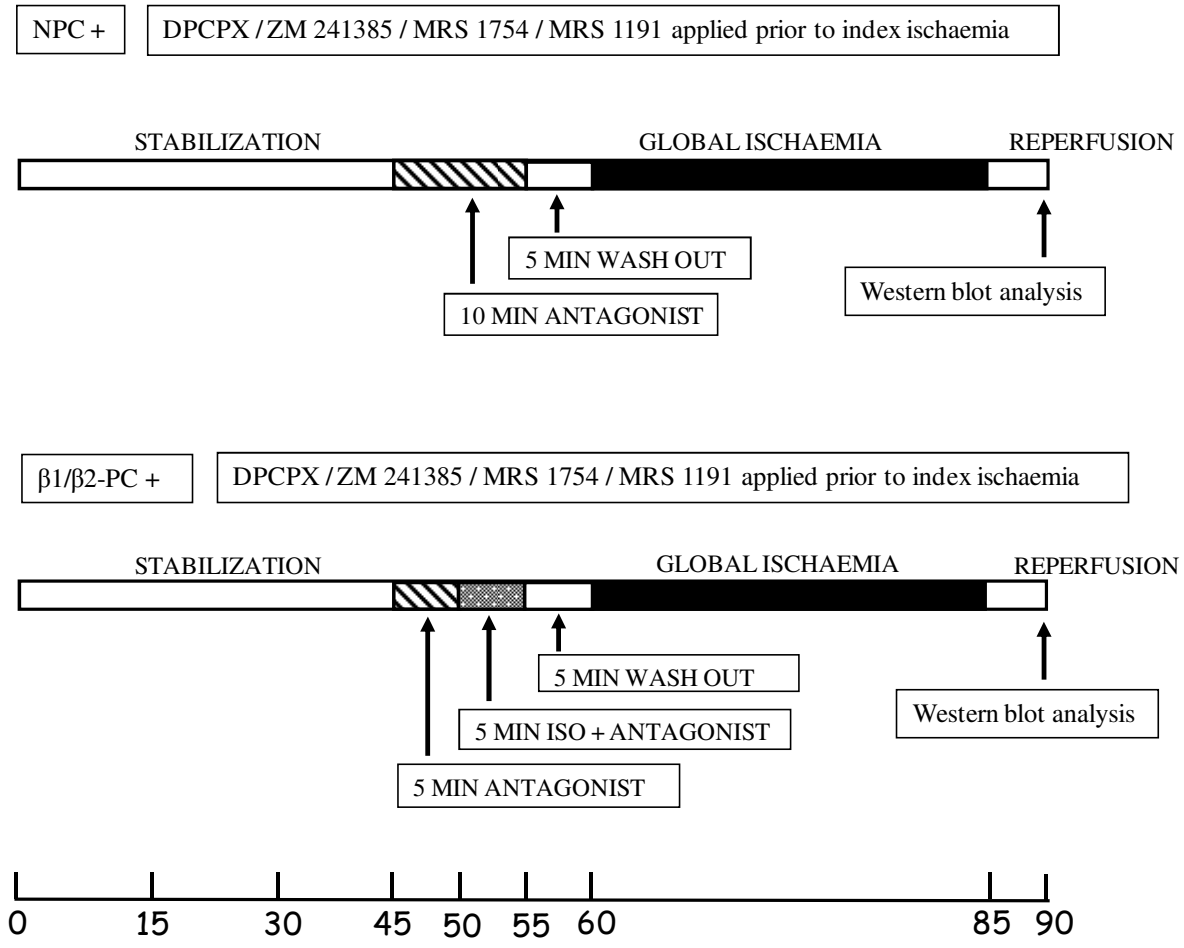


5.1.2 To investigate whether adenosine and adenosine A_1, A_{2A}, A_{2B} and A_3 receptors affect PKB and ERKp42/p44 MAPKinase activation in β1/β2-PC (Fig. 5.2 A and B)

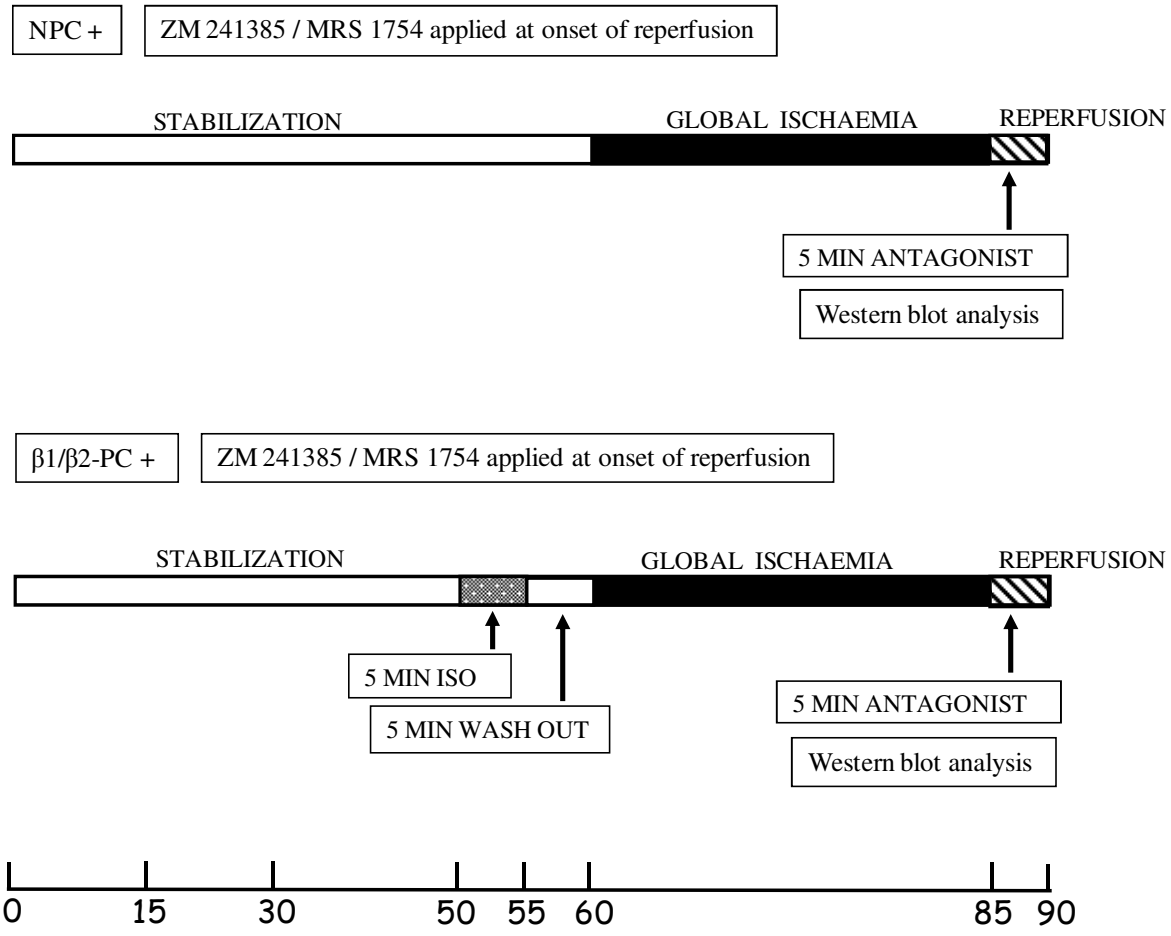
The adenosine A_1 (DPCPX) (1μM), A_{2A} (ZM241385) (1 μM), A_{2B} (MRS1754) (1μM) or the A_3 (MRS 1191) (1μM) receptor antagonist was administered prior to global ischaemia to NPC and β1/β2-PC hearts as indicated in Fig. 5.2 A. These hearts were freeze-clamped at 5 min reperfusion after 25 min global ischaemia and analysed using Western blot analysis to investigate the expression of total and phosphorylated PKB/Akt as well as total and phosphorylated ERK p44/p42 MAPK.

In separate groups of experiments the A_{2A} (ZM241385) (1 μM) or A_{2B} (MRS1754) (1μM) receptor antagonist was applied at the onset of reperfusion for 5 minutes, after which hearts were freeze-clamped and analysed using Western blot analysis for total and phosphorylated PKB/Akt and ERK p44/p42 MAPKinase (Fig 5.2 B).

Experimental protocol: (Fig. 5.2 A)



Experimental protocol: (Fig. 5.2 B)



5.2 Results

5.2.1 a The involvement of adenosine in β1/β2-PC (Table 5.1)

To assess the involvement of adenosine in β-PC, a preliminary study was done using adenosine deaminase (at one concentration only). Adenosine deaminase (ADA) (0.3 U/ml) applied prior to RI (trigger phase) of NPC or hearts exposed to β1/β2-PC (0.1 μM) had no significant effect on any of the haemodynamic parameters at the end of reperfusion and hearts subjected to β1/β2-PC still showed significant increases in AO, CO and total work in the presence of adenosine deaminase.

Table 5.1: Effect adenosine deaminase on mechanical recovery of β1/β2-PC hearts

β-AR agonist: Isoproterenol

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	16.28±0.36	39.78±0.85	55.94±0.84	269±4.00	107.0±1.38	13.39±0.27
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI (n=18)	16.61±0.25	40.25±1.01	56.78±1.18	252±3.57	107.5±1.66	13.99±0.45
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

# P< 0.05 vs β1/β2-PC After RI

Adenosine deaminase

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+ADA After RI (n=6)	8.00±3.62	5.23±2.64	13.58±6.23	126±57.75	44.93±20.16	2.72±1.23
β1/β2-PC+ADA After RI (n=6)	15.17±1.50	14.17±2.54	29.33±3.34	245±5.63	88.78±1.58	5.89±0.82

5.2.1 b The effect of adenosine deaminase on IS in β1/β2-PC (Fig. 5.3)

Adenosine deaminase (0.3 U/ml) applied to NPC hearts did not show significant change of IS. However, the application of adenosine deaminase (0.3 U/ml) in the same experimental setting prior to regional ischemia of β1/β2-PC hearts, illustrated a significantly increased infarct size (β1/β2-PC: 16.39±0.72% vs 27.24±1.30%, p<0.01), which clearly illustrates the involvement of adenosine in β-adrenergic preconditioning, despite the fact that mechanical recovery of these hearts were not affected.

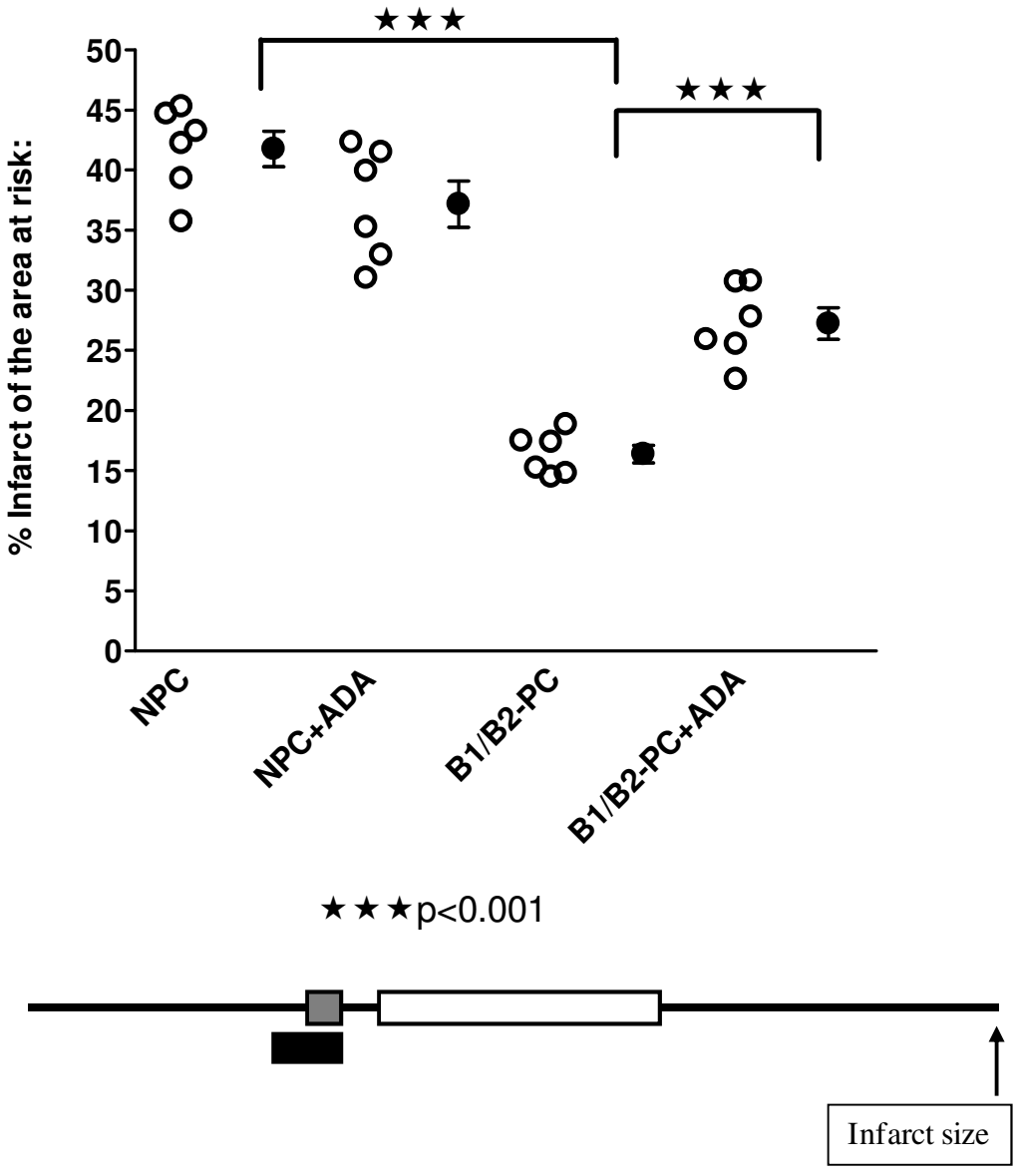


Fig. 5.3: The effect of adenosine deaminase on infarct size in β1/β2-PC

5.2.1 c The effect of adenosine inhibition on PKB/Akt and ERK p44 / p42 MAPKinase (Fig. 5.4 A and B)

Western blot analysis for phosphorylated PKB/Akt of hearts subjected to β1/β2-PC showed a significant increase (fold increase: 2.36±0.12, $p<0.001$ vs control), after 5 min reperfusion, which was not affected by the application of adenosine deaminase prior to global ischaemia (Fig. 5.4 A).

Similarly, Western blot analysis for phosphorylated ERK p44/p42 MAPKinase of hearts subjected to β1/β2-PC exhibited significant increase in ERK p44 (fold increase: 2.78±0.24, $p<0.001$ vs control) and p42 (2.53±0.26, $p<0.001$ vs control), after 5 min reperfusion, which was not significantly altered by the application of adenosine deaminase prior to global ischaemia (Fig. 5.4 B). Total PKB/Akt and ERK p44/p42 MAPKinase were similar in all groups studied.

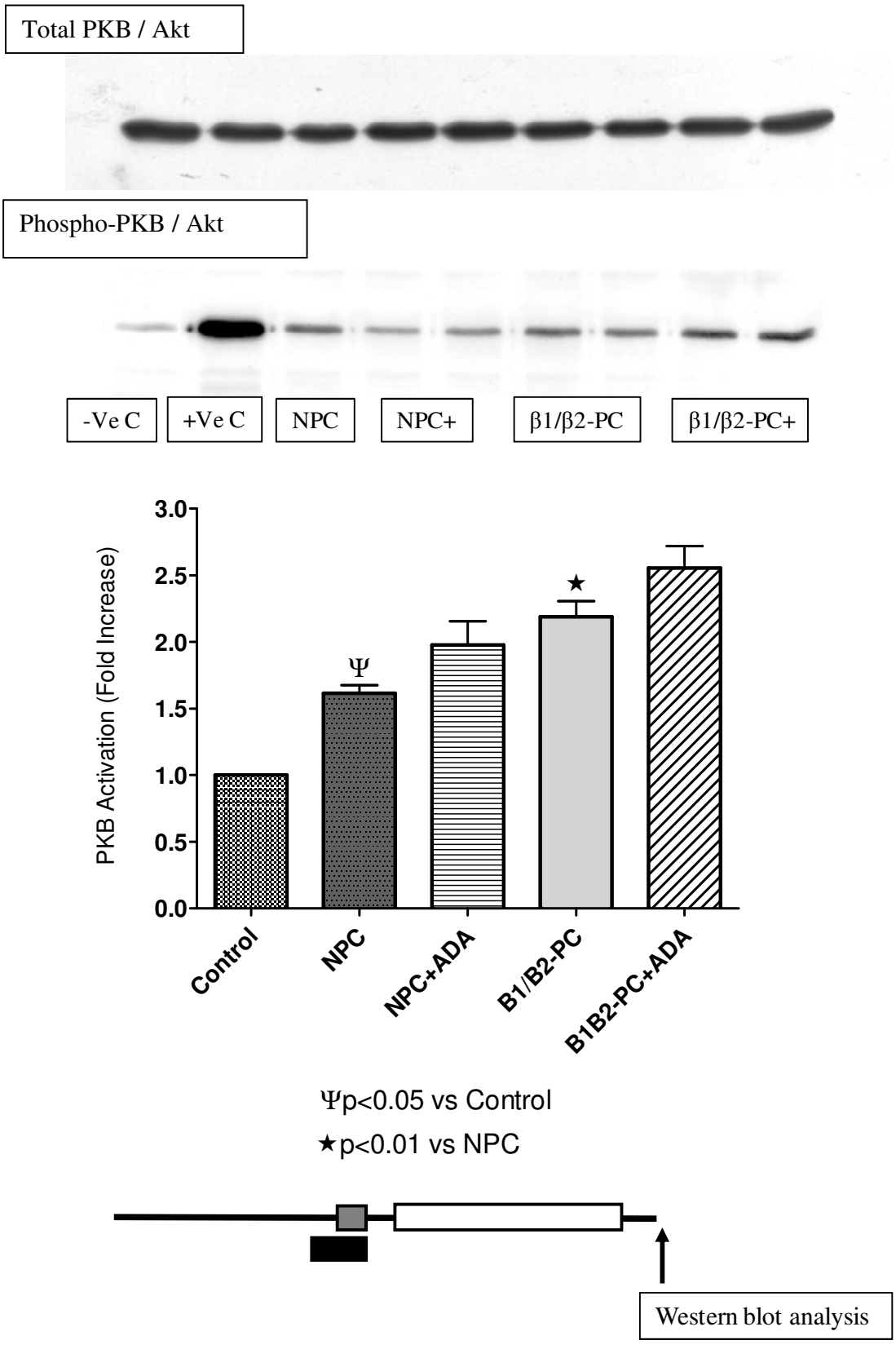


Fig. 5.4 A: The effect of adenosine deaminase on PKB/Akt expression during early reperfusion

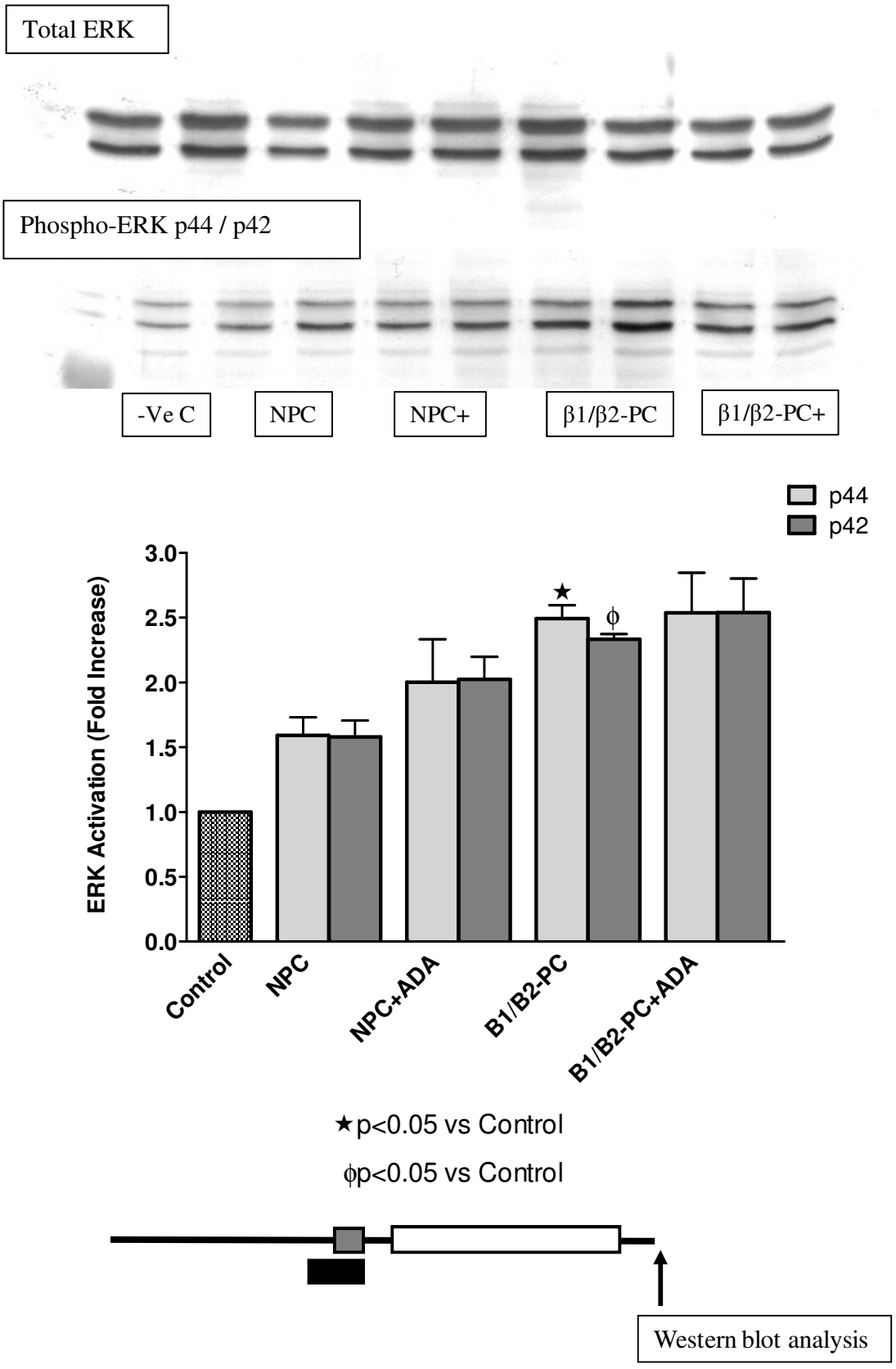


Fig. 5.4 B: The effect adenosine deaminase on ERK p44 / p42 MAPKinase expression during early reperfusion

5.2.2 a The involvement of A_1-AdoR in β1/β2-PC (Table 5.2)

The A_1-AdoR antagonist, DPCPX (1μM), applied prior to RI (trigger phase) or during reperfusion of NPC or hearts exposed to β1/β2-PC (0.1 μM) had no significant effect on any of the haemodynamic parameters at the end of reperfusion.

Table 5.2: Effect of A_1-AdoR antagonist, DPCPX on mechanical recovery during reperfusion of β1/β2-PC hearts

β-AR agonist: Isoproterenol

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.08±0.37	39.17±1.09	53.88±1.05	269±4.04	102.0±4.04	12.46±0.35
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI (n=18)	15.89±0.21	39.33±0.51	55.17±0.68	274±7.41	104.70±1.27	13.25±0.24
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

# P< 0.05 vs β1/β2-PC After RI

A_1-AdoR antagonist: DPCPX (Trigger)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+DPCPX After RI (n=6)	14.85±0.95	4.66±1.28	19.25±1.83	273±7.86	84.52±1.45	4.15± 0.20
β1/β2-PC +DPCPX After RI (n=6)	13.50±0.01	14.90±2.86	26.50±3.14	270±10.27	88.30±1.51	5.67±0.63

A_1-AdoR antagonist: DPCPX (Reperfusion)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+DPCPX After RI (n=6)	11.50±1.38	9.58±1.77	20.92±2.83	240±9.65	85.36±2.87	4.10± 0.63
β1/β2-PC +DPCPX After RI (n=6)	13.33±1.07	12.33±1.99	25.58±2.67	260±19.53	89.08±1.95	5.18±0.25

5.2.2 b The effect of DPCPX on infarct size in β1/β2-PC (Fig. 5.5)

A_1-AdoR inhibition with DPCPX (1μM), applied to NPC or β1/β2-PC hearts, prior to RI or at the onset of reperfusion did not show significant changes in IS, in both instances. This indicated clearly that this receptor subtype is not involved in the protective effect of β1/β2-PC.

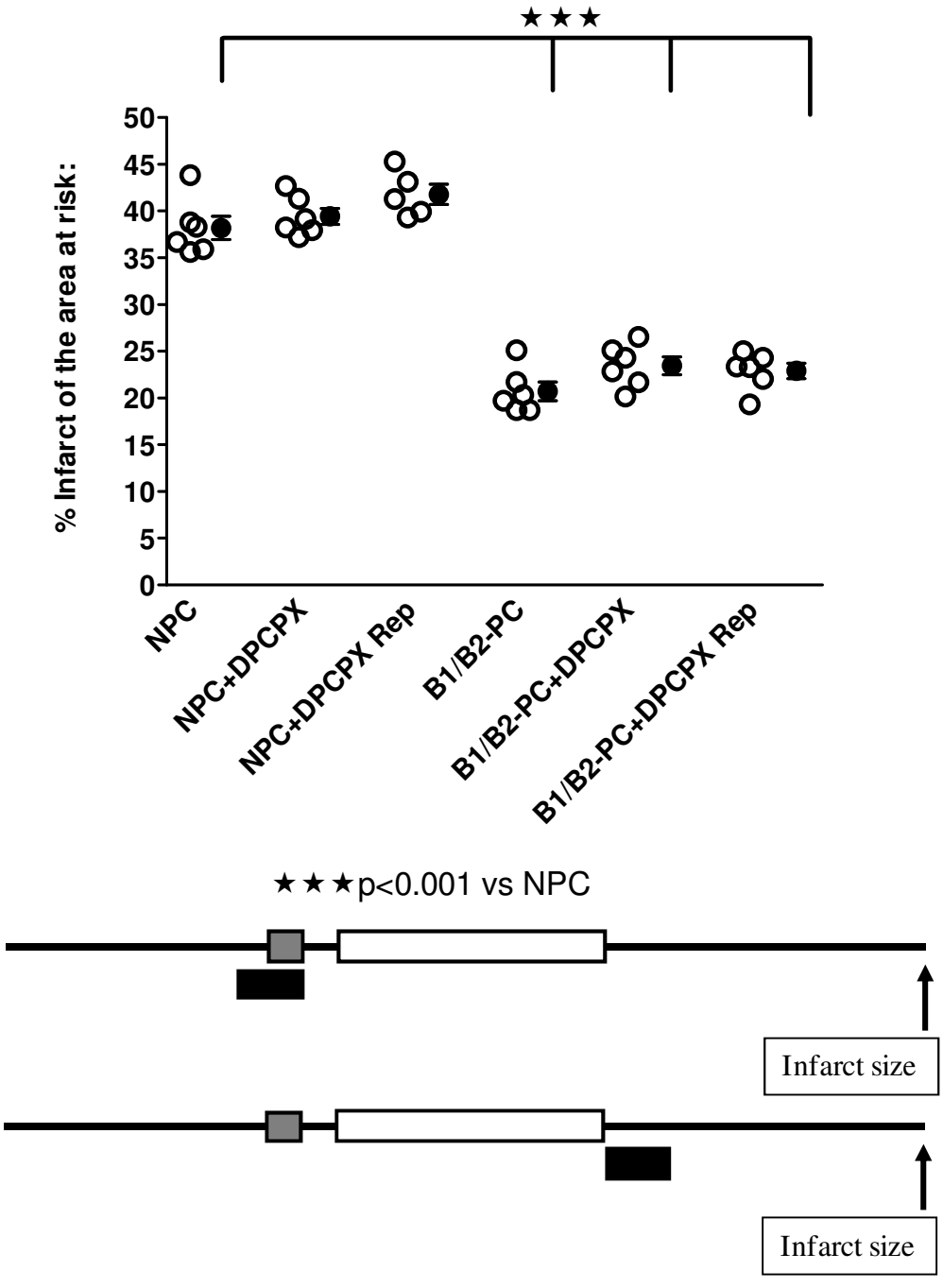


Fig. 5.5: The effect of A_1-AdoR inhibition with DPCPX on infarct size in β1/β2-PC

5.2.2 c The effect of A_1-AdoR inhibition with DPCPX on PKB/Akt and ERK p44 / p42 MAPKinase (Fig 5.6 A and B)

Western blot analysis for phosphorylated PKB/Akt of hearts subjected to β1/β2-PC exhibited a significant increase (fold increase: 2.18±0.21, $p<0.001$ vs control) during early reperfusion, which was not affected by the application DPCPX (2.29±0.23) prior to global ischaemia (Fig. 5.6 A).

Similarly, Western blot analysis for phosphorylated ERK p44/p42 MAPKinase of hearts subjected to β1/β2-PC showed a significant fold increase of ERK p44 (fold increase: 2.49±0.10, $p<0.001$ vs control) and p42 (2.13±0.04, $p<0.001$ vs control), which was not altered by the administration of DPCPX prior to global ischaemia (Fig. 5.6 B).

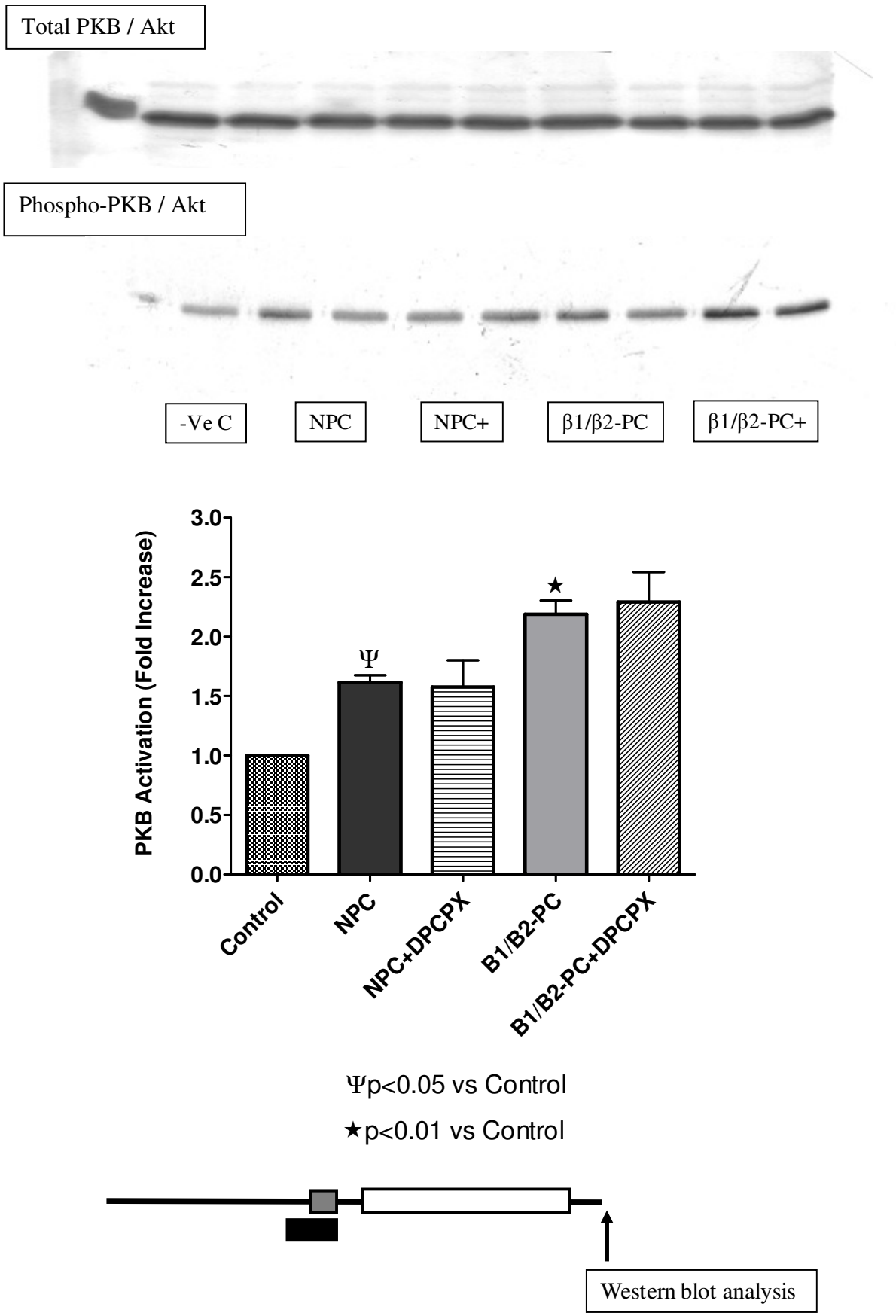


Fig. 5.6 A: The effect of DPCPX on PKB/Akt expression during early reperfusion

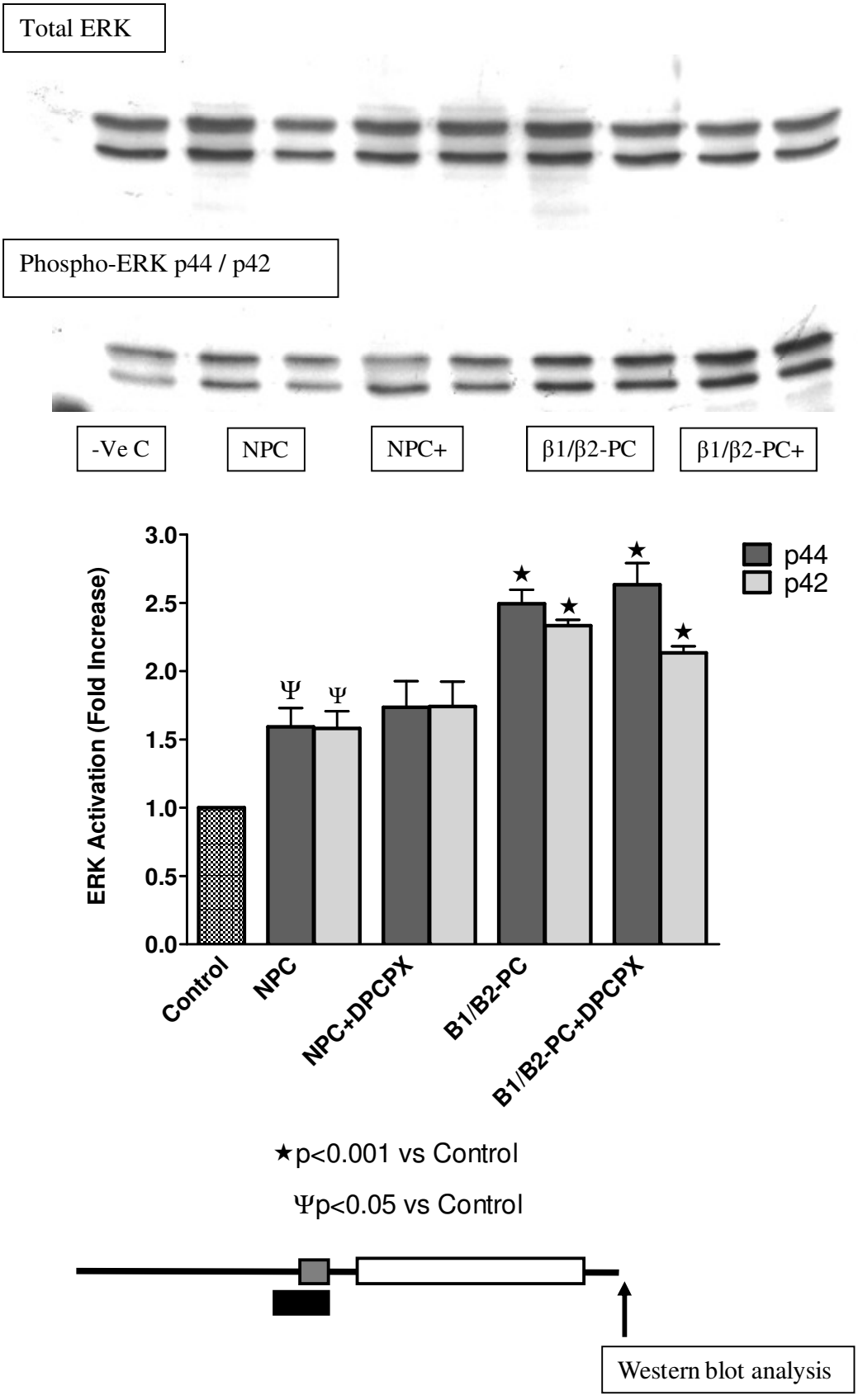


Fig. 5.6 B: The effect of DPCPX on ERK p44 / p42 MAPKinase expression during early reperfusion

5.2.3 a The involvement of A_{2A}-AdoR in β1/β2-PC (Table 5.3)

The A_{2A}-AdoR antagonist, ZM 241385 (1 μM), applied prior to regional ischaemia (trigger phase) or at the onset of reperfusion of hearts exposed to β1/β2-PC (0.1 μM) had no significant effect on haemodynamic parameters as measured during reperfusion. However, the CF, AO, CO and total work of hearts receiving ZM 241385 at the onset of reperfusion were significantly lower than those treated with the drug during the trigger phase of β1/β2-PC.

Table 5.3: Effect of A_{2A}-AdoR antagonist, ZM 241385 on mechanical recovery during reperfusion of β1/β2-PC hearts

β-AR agonist: Isoproterenol

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.33±0.33	40.83±0.63	55.79±0.55	263±7.65	100.80±1.52	12.79±0.29
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI (n=18)	16.17±0.16	41.67±0.82	57.77±0.85	258±5.12	100.0±2.64	13.67±0.31
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.53±0.70

# P< 0.05 vs β1/β2-PC After RI

A_{2A}-AdoR antagonist: ZM 241385 (Trigger) (1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+ZM After RI (n=6)	13.90±0.90	11.70±2.21	25.50±1.48	261±9.87	85.29±1.44	4.87±0.36
β1/β2-PC +ZM After RI (n=6)	15.17±0.52	22.33±2.27	37.50±2.27	257±12.69	93.84 ±0.92	7.98±0.22

A_{2A}-AdoR antagonist: ZM 241385 (Reperfusion) (1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+ZM After RI (n=6)	10.92±2.49	8.41±2.14	19.83±4.47	235±49.33	74.38±15.07	3.81±0.83
β1/β2-PC +ZM After RI (n=6)	10.25±2.17 ¥	10.92±2.27 ¥	20.83±4.29 ¥	229±47.49	75.00±15.29	4.25±0.88 ¥

¥ P<0.05 vs β1/β2-PC After RI

5.2.3 b The effect of ZM 241385 on IS in β1/β2-PC (Fig. 5.7)

A_{2A}-AdoR inhibition with ZM 241385 (1 μM), prior to regional ischaemia of hearts exposed to β1/β2-PC, did not increase infarct size (22.10±0.68%) but when applied at the onset of reperfusion, infarct size was significantly increased in the β1/β2-PC +ZM241385 R10 group (40.95±0.52%, $p<0.001$ vs β1/β2-PC). This means loss of cardioprotection and illustrates the involvement of this receptor only at the onset of reperfusion in β1/β2-PC.

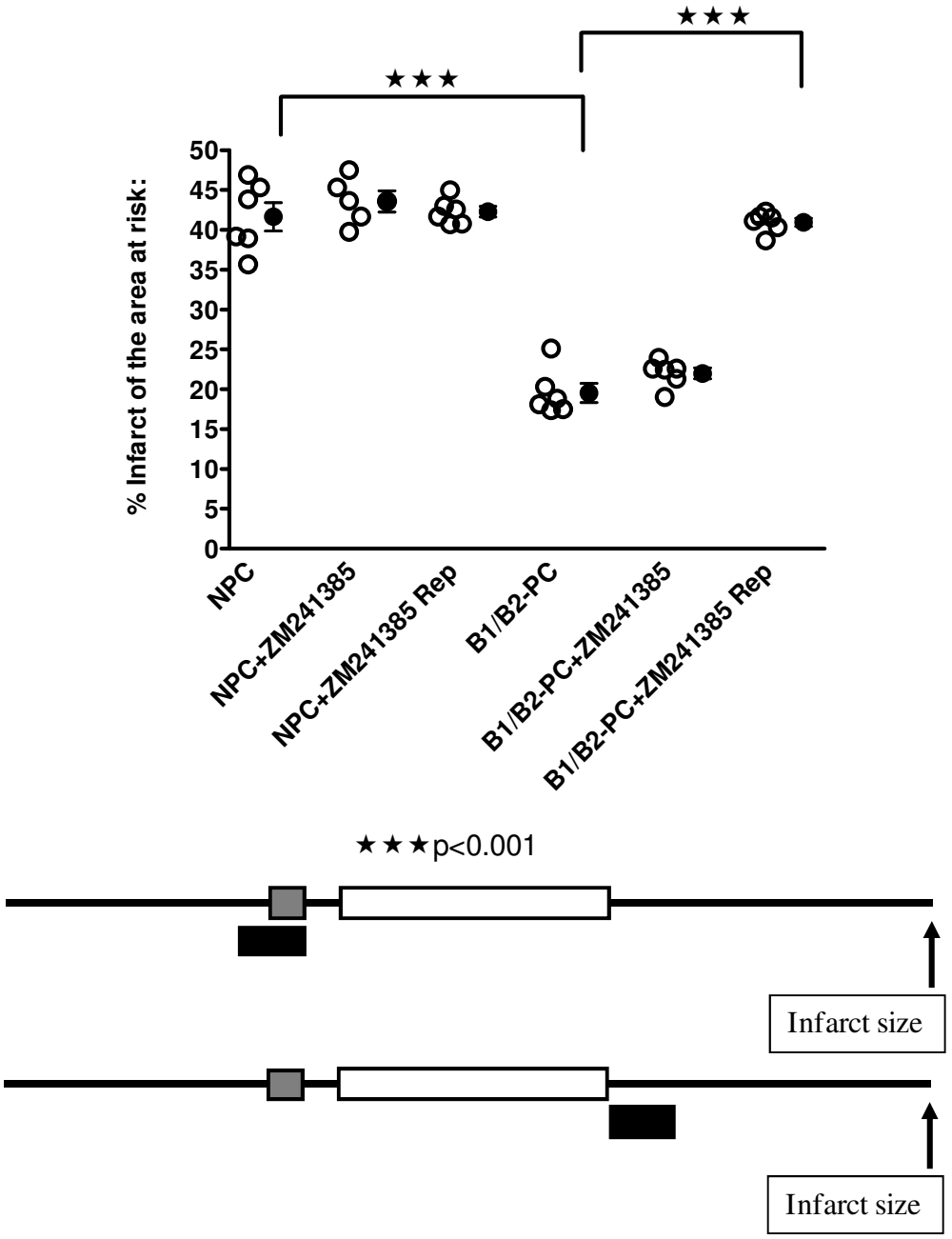


Fig. 5.7: The effect of A_{2A}-AdoR inhibition with ZM 241385 on infarct size in β1/β2-PC

5.2.3 c The effect of A_{2A}-AdoR inhibition with ZM 241385 on PKB/Akt and ERK p44 / p42 MAPKinase (Fig. 5.8 A, B, C and D)

Western blot analysis for phosphorylated PKB/Akt of hearts subjected to β1/β2-PC (fold increase: 2.18±0.16, <0.001 vs control), was significantly altered (fold increase:1.49±0.49, p<0.01 vs β1/β2-PC) by the application of the adenosine A_{2A} receptor antagonist, ZM 241385 (1 μM) prior to global ischaemia (Fig. 5.8 A). In addition, the activation of PKB/Akt was found to be significantly reduced and to a greater extent (0.43±0.02, p<0.001 vs β1/β2-PC), when the A_{2A}-AdoR antagonist, ZM 241385 (1 μM) was applied at the onset of reperfusion (Fig. 5.8 B). Interestingly, the drug also caused a significant inhibition of PKB/Akt phosphorylation when added to NPC hearts during reperfusion.

Western blot analysis for phosphorylated ERK p44/p42 MAPKinase of hearts subjected to β1/β2-PC, illustrated the characteristic significant increase of ERK p44 (fold increase: 2.49±0.10, p<0.001 vs control) and p42 (2.33±0.04, p<0.001 vs control) MAPKinase, respectively. Only ERK p44 MAPKinase was appreciably altered by the administration of ZM 241385 prior to global ischaemia (fold increase: 1.85±0.07, p<0.05 vs β1/β2-PC), whereas the ERK p42 MAPKinase showed no significant change (Fig. 5.8 C).

In contrast, inhibition of the A_{2A}-AdoR at the onset of reperfusion, reversed the significant increase of ERK p44 (2.44±0.08, p<0.001 vs control) and p42 (2.33±0.05, p<0.001 vs control) MAPKinase observed in β1/β2-PC hearts. ERK p44 (1.04±0.15, p<0.001 vs β1/β2-PC) and p42 (1.56±0.19, p<0.001 vs β1/β2-PC), phosphorylation was significantly lowered in the presence of A_{2A}-AdoR blockade (Fig. 5.8 D). As previously observed, the expression of total PKB/Akt and ERK p44/p42 MAPKinase remained unchanged in all experiments.

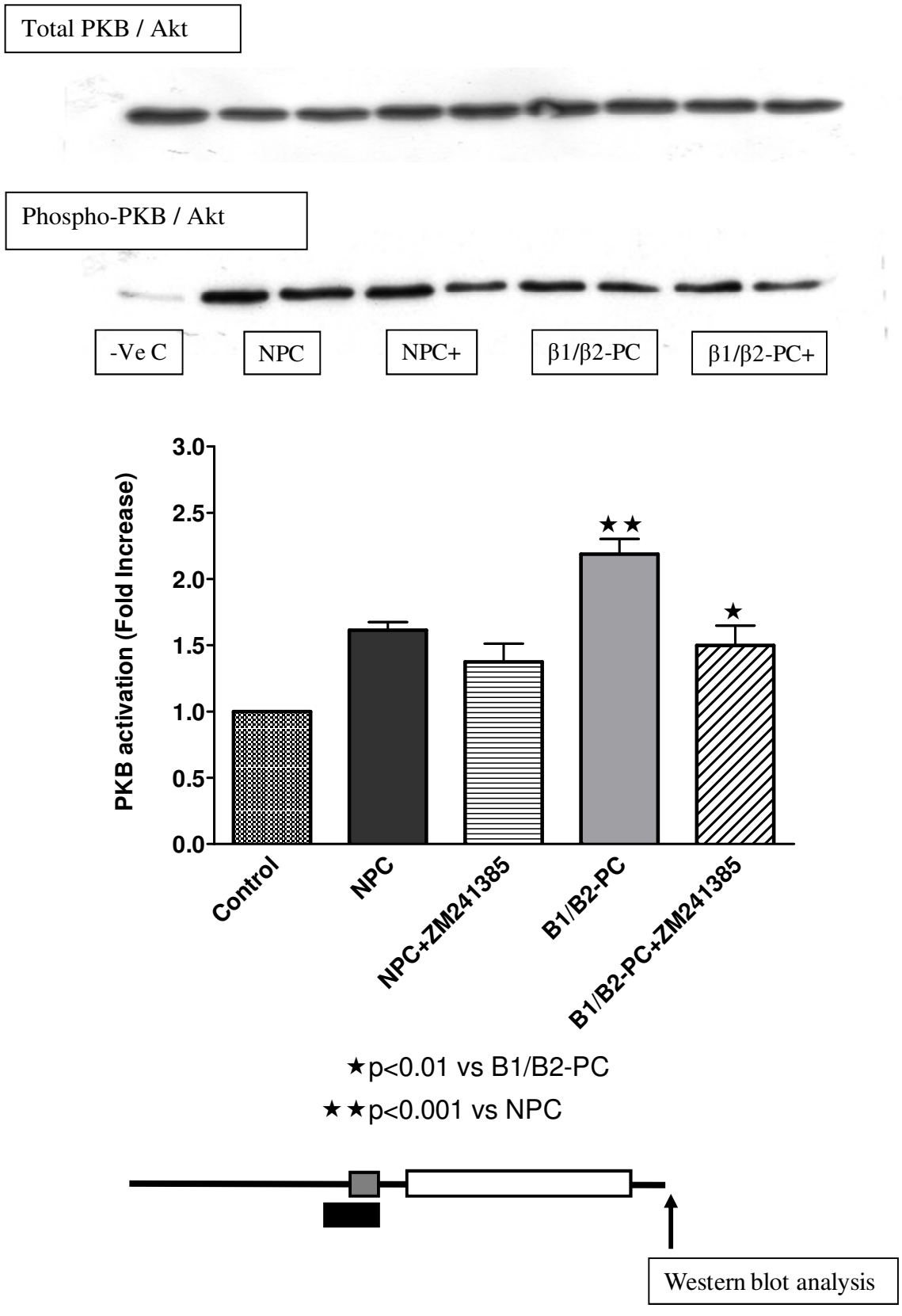


Fig. 5.8 A: The effect of ZM 241385 applied prior to global ischaemia on PKB/Akt expression during early reperfusion

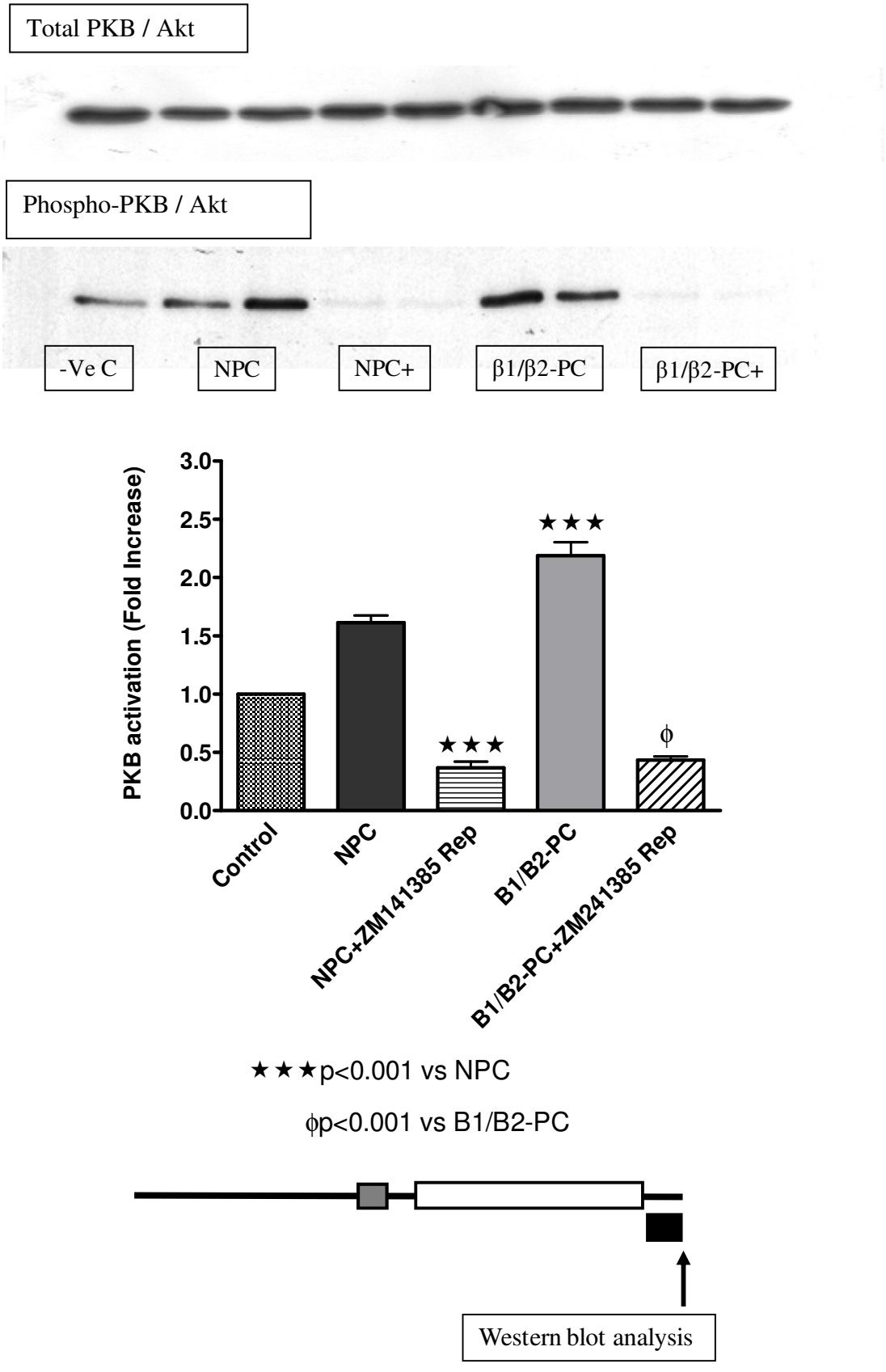


Fig. 5.8 B: The effect ZM 241385 applied after global ischaemia on PKB/Akt expression during early reperfusion

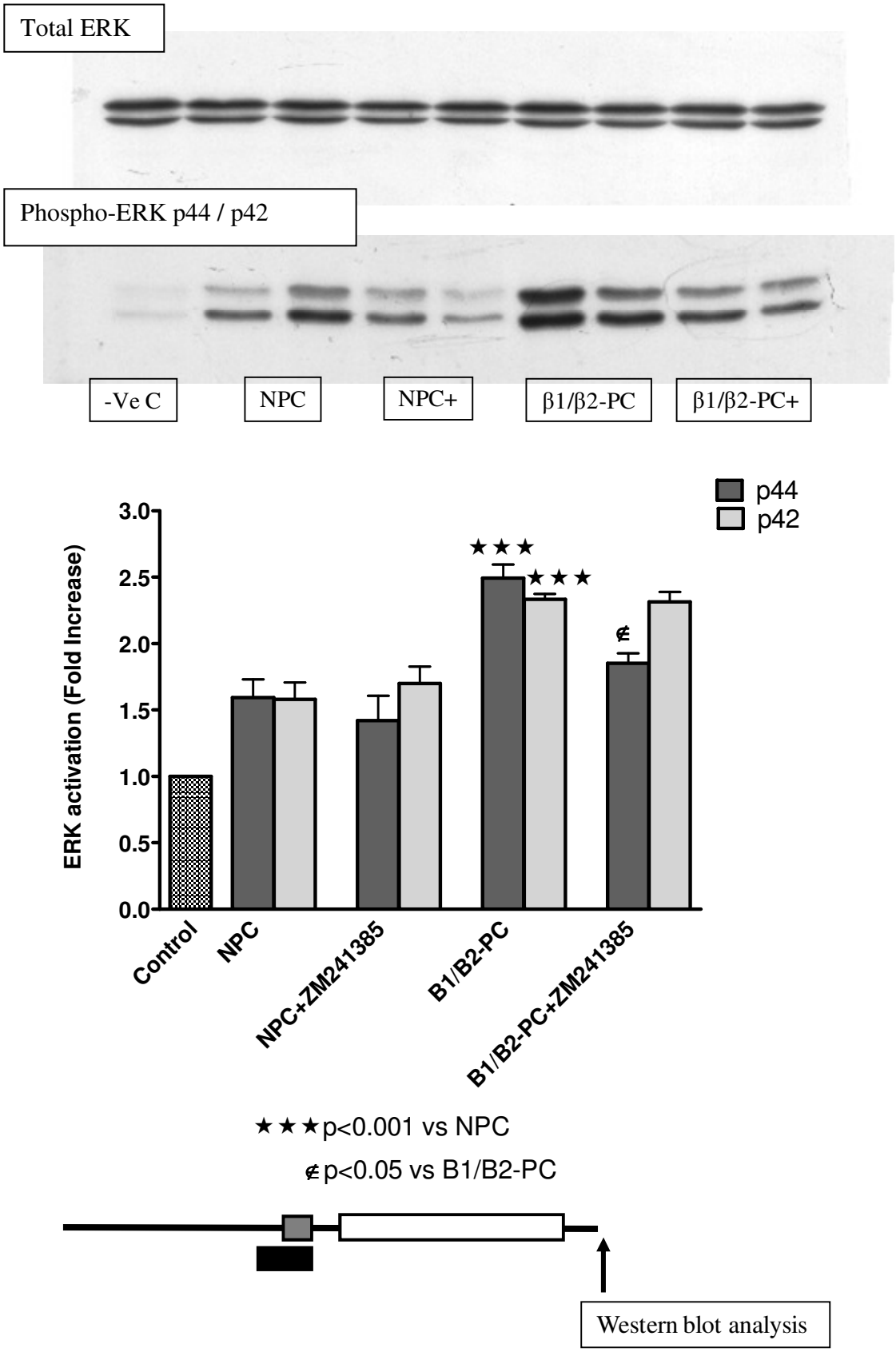


Fig. 5.8 C: The effect of ZM 241385 applied prior to global ischaemia on ERK p44 / p42 MAPKinase expression during early reperfusion

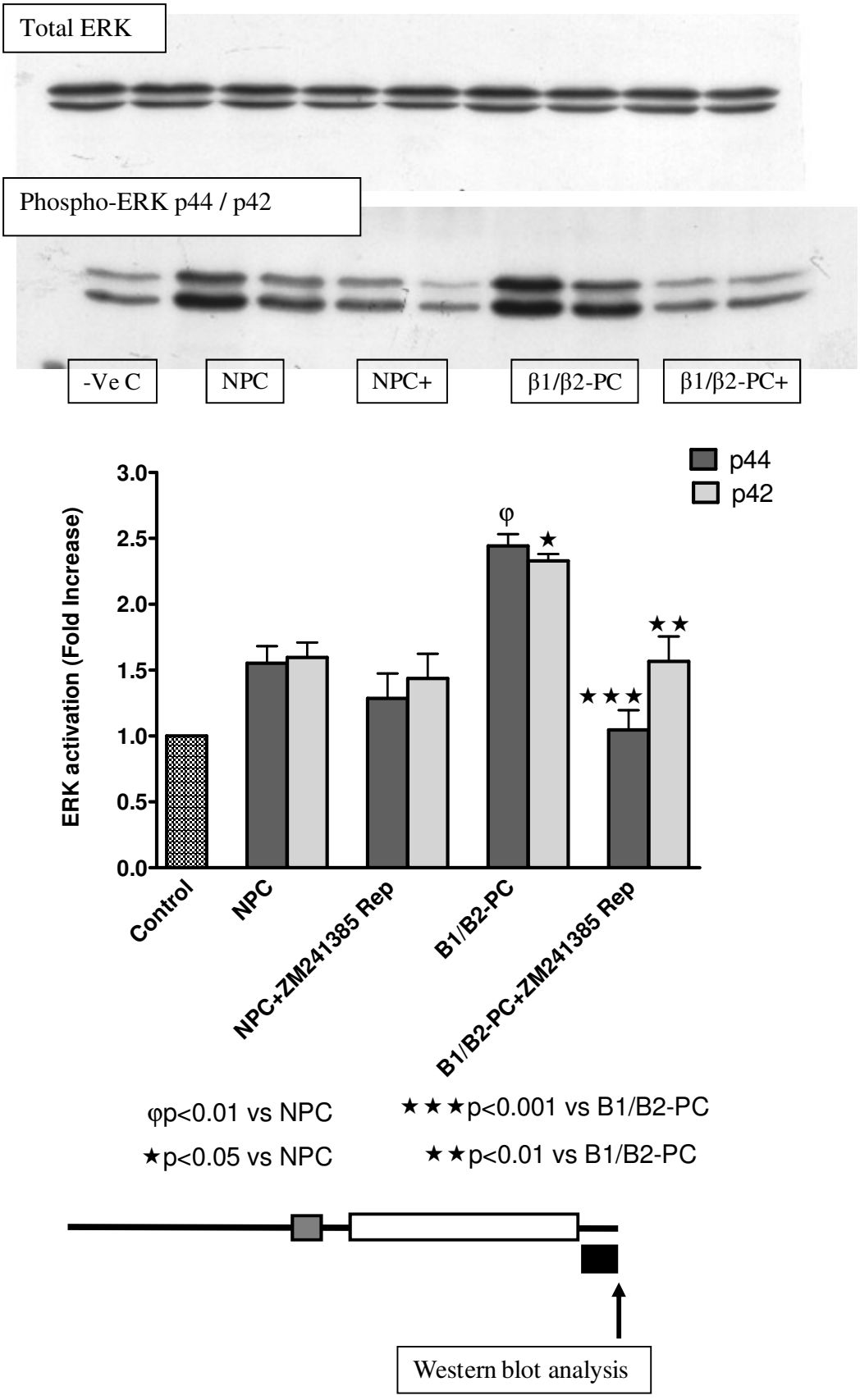


Fig. 5.8 D: The effect of ZM 241385 applied after global ischaemia on ERK p44 / p42 MAPKinase expression during early reperfusion

5.2.4 a The involvement of A_{2B}-AdoR in β1/β2-PC

The A_{2B}-AdoR antagonist, MRS1754 (1μM), applied prior to regional ischaemia (trigger phase) caused a small, but significant decline in functional recovery of β1/β2-PC hearts. Inhibition of this receptor at the start of reperfusion of hearts exposed to β1/β2-PC, significantly reduced haemodynamic parameters such as AO, CO and total work, illustrating a definite role of this receptor in the trigger phase as well as at the onset of reperfusion of β1/β2-PC (Table 5.4).

Table 5.4: Effect of A_{2B}-AdoR antagonist, MRS1754, on mechanical recovery during reperfusion of β1/β2-PC hearts

β-AR agonist: Isoproterenol

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.00±0.0.40	40.00±1.18	54.72±0.96	273±4.15	101.80±2.00	12.60±0.47
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI (n=18)	15.71±0.22	39.24±1.06	55.00±1.16	247±9.07	98.97±1.76	12.34±0.35
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.53±0.70

# P< 0.05 vs β1/β2-PC After RI

A_{2B}-AdoR antagonist: MRS 1754 (Trigger) (1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+MRS1754 After RI (n=6)	5.53±2.39	6.60±2.94	12.00±5.27	217.10±19.18	62.49±2.30	3.72±0.75
β1/β2-PC+MRS1754 After RI (n=6)	10.00±1.00 ★	12.50±1.97 ★	22.50±2.77 ★	239±20.77	85.94±1.93	4.35±0.62

★p<0.05 vs β1/β2-PC After RI

Table 5.4: (continued)

A_{2B}-AdoR antagonist: MRS 1754 (Reperfusion) (1 µM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+MRS1754 After RI (n=6)	8.75±1.87	4.83±1.79	13.53±2.87	223±50.47	70.95±14.28	2.59±0.55
β1/β2-PC+MRS1754 After RI (n=6)	7.75±2.43 ¥	6.83±2.78 ¥	14.58±5.10 ¥	170.00±54.27	85.03±18.38	2.69±0.95 €

¥ P< 0.05 vs β1/β2-PC After RI

€ P<0.01 vs β1/β2-PC After RI

5.2.4 b The effect of MRS 1754 on IS in β1/β2-PC (Fig. 5.9)

The small infarct size of hearts exposed to β1/β2-PC (19.55±1.19%, p<0.001 vs NPC) was significantly increased (24.86±1.17, p<0.05 vs β1/β2-PC) by the application of the A_{2B}-AdoR antagonist, MRS 1754 (1 μM) prior to regional ischaemia, as well as at the onset of reperfusion (32.55±1.17%, p<0.001 vs β1/β2-PC).

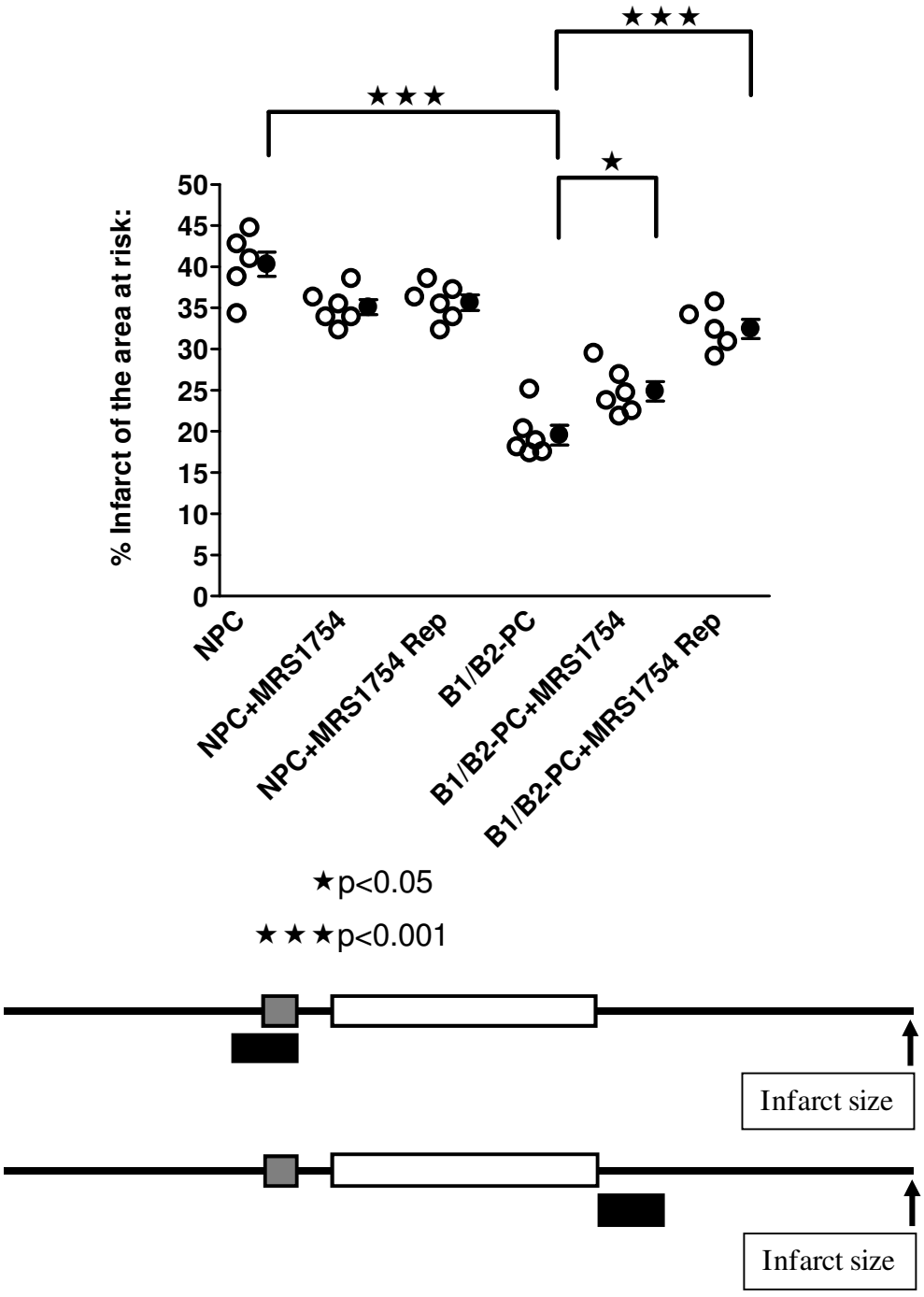


Fig. 5.9: The effect of A_{2B}-AdoR inhibition with MRS 1754 on infarct size in β1/β2-PC

5.2.4 c The effect of A_{2B}-AdoR inhibition with MRS 1754 on PKB/Akt and ERK p44 / p42 MAPKinase (Fig. 5.10 A, B, C and D)

Phosphorylated PKB/Akt expression of hearts subjected to β1/β2-PC (2.18±0.11, $p<0.001$ vs control) was significantly reduced by the application of the adenosine A_{2B} receptor antagonist, MRS1754 (1 μM) prior to global ischaemia (1.43±0.07, $p<0.001$ vs β1/β2-PC) (Fig. 5.10 A). Similarly, when this antagonist was applied at the onset of reperfusion, the activation of PKB/Akt of hearts subjected to β1/β2-PC (2.18±0.11, $p< 0.001$ vs control), was significantly altered and to a greater extent than when the antagonist was applied prior to sustained ischaemia (0.65±1.18, $p<0.001$ vs β1/β2-PC) (Fig. 5.10 B).

Western blot analysis for phosphorylated ERK p44/p42 MAPKinase of hearts subjected to β1/β2-PC , illustrated a significant fold increase of ERK p44 (2.49±0.10, $p<0.001$ vs control) and p42 (2.33±1.52, $p<0.001$ vs control) MAPKinase, which was appreciably reduced by the administration of MRS1754 prior to global ischaemia (ERK p44 1.59±0.05, $p<0.001$ vs β1/β2-PC) and ERK p42 (1.52±0.08, $p<0.01$ vs β1/β2-PC), (Fig. 5.10 C). Likewise, with the application of MRS1754 at the onset of reperfusion (Fig. 5.10 D), ERK p44 (1.53±0.10, $p<0.001$ vs β1/β2-PC) and ERK p42 (1.59±0.06, $p<0.001$ vs β1/β2-PC) MAPKinase, revealed a similar trend, respectively. As seen previously, expression of total PKB/Akt and ERK p44/p42 MAPKinase, was similar in all experimental groups.

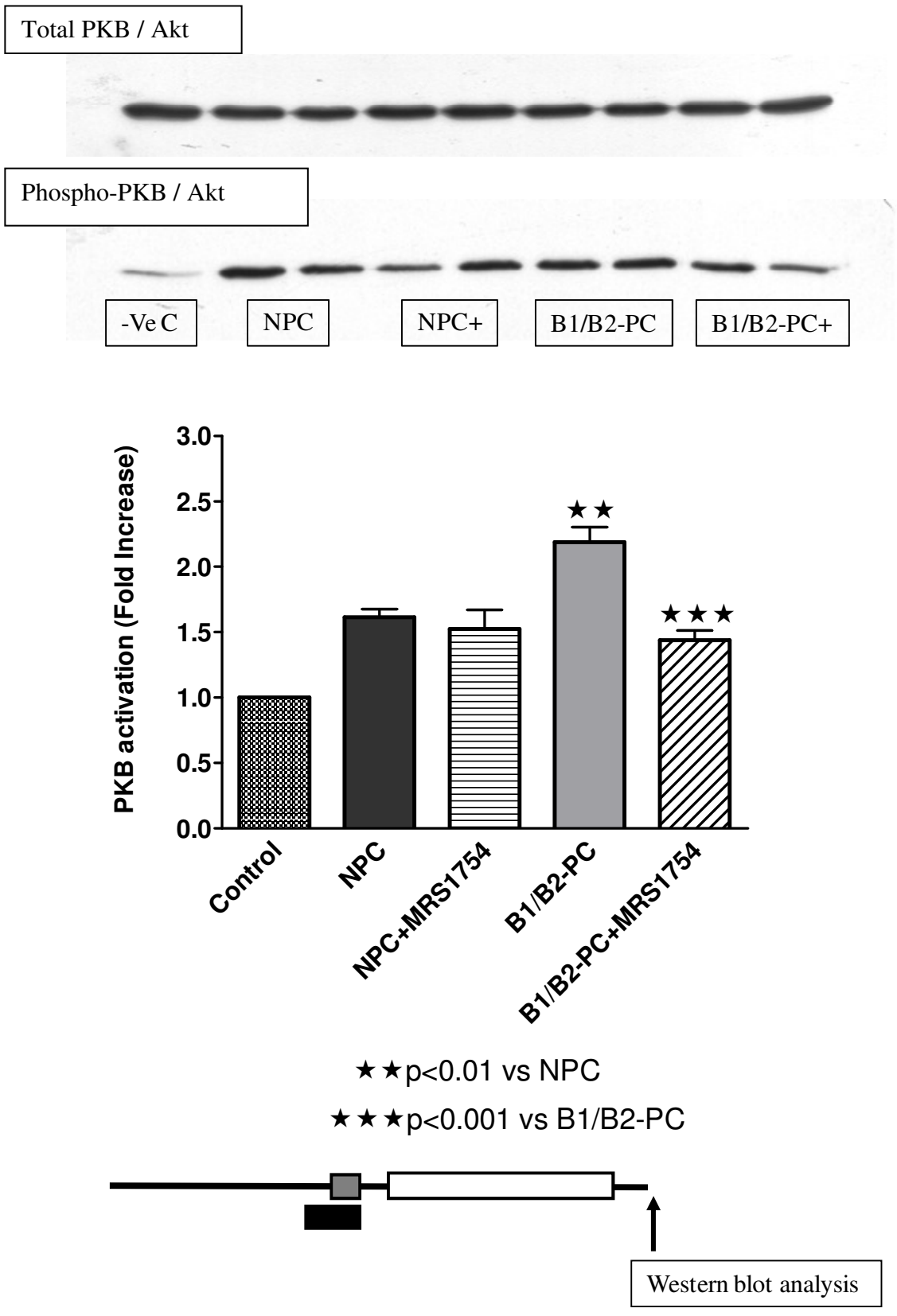


Fig. 5.10 A: The effect of MRS 1754 applied prior to global ischaemia on PKB/Akt expression during early reperfusion

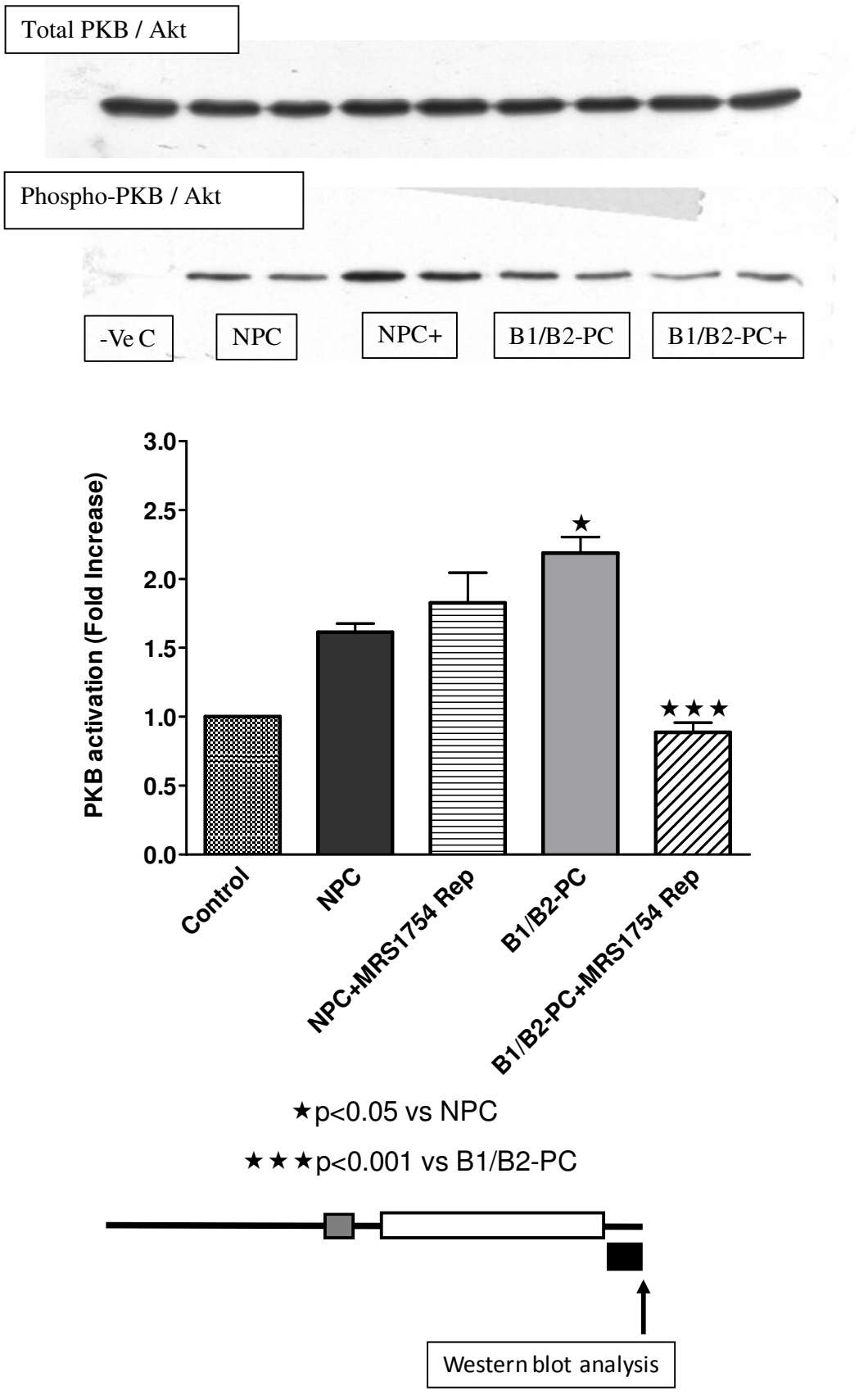


Fig. 5.10 B: The effect of MRS 1754 applied after global ischaemia on PKB/Akt expression during early reperfusion

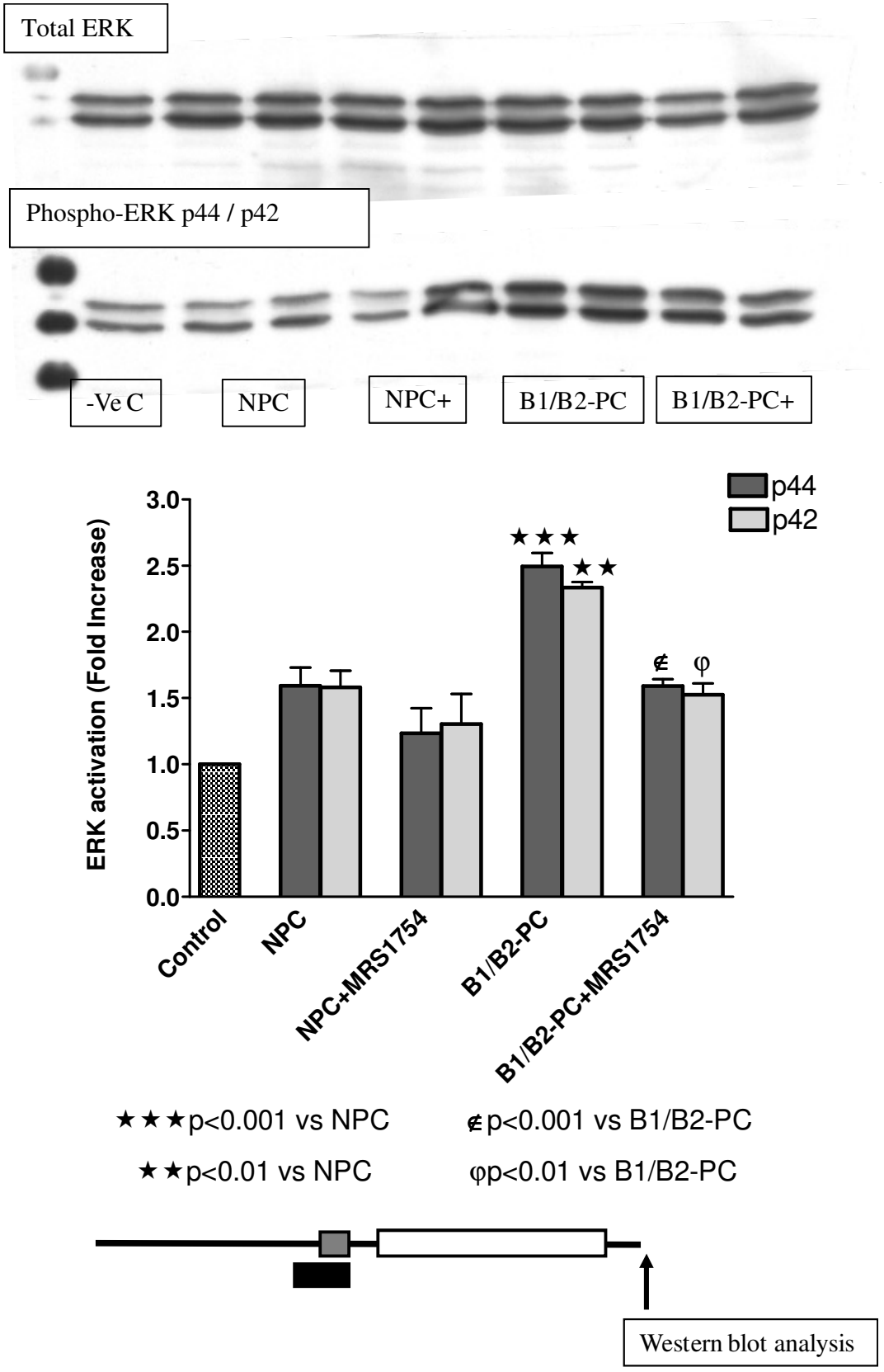


Fig. 5.10 C: The effect of MRS 1754 applied prior to global ischaemia on ERK p44 / p42 MAPKinase expression during early reperfusion

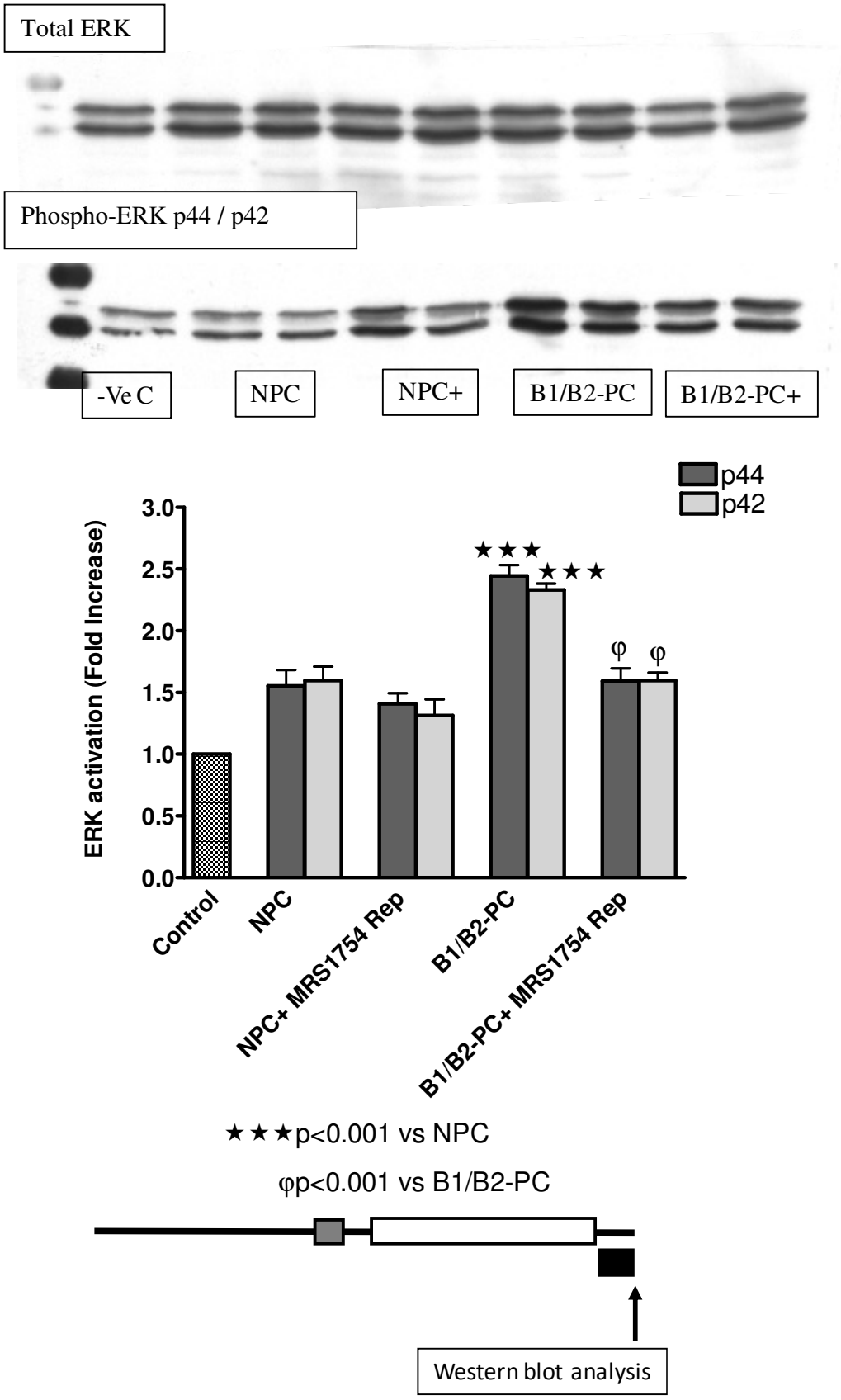


Fig. 5.10 D: The effect of MRS 1754 applied after global ischaemia on ERK p44 / p42 MAPKinase expression during early reperfusion

5.2.5 a The involvement of A_3-AdoR in β1/β2-PC (Table 5.5)

The A_3-AdoR antagonist, MRS1191 (1μM), applied prior to regional ischaemia (trigger phase) or at the onset of reperfusion of hearts exposed to β1/β2-PC, significantly reduced haemodynamic parameters such as AO, CO and total work, illustrating a definite role of this receptor in the cardioprotection of β1/β2-PC.

Table 5.5: Effect of A_3-AdoR antagonist, MRS1191 on mechanical recovery during reperfusion of β1/β2-PC hearts

β-AR agonist: Isoproterenol

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.38±0.34	41.83±0.68	56.33±0.64	275±4.33	102.2±1.50	13.24±026
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI (n=18)	16.11±0.19	41.55±0.80	57.56±0.85	268±5.18	102.4±1.11	13.36±0.28
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

# P< 0.05 vs β1/β2-PC After RI

A_3-AdoR antagonist: MRS1191 (Trigger) (1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+MRS1191 After RI (n=6)	12.92±0.76	2.33±0.33	14.33±0.45	300±3.45	83.05±1.47	2.75±0.08
β1/β2-PC + MRS1191 After RI	9.00±2.84	3.33±1.11 Φ	12.67±4.03 Φ	176±57.39	62.26±19.69	2.61±0.83 Φ

Φp<0.001 vs β1/β2-PC After RI

Table 5.5: (continued)

A_3-AdoR antagonist: MRS1191 (reperfusion) (1 µM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+MRS1191 After RI (n=6)	12.92±0.76	2.33±0.33	14.33±0.45	300±3.45	83.05±1.47	2.75±0.08
β1/β2-PC + MRS1191 After RI	9.41±3.00	3.00±1.23 ¥	12.38±3.96 ¥	191±60.77	54.15±17.66	2.45±0.78 ¥

¥p<0.001 vs β1/β2-PC After RI

5.2.5 b The effect of MRS 1191 on IS in β1/β2-PC (Fig. 5.11)

The infarct size of NPC hearts indicates a large IS of 39.20±1.24% compared to the significantly reduced infarct size of hearts exposed to β1/β2-PC (20.72±0.99%, p<0.001). A_3-AdoR inhibition, with MRS 1191 (1 μM) of hearts exposed to β-adrenergic preconditioning, prior to regional ischaemia or at the onset of reperfusion, significantly increased infarct size in both experimental settings (40.32±2.36% and 42.12±1.17%, p<0.001, respectively).

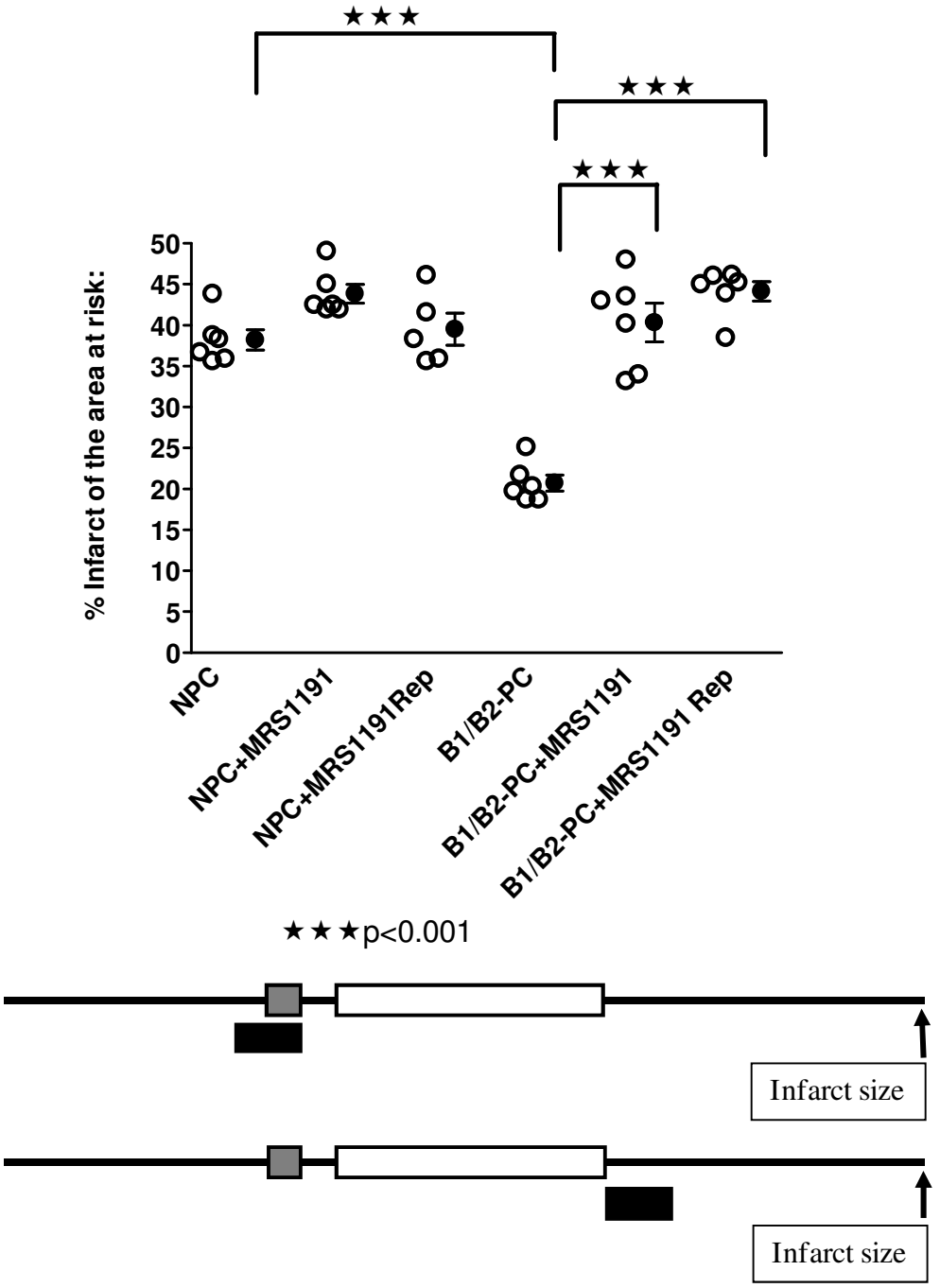


Fig. 5.11: The effect of A_3-AdoR inhibition with MRS 1191 on infarct size in β1/β2-PC

5.2.5 c The effect of A_3-AdoR inhibition with MRS 1191 on PKB/Akt and ERK p44 / p42 MAPKinase (Fig. 5.12 A and B)

Western blot analysis for phosphorylated PKB/Akt of hearts subjected to β1/β2-PC (2.18±0.11, p< 0.001 vs control), showed that it was significantly altered (1.43±0.07, p<0.001 vs β1/β2-PC) by the application of the A_3-AdoR antagonist, MRS 1191 (1 μM) prior to global ischaemia (Fig. 5.12 A).

Similarly, Western blot analysis for phosphorylated ERK p44/p42 MAPKinase of hearts subjected to β1/β2-PC, illustrated a significant fold increase of ERK p44 (2.49±0.10, p<0.001 vs control) and p42 (2.33±0.04, p<0.001 vs control) MAPKinase. This was significantly altered by the administration of the A_3-AdoR antagonist prior to global ischaemia, ERK p44 (1.49±0.05, p<0.01 vs β1/β2-PC) and p42 (1.56±0.04, p<0.05 vs β1/β2-PC) (Fig. 5.12 B).

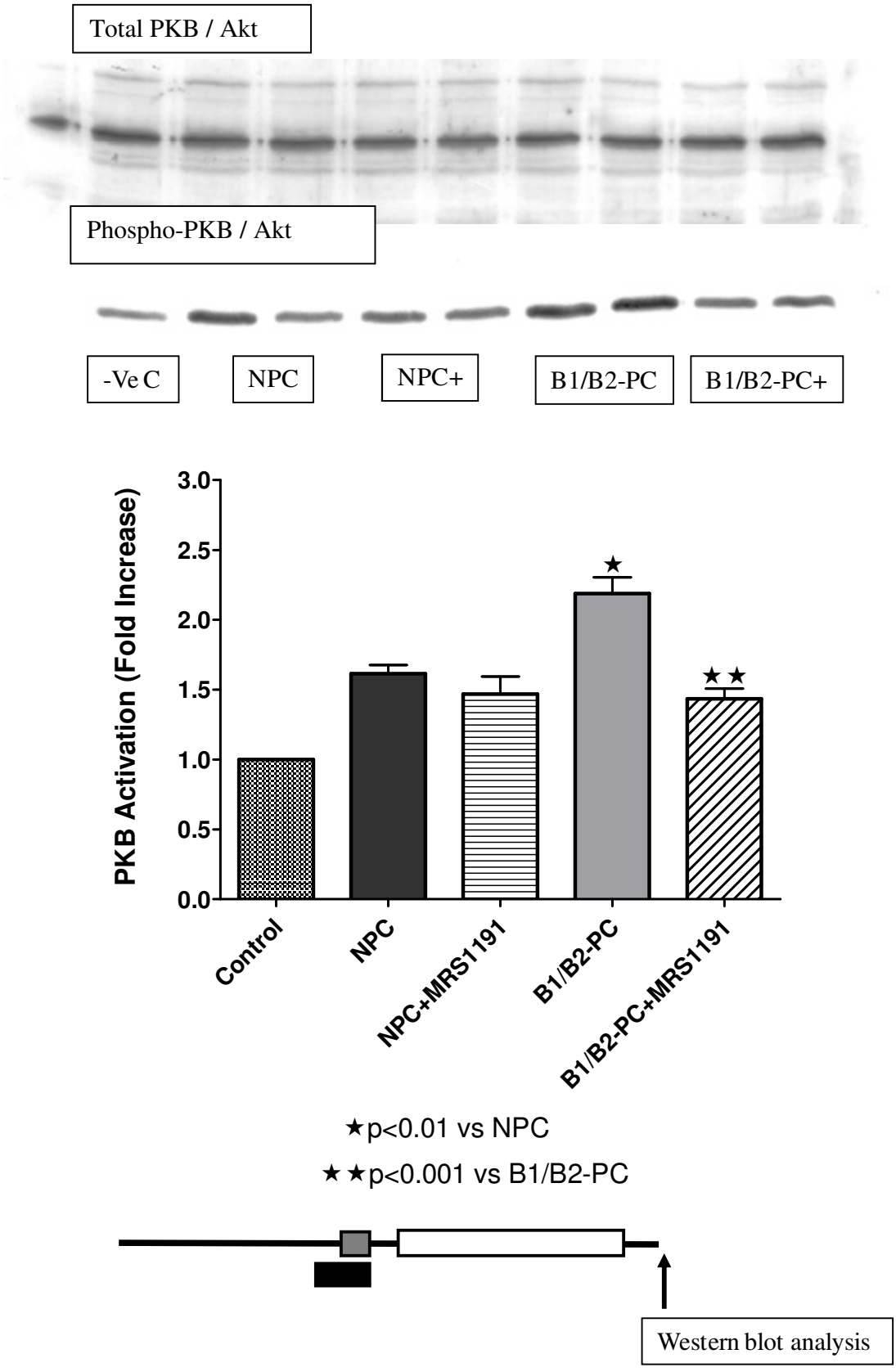


Fig. 5.12 A: The effect of MRS 1191 applied prior to global ischaemia on PKB/Akt expression during early reperfusion

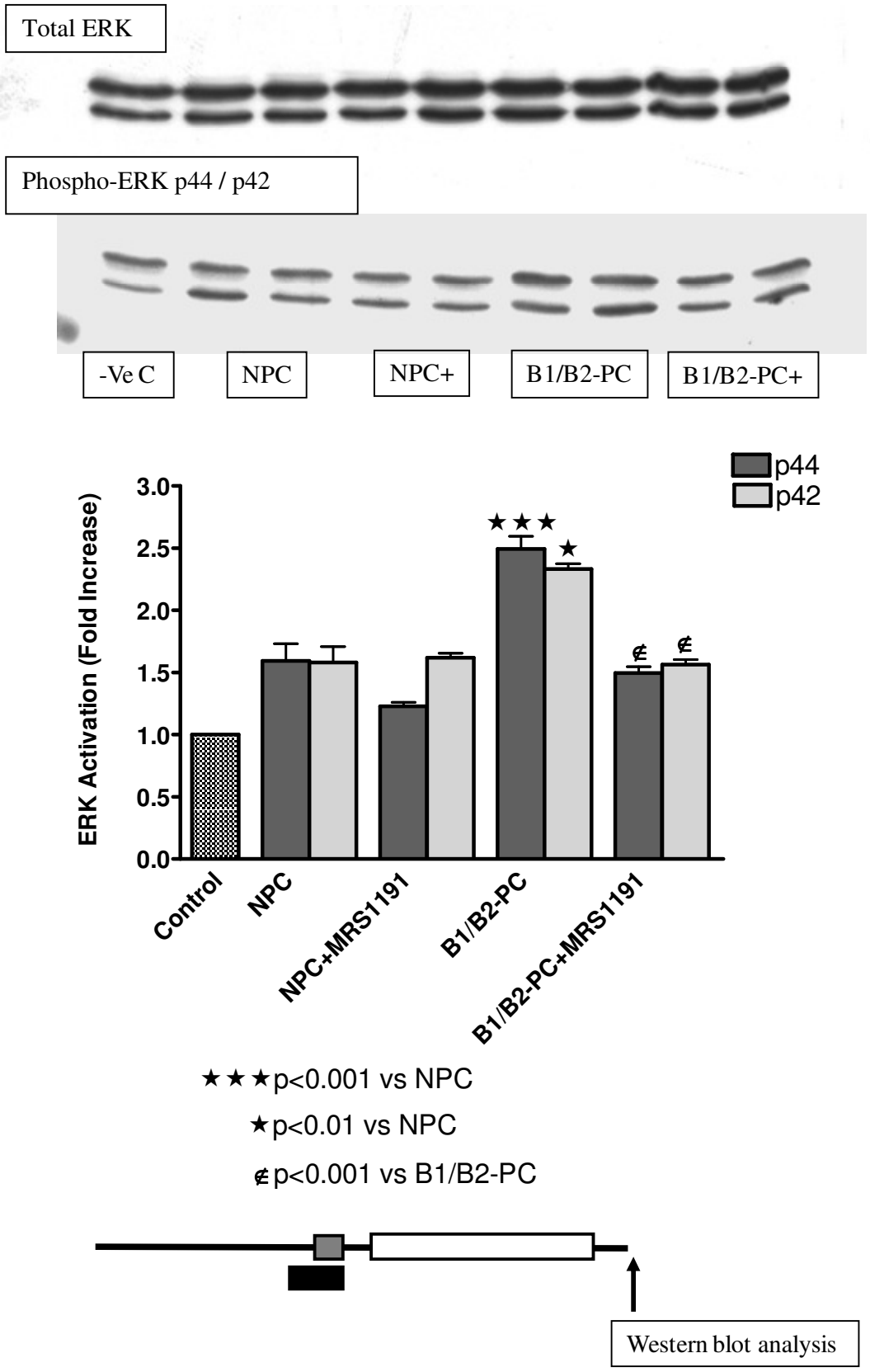


Fig. 5.12 B: The effect of MRS 1191 applied prior to global ischaemia on ERK p44 / p42 MAPKinase expression during early reperfusion

5.3 Discussion

The role of adenosine in the mechanism of ischaemic preconditioning has been studied by many workers [for reviews see Downey, Krieg and Cohen, 2008; Cohen and Downey, 2008; Yang, Cohen, and Downey, 2010]. It is released by short episodes of ischaemia in sufficient quantities to trigger cardioprotection. β-adrenergic stimulation of hearts with isoproterenol has also been shown to cause adenosine release, suggested to be due to an imbalance between oxygen supply and demand (demand ischaemia) [Deussen and Schrader, 1991]. The results obtained in this study suggest that sufficient adenosine was released during isoproterenol stimulation to elicit cardioprotection via its receptor subtypes. A preliminary study showed that elimination of adenosine by adenosine deaminase, significantly increased infarct size and reduced cardioprotection (Fig. 5.3), suggesting the involvement of adenosine in this cardioprotective phenomenon. Although these observations were not associated with significant changes in functional recovery, it was decided to evaluate the respective roles of the Ado-R subtypes in β-adrenergic preconditioning, on the premise that infarct size is perhaps the better indicator of cardioprotection.

The salient findings were: (i) the triggering of isoproterenol-induced protection is mainly dependent on endogenous adenosine acting on the adenosine A_3 receptor, with the A_{2B} receptor making a minor contribution; (ii) the mediator phase is dependent on the activation of the adenosine A_3 receptor in conjunction with mainly the adenosine A_{2A} receptor during reperfusion and to a lesser extent the adenosine A_{2B} receptor; (iii) the signal transduction pathways during both phases involve the PKB/Akt and ERK p44 / p42 pathways.

5.3.1 The role of A_1-AdoR in β-adrenergic preconditioning

The adenosine A_1 receptor is the most extensively studied adenosine receptor subtype within the context of cardioprotection and cardioprotection due to A_1 receptor agonism has been observed in many species examined [Morrison et al., 2006; for review see Headrick and Lasley, 2009]. Although there is some controversy regarding the role of this receptor in ischaemic preconditioning, there is very convincing evidence for a crucial role for adenosine in ischaemic preconditioning in the rat [Headrick et al., 1996] and it is generally accepted that adenosine and

the adenosine A_1 receptor are essential in the mechanism of cardioprotection in multiple species [for review see Peart and Headrick, 2007]. It must be noted that this receptor triggers both PKB/Akt and ERK p44/p42 MAPK activation, yet only ERK p44/p42 and not PKB/Akt was shown to be involved in cardioprotection such as IPC [Germack et al., 2004; Germack and Dickenson, 2005].

In contrast to its proven role in ischaemic preconditioning, the adenosine A_1 receptor is not involved in β1/β2-PC with isoproterenol: the infarct size and haemodynamic parameters of hearts exposed to β-adrenergic preconditioning (β1/β2-PC) were not affected by inhibition of the adenosine A_1 receptor with DPCPX, a highly selective A_1-AdoR blocker, prior to index ischemia or at the onset of reperfusion (Table 5.2 and Fig. 5.5). In addition, adenosine A_1 receptor inhibition during preconditioning prior to index ischaemia had no significant effect on PKB/Akt or ERK p44/p42 MAPK activation of β1/β2-PC hearts during reperfusion, which further indicates that this receptor did not contribute to the cardioprotective effects of β1/β2-PC (Fig. 5.6 A and B).

5.3.2 The involvement of A_{2A}-AdoR in β-adrenergic preconditioning

To elucidate the role of the A_{2A}-AdoR in β1/β2-PC, use was made of the selective A_{2A}-AdoR antagonist ZM 241385, which is >200 more potent for the A_{2A}- than the A_{2B}-Ado receptor [Kis, Baxter and Yellon, 2003]. In this study, it was illustrated that ZM 241385 administered prior to index ischaemia (trigger phase) had no effect on mechanical recovery or infarct size in β1/β2-PC hearts (Table 5.3 and Fig. 5.7): showing that the A_{2A}-AdoR was not involved in the triggering stage of β1/β2-PC.

On the other hand, in this study it was illustrated that A_{2A}-AdoR inhibition at the start of reperfusion caused significant inhibition of functional recovery when compared with β1/β2-PC (Table 5.3), associated with an increase in infarct size (Fig. 5.7). Interestingly, A_{2A}-AdoR inhibition resulted in a significant inhibition of the PKB/Akt as well as ERK p44/p42 MAPK phosphorylation during reperfusion, regardless of the time of administration.

The finding that the A_{2A}-AdoR antagonist, when added prior to ischaemia, had no effect on the reduction in infarct size but was associated with a reduction in the activation of the RISK pathway, was surprising and in contrast with our other data where a reduction in infarct size was found to occur concomitantly with the activation of the RISK pathway.

Although there are many studies confirming the association between cardioprotection and activation of PKB/Akt and ERK p44/p42 MAPK, the question has been raised whether activation of the RISK pathway is indeed mandatory for cardioprotection. There are several studies that could not confirm this association and this problem has been the topic of a recent editorial by Heusch, 2009, namely: "No RISK, no cardioprotection ? A critical perspective". However, apart from the above mentioned discrepancy, the results obtained in the present study showed a good correlation between cardioprotection and activation of the RISK pathway, as well as visa versa.

Generally, the A_2-AdoRs are located on smooth muscle and endothelial cells of blood vessels mediating the vascular effects of adenosine (Li and Fredholm, 1985). Thus, A_2-AdoR agonism in an experimental setting [Maddock et al., 2001] would lead to enhanced vasodilation. Vinten-Johansen et al., 1999 identified a role for the this receptor subtype in the phenomenon of postconditioning [Kin et al., 2005], the protection of which was evident in the presence and absence of blood cells [Zhao et al., 2003; Kin et al., 2005; Yang et al., 2005]. The possibility that the adenosine A_{2A} receptor has a more prominent role in cardioprotection at reperfusion is more likely, because it was shown that this receptor subtype regulates inflammatory tissue damage and remodeling associated with ischaemia and reperfusion [Peart and Headrick, 2007].

However, controversy also highlights the adenosine A_{2A} receptor downstream signaling and activation of PKB/Akt and ERK p44/p42 MAPK. A_{2A}-AdoRs are coupled to adenylyl cyclase-cAMP- PKA pathway via the stimulatory Gs protein and is consequently linked to the modulation of contractility [Cannell et al., 1995; Lindemann et al., 1983; Zhang et al., 1995]. PKA in turn, could activate PKB. However, the connection between cAMP – PKA and PKB/Akt activation is another subject of controversy since it was shown that cAMP could activate PKB/Akt independent of PKA [Meroni et al., 2002]. Recently, another cAMP binding protein was cloned and named Epac (Exchange protein directly activated by cAMP) [de Rooij

et al., 1998; Kawasaki, 1998]. Epac is a GTPase factor activating Rap1, and there is growing evidence that cAMP-mediated PKB activation requires the presence of Epac [Mei et al., 2002].

Similarly, the connection between cAMP - PKA and ERK p44/p42 and the role of small GTPase proteins are subjects of intense discussion and disparity [Vossler et al., 1997; Stork and Schmitt, 2002; Norum et al., 2003]. It was proposed that the $A2_A$ adenosine receptor couples to the $G_{12/13}$ protein instead of Gs [Sexl V et al., 1997]. Thus, the A_{2A} receptor-mediated activation on ERK p44/p42 may occur also via the involvement of the Ras –Ras– Sos pathway [Seidel et al., 1999].

5.3.3 The role of A_{2B}-AdoR in β-adrenergic preconditioning

Presently, there is no definitive evidence that functional A_{2B} or A_3 adenosine receptors are expressed in adult mammalian myocytes [Marala and Mustafa, 1998; Kilpatrick et al., 2002]. Thus, the effects of the adenosine receptors on myocardial responses to ischaemia may not necessarily reflect direct myocyte responses but indirect actions of other cell types [Peart and Headrick, 2007]. The adenosine A_{2B} receptors are defined as the low affinity receptor [Beukes, 2000] and thus, significant adenosine A_{2B} receptor activation will occur only at times of excessive adenosine accumulation, such as during ischemia, which is discharged at the onset of reperfusion.

In this study, it was illustrated that the selective adenosine A_{2B} receptor blocker, MRS 1754 administered prior to index ischaemia had no effect on functional recovery during reperfusion, whereas infarct size was significantly increased, when compared with the values obtained with β1/β2-PC (Table 5.4 and Fig. 5.9). In addition, it was found that inhibition of this receptor prior to sustained ischaemia, significantly reduced PKB/Akt (Fig. 5.10A) and ERK p44/p42 MAPK (Fig. 5.10 D) activation during reperfusion. Thus, it was concluded that activation of the adenosine A_{2B} receptor plays a role in the trigger phase of β-adrenergic preconditioning.

Similarly, in the present study it was shown that inhibition of the adenosine A_{2B} receptor at the beginning of reperfusion, significantly decreased mechanical recovery and elevated infarct size (Table 5.4 and Fig. 5.9), illustrating that the this receptor may have a more prominent role at this stage of β1/β2-PC. The significance of A_{2B}-AdoR activation at the onset of reperfusion was

further demonstrated by the finding that the inhibitor MRS 1754 also abolished the activation of PKB/Akt and ERK p44/p42 MAPK, characteristic of the cardioprotection of β1/β2-PC.

The adenosine A_{2B} receptor is generally coupled to the Gs-AC-PKA pathway [Mutafova-Yambolieva and Keef, 1997] and modulates contractility. Several other studies implicated A_{2B}-AdoR signaling to the phosphoinositide metabolism via $Gq_{/11}$ [Yakel et al., 1993; Feoktisov and Biaggioni, 1995]. In studies of IPC and postconditioning, it was illustrated that activation of this receptor subtype during early reperfusion lead to the activation of PKC. It was noted that PKC activation lowered the threshold of the A_{2B} receptor for adenosine [Kuno et al., 2007] and it has been suggested that these receptors can only respond to the heart's endogenous adenosine after sensitization by PKC. In summary the data obtained suggest a definite role for the adenosine A_{2B} receptor during both the triggering and mediator phases of β1/β2-PC.

5.3.4 The contribution of the adenosine A_3 receptor to the cardioprotection of β1/β2-PC

It has almost uniformly been shown that the adenosine A_3 receptor mediates cardioprotection in multiple species and models [for review see Peart and Headrick, 2007]. Also, in the present study, it was illustrated that inhibition of the adenosine A_3 receptor subtype with the specific A_3 inhibitor, MRS 1191 either prior to index ischaemia or at the start of reperfusion, caused significant inhibition of cardiac function during reperfusion and significantly increased infarct size as compared with the data obtained after β1/β2-PC. Thus, cardioprotection of β1/β2-PC was abolished by administration of the inhibitor at both time intervals of the experimental protocol (Table 5.5 and Fig. 5.11). This is in contrast with ischaemic preconditioning where roles for both A_1- and A_3-AdoR during the triggering phase only was reported in the rat heart [de Jonge et al., 2002; Peart and Headrick, 2007].

The results obtained with adenosine A_3 receptor blockade, suggest a major role for the activation of the RISK pathway in β1/β2-PC: activation of PKB/Akt and ERK p44/p42 MAPKinase at the onset of reperfusion of hearts subjected to β1/β2-PC was significantly reduced by adenosine A_3 receptor inhibition either prior to global ischaemia or at the onset of reperfusion (Fig. 5.12 A and B). This finding emphasizes the role of the adenosine A_3 receptor

mediated activation of PKB/Akt and ERK p44/p42 MAPKinase in the cardioprotective response of β-adrenergic preconditioning as well as the required pre-ischaemic A_3 receptor agonism required for cardioprotection [Thourani et al., 1999; Flood et al., 2003].

Interestingly, the work of Germack et al (2004, 2005) suggests that although the adenosine A_3 receptor can trigger both PKB/Akt and ERK p44/p42 MAPKinase activation, only the latter is required for the protective actions of this receptor during hypoxia-reoxygenation. Results presented in chapter 4 also confirm the importance of ERK p44/p42 MAPKinase activation during reperfusion of β1/β2-PC hearts, since inhibition of this MAPKinase completely abolished cardioprotection induced by isoproterenol.

In view of the significant role that the A_3-AdoR plays in both ischaemia and β1/β2-PC, it is surprising that the myocardial expression of this receptor appears to be exceedingly low in murine myocardium [Black et al., 2002]. It was suggested to be present in dog, rabbit and rat cardiac tissues [Auchampach et al., 1997; Takano et al., 2001], but there is presently very little direct evidence of its expression in mammalian cardiomyocytes [Headrick and Peart, 2005]. Thus, precisely how and where it mediates its protective effects remains unclear.

Earlier studies, illustrated that selective A_3-AdoRs agonists could trigger a potent protective response in isolated cardiomyocytes [Armstrong and Genote, 1994; Lee et al., 2001; Chaudary et al., 2004; Germack et al., 2004]. It was also indicated that acute treatment with A_3-AdoR agonists produced cardioprotection characterized by reduced infarct size [Auchampach et al., 1997; Maddock et al., 2001], decreased apoptotic death [Maddock et al., 2002] and enhanced contractile function [Maddock et al., 2003; Gardner et al., 2004]. Some reports suggested that the A_3-AdoRs mediated protection occur post-ischaemia [Jordan et al., 1999; Maddock et al., 2002], while others indicate that pre-ischaemic agonism is required for cardioprotection [Thourani et al., 1999; Flood et al., 2003].

Research has also shown that resveratrol-induced cardioprotection was A_3-AdoR dependent, associated with phosphorylation of PKB/Akt and cAMP response element binding protein (CREB) [Das et al., 2005]. The individual inhibition of PI3-Kinase or MEK1/2 only partially limited CREB activation and protection, whereas simultaneous inhibition of PI3-Kinase and MEK completely blocked CREB activation and protection [Das et al., 2005].

In both A_1- and A_3-AdoRs mediated responses, there is evidence of convergence on common mediators / end-effectors such as the mitochondrial K_{ATP} channel [Tracey et al., 1998; Thourani et al., 1999]. In summary, results indicate a major role for both the A_3- and A_{2B}-AdoR subtype, in the trigger as well as mediatory phases, while the A_{2A} subtype only becomes important during the onset of reperfusion. The data in this chapter strongly suggests a role for endogenous adenosine produced during the triggering phase, as well as during reperfusion, in the cardioprotection elicited by transient administration of isoproterenol. At least three AdoR subtypes appear to be involved in different stages of this cardioprotective phenomenon.

Chapter 6

Investigation of the roles of the mitochondrial K_{ATP} channel, reactive oxygen species (ROS) and nitric oxide in β-adrenergic preconditioning

The mitochondrial K_{ATP} channel (mitoK_{ATP} channel) and the production of reactive oxygen species have been shown to be involved in the cardioprotection of ischaemic preconditioning [for reviews see Downey, Krieg and Cohen, 2008; Cohen MV and Downey, 2008]. Non-adenosine mediated activators of ischaemic preconditioning, such as opioids and bradykinin, have been shown to utilize a signaling pathway that involves endothelial nitric oxide synthase (eNOS) activation. The resulting nitric oxide (NO) activates soluble guanylyl cyclase resulting in cGMP-dependent protein kinase (PKG) activation through the production of cyclic guanosine monophosphate. PKG initiates opening of the mitoK_{ATP} channels, which in turn results in ROS generation and activation of protein kinase C (PKC) during the triggering phase of ischaemic preconditioning for [Downey, Krieg and Cohen, 2008; Cohen and Downey, 2008]. Thus, the production of ROS in the context of ischaemic preconditioning acted as a second messenger to activate PKC and it is thought that PKC activation could be the end of the trigger phase and the first step of the mediator phase of ischaemic preconditioning. Both ROS and NO have been shown to lead to the cardioprotective state of ischaemic preconditioning (Garlid et al., 2003). Interestingly, in contrast to opioids and bradykinin, adenosine has a second direct coupling to PKC that bypasses the mitochondrial or redox signaling pathway [Downey, Krieg and Cohen, 2008]. The mediator phase occurs during the first minutes of reperfusion following lethal ischaemic insult and is still poorly defined.

Based on the knowledge that is available regarding events during ischaemic preconditioning and the similarities between ischaemic and pharmacological preconditioning, the question arose whether ROS, NO or the mitoK_{ATP} channels are involved in beta-adrenergic receptor stimulated cardioprotection with isoproterenol.

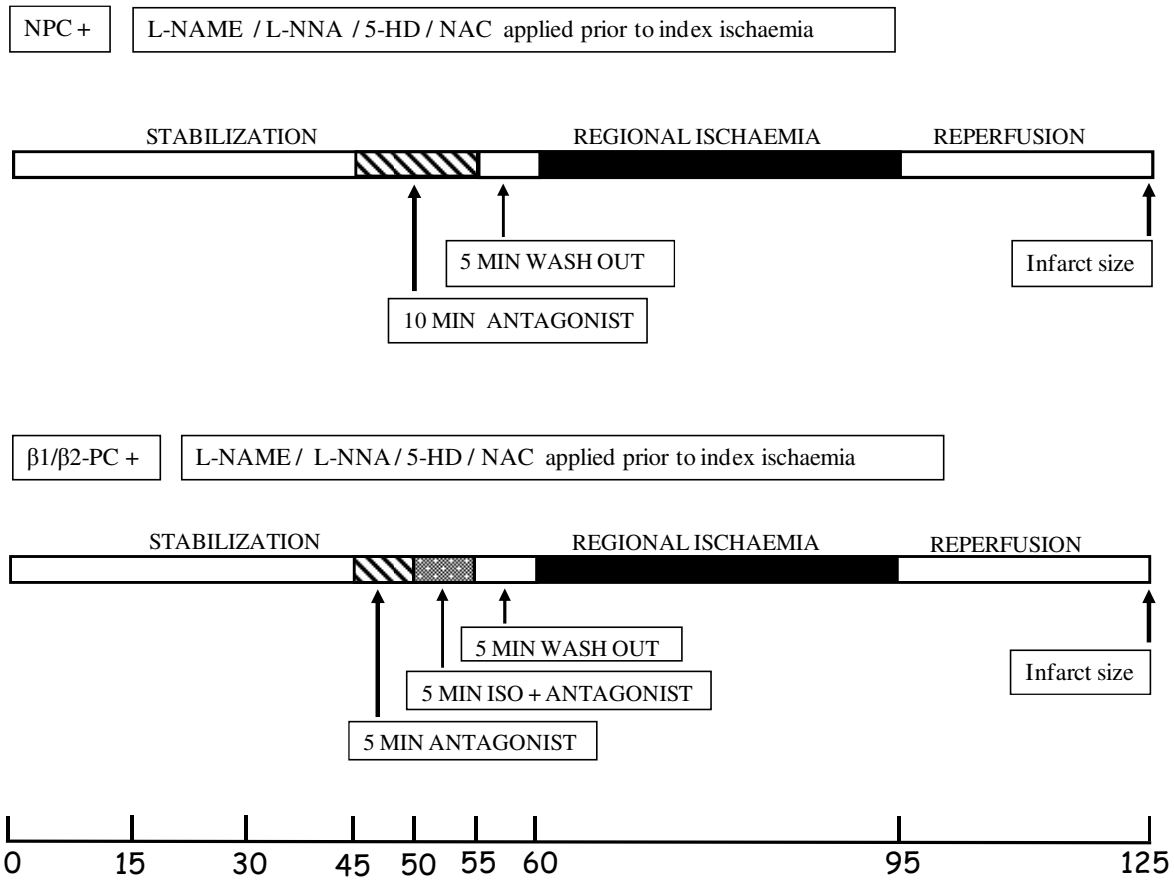
Evidence exists that both the β1- and β2-receptor subtypes could be involved in ROS production [Opie, Thandroyen and Muller, 1979; Zhang et al., 2005].

β-adrenergic mechanisms have been shown to be involved in the control of NO generation in cardiomyocytes [Slezak et al., 2004] and isoproterenol is capable of activating eNOS via Gαi [Balligand et al., 1999]. As far as we know, the role of the mitochondrial K_{ATP} channel in β1/β2-PC has never been investigated. In view of the above, in this chapter the respective roles of ROS, NO and the mitoK_{ATP} channels in β1/β2-PC with isoproterenol were investigated.

6.1 Methods

Inhibitors of NOS, L-NAME (N-nitro-L-arginine methyl ester hydrochloride) (50 uM) and L-NNA (N-nitro-L-arginine) (50 uM), the mitochondrial K_{ATP}-channel blocker 5-HD (5-hyroxydecanoate) (100 μM) or the oxygen radical scavenger NAC, (N-acetyl cysteine) (300 μM), were dissolved in distilled water and applied in Krebs-Henseleit buffer for 5 min prior to and during the 5 minute isoproterenol administration which was followed by a 5 min washout episode. Hearts were then subjected to 35 min regional ischaemia and reperfusion after which the infarct size was determined, as previously described in chapter 2. In a separate group of experiments, NAC was infused at the onset of reperfusion for 10 minutes, following regional ischemia. Haemodynamic parameters were recorded at the end of the 15 minute working heart mode prior to regional ischaemia and compared with haemodynamic parameters and infarct size at the end of reperfusion following regional ischaemia (Fig. 6.1).

Experimental protocol: (Fig. 6.1)



6.2 Results

6.2.1 a The role of nitric oxide in β1/β2-PC (Table 6.1)

Haemodynamic parameters such as AO, CO and total work were significantly reduced after the application of NOS inhibitors, L-NNA or L-NAME, during the triggering phase of β1/β2-PC, prior to regional ischaemia.

Table 6.1: Effect of NOS inhibitors on mechanical recovery during reperfusion of β1/β2-PC hearts

β-AR agonist: Isoproterenol (0.1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.39±0.31	41.50±0.77	56.08±0.78	251±6.91	103.0±1.34	13.03±0.31
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before R I (n=18)	15.67±0.18	40.67±0.68	56.25±0.73	245±7.39	101.7±1.24	12.92±0.31
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

# P< 0.05 vs β1/β2-PC After RI

NOS inhibitor: L-NAME (50 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+LNAME After RI (n=6)	8.60±2.07	9.58±3.73	19.17±5.63	196±40.22	70.01±14.20	3.29±0.89
β1/β2-PC+ LNAME After RI (n=6)	8.27±1.97	7.91±3.15 ★	16.25±4.69 ★	152±36.79	69.90±14.18	3.20±0.88 ⌖

★p<0.05 vs β1/β2-PC After RI

⌖p<0.01 vs β1/β2-PC After RI

Table 6.1: (continued)

NOS inhibitor: L-NNA (50 µM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+LNNA After RI (n=6)	11.92±1.16	14.00±2.96	27.83±4.54	246±3.21	88.61±1.59	4.88±0.67
β1/β2-PC+LNNA After RI (n=6)	8.58±1.95	5.00±2.29 Φ	13.58±3.81 Φ	189±43.53	70.60±14.23	2.76±0.76 Φ

Φ $p<0.01$ vs β1/β2-PC After RI

6.2.1 b The effect of nitric oxide inhibition on infract size in β1/β2-PC (Fig. 6.2)

The small infarct size of hearts exposed to β1/β2-PC (20.30±1.510%) was not affected when the NOS inhibitors L-NNA or L-NAME were applied prior to regional ischemia (15.18±1.43% and 18.65±1.59%, respectively) which suggests that nitric oxide did not play a role in the cardioprotective effects of in β1/β2-PC.

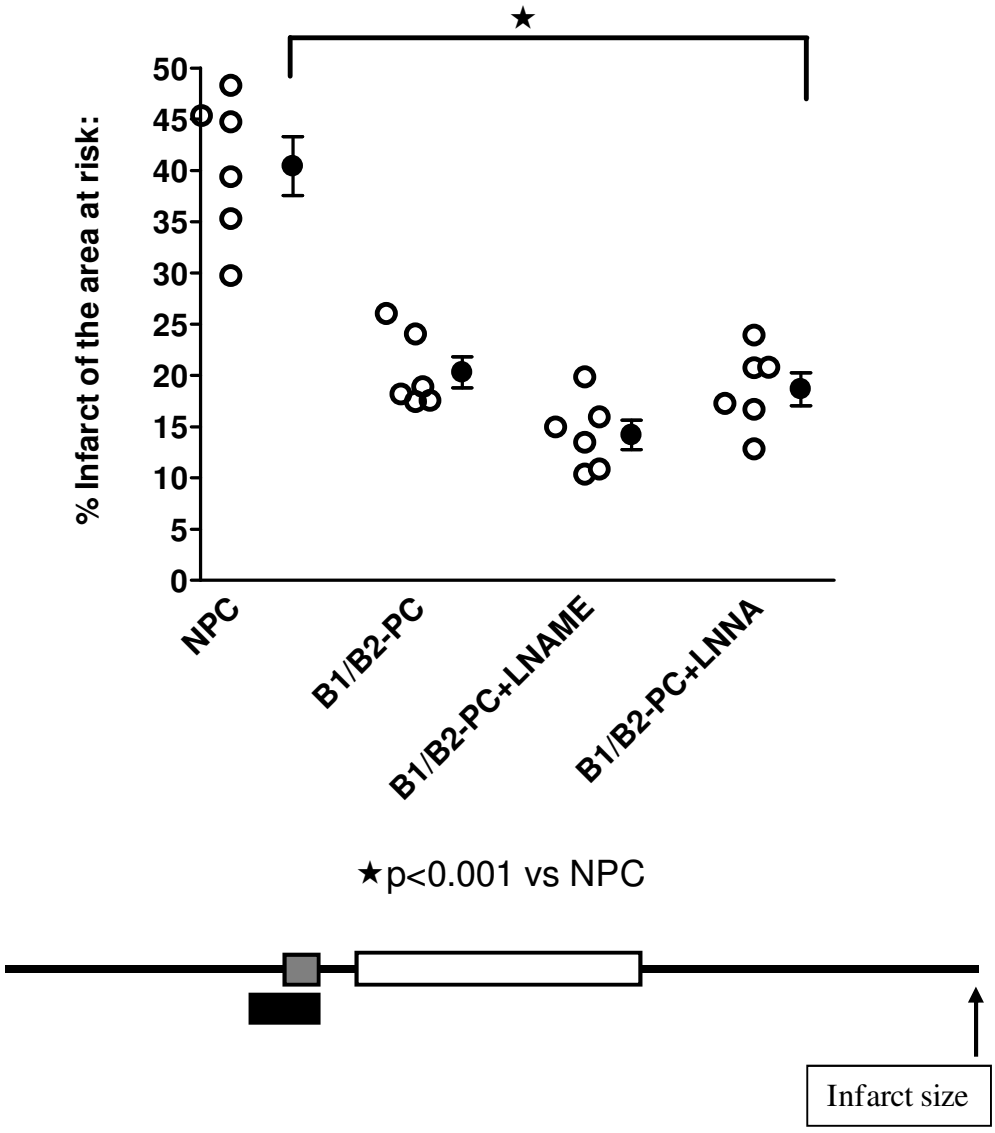


Fig. 6.2: The effect of NOS inhibitors, L-NNA or L-NAME on infarct size in β1/β2-PC

6.2.2 a Role of the mitochondrial K_{ATP} channel in β1/β2-PC (Table 6.2)

The mitoK_{ATP} channel blocker, 5-HD applied prior to RI had no significant effect on any of the haemodynamic parameters at the end of reperfusion, when compared with those of hearts preconditioned with isoproterenol. In this study 5-HD was administered during the trigger phase only, since the mitoK_{ATP} channel have been shown to be involved during the triggering phase only of ischaemic preconditioning.

Table 6.2: Effects of the mitoK_{ATP} channel blocker on mechanical recovery during reperfusion of β1/β2-PC hearts

β-AR agonist: Isoproterenol (0.1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI	14.67±0.55	42.00±0.77	55.30±1.41	270±5.41	104.1±2.10	13.27±0.49
NPC After RI (n=6)	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI	15.83±0.47	39.33±2.73	55.25±1.30	253±9.07	101.8±2.64	12.95±1.30
β1/β2-PC After RI (n=6)	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

# P< 0.05 vs β1/β2-PC After RI

mitoK_{ATP} channel blocker: 5-HD (100 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+5-HD After RI (n=6)	6.90±2.90	5.90±2.95	12.80±5.34	232.2±15.95	83.51±2.96	2.93±1.17
β1/β2-PC+ 5-HD After R(n=6)	11.42±1.26	13.50±1.29	24.92±1.63	240±8.65	89.70±1.98	4.95±0.19

6.2.2 b The effect of mitochondrial K_{ATP} channel inhibition on infarct size in β1/β2-PC (Fig. 6.3)

The mK_{ATP} channel blocker, 5-HD applied prior to regional ischemia in β1/β2-PC had no significant effect on the infarct size reducing capabilities of β1/β2-PC. This suggests that the mitochondrial K_{ATP} channel does not play a role in the protective effects of β-adrenergic preconditioning.

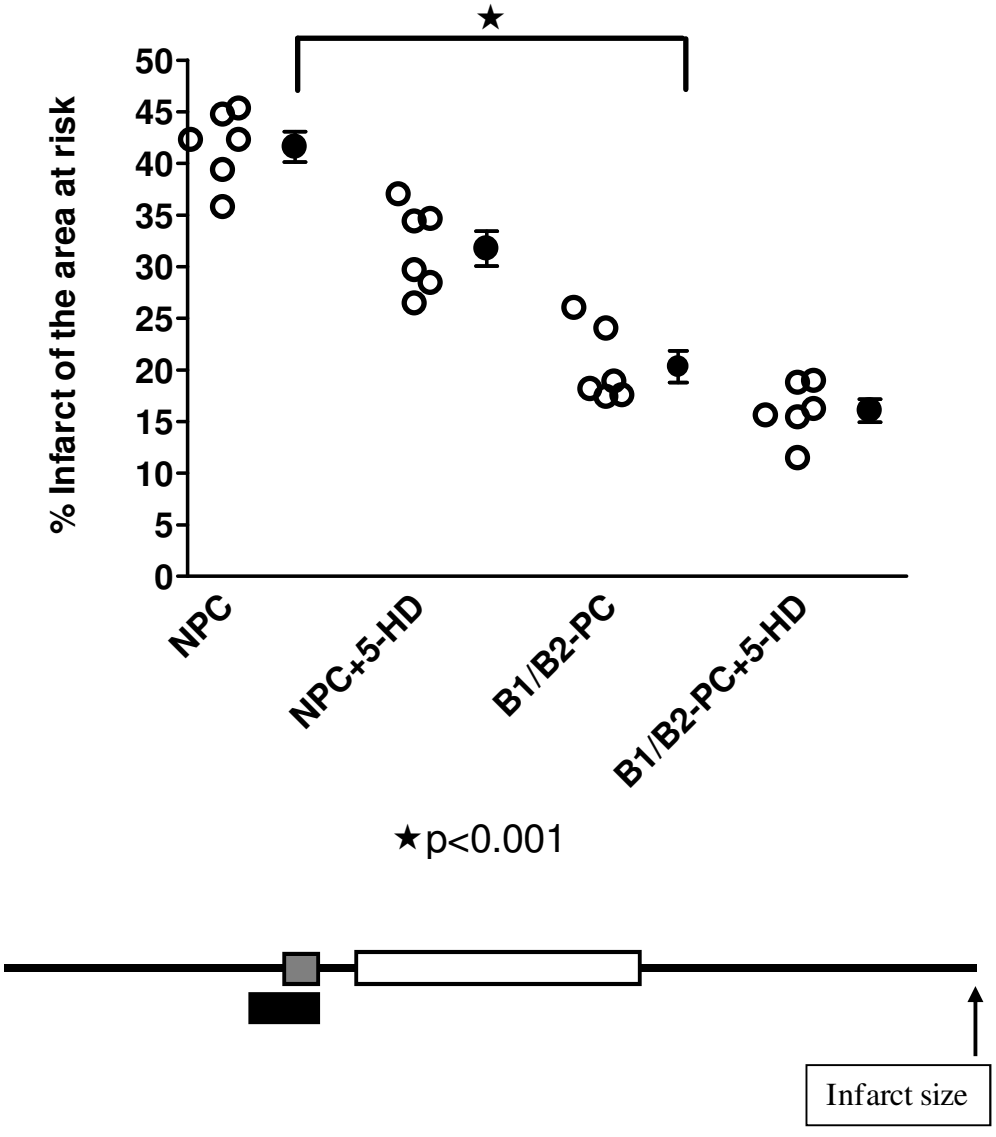


Fig. 6.3: The effect of the mitochondrial K_{ATP} channel blocker, 5-HD on infarct size in β1/β2-PC

6.2.3 a The role of reactive oxygen species in β1/β2-PC (Table 6.3)

The oxygen radical scavenger NAC, (N-acetyl cysteine) applied prior to RI (trigger phase) or at the onset of reperfusion had no significant effect on any of the haemodynamic parameters at the end of reperfusion, when compared with those obtained in hearts pre-treated with isoproterenol (0.1 μM).

Table 6.3: Effect of the ROS scavenger NAC on mechanical recovery during reperfusion of β1/β2-PC hearts

β-AR agonist: Isoproterenol (0.1 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC Before RI (n=18)	15.53±0.28	39.10±0.92	54.43±0.81	271±5.10	102.3±1.52	12.63±0.38
NPC After RI	10.25±0.90	7.250±1.01 #	19.01±1.02 #	235±15.30	86.80±2.13	3.61±0.22 #
β1/β2-PC Before RI (n=18)	16.28.±0.24	40.50±0.70	56.69±0.80	268±5.48	105.2±1.98	13.54±0.31
β1/β2-PC After RI	13.58±1.11	18.00±2.78	31.58±3.53	240±19.69	87.36±1.81	6.43±0.70

# P< 0.05 vs β1/β2-PC After RI

ROS scavenger: NAC (Trigger) (300 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+NAC After RI (n=6)	8.00±4.09	4.00±2.30	12.67±6.43	169±54.87	55.29±27.67	2.36±1.21
β1/β2-PC +NAC After RI (n=6)	11.17±2.55	13.50±3.50	24.58±6.00	201±41.56	76.57±13.37	4.98±1.30

ROS scavenger: NAC (Reperfusion) (300 μM)

	CF (ml/min)	AO (ml/min)	CO (ml/min)	Heart rate (beats/min)	PSP (mmHg)	Total work (mW)
NPC+NAC After RI (n=6)	9.25±2.90	4.33±4.27	13.58±4.50	168±53.60	59.47±18.11	2.70±0.59
β1/β2-PC +NAC After RI (n=6)	14.17±.0.88	12.58±1.50	27.58±1.96	280.2±12.29	88.74±2.81	5.46±0.40

6.2.3 b The effect of reactive oxygen species inhibition on infarct size in β1/β2-PC (Fig. 6.4)

The role of ROS in β-adrenergic preconditioning was clearly illustrated when the ROS scavenger, NAC was applied, prior to or at the onset of reperfusion of hearts preconditioned with isoproterenol. At both time intervals infarct size was significantly increased (25.97±2.41%, $p<0.05$ and 33.99±2.80% vs β1/β2-PC 16.71±.088, $p<0.001$, respectively) suggesting that ROS generation plays a role in both the triggering and mediator phases of β1/β2-PC.

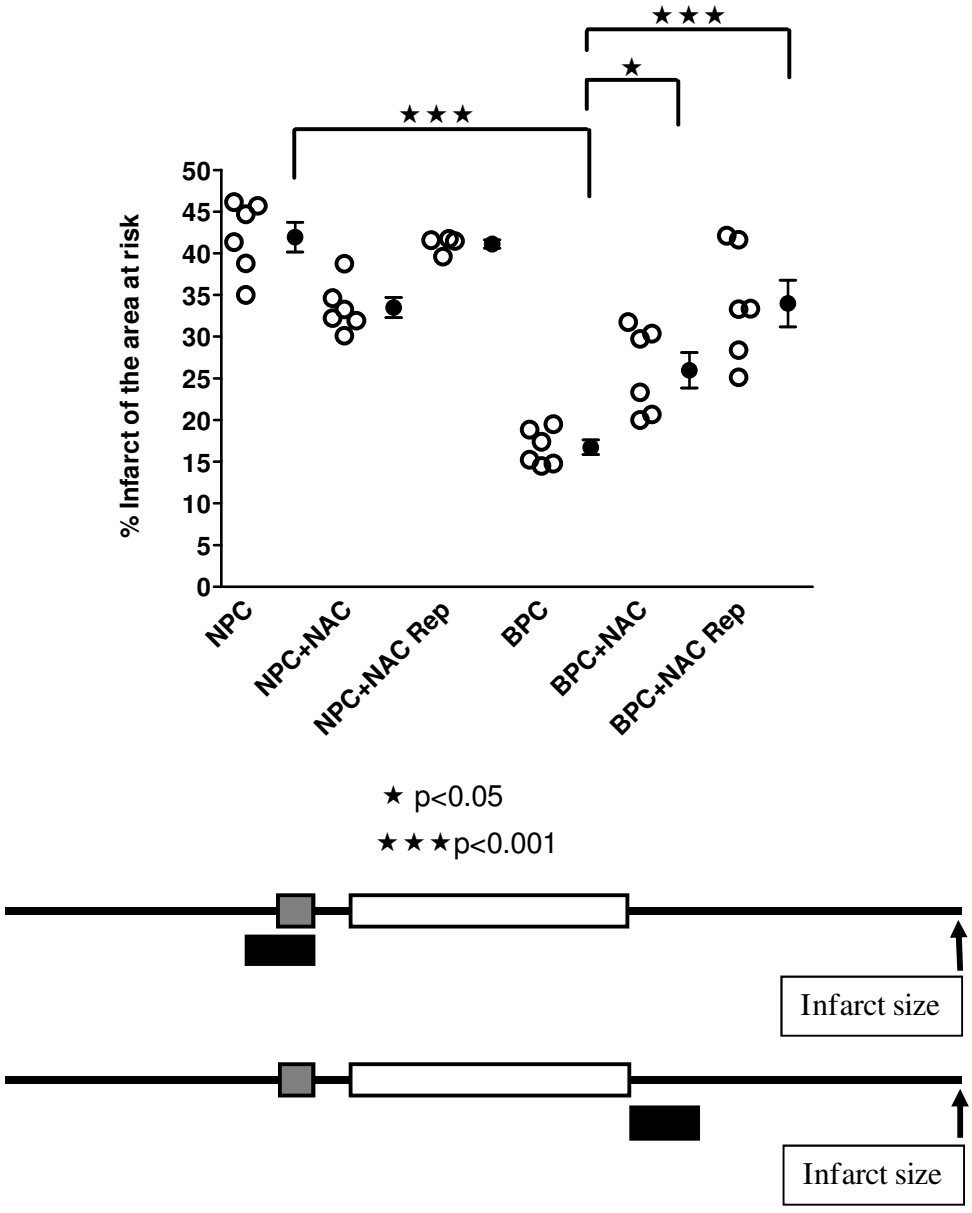


Fig. 6.4: The effect of ROS scavenger, NAC on infarct size in β1/β2-PC

6.3 Discussion

The results obtained in this chapter indicated that (i) NO plays a role in improving functional recovery in β-PC, while having no effect on infarct size; (ii) the mitoK_{ATP} channel has no role in β-PC and (iii) blocking ROS generation either during the triggering phase or reperfusion was without effect on functional recovery, but significantly increased infarct size.

6.3.1 The role of Nitric Oxide (NO) in the cardioprotective effects of β1/β2-PC

The effect of NO on a given cell type will largely depend on the NO concentration, the duration of NO exposure and the composition of the microenvironment. As stated in the introduction, cardioprotection can be elicited by the administration of NO donors. Thus, pharmacological mimicking with NO donors can bring about the same cardioprotection as present in ischaemic preconditioning (IPC) (Lochner et al., 2000). From this study of β1/β2-PC, it has emerged that the application of NOS inhibitors L-NAME / L-NNA during the triggering phase of β1/β2-PC had no effect on the infarct limiting effect of β1/β2-PC (Fig. 6.2). On the other hand, haemodynamic parameters obtained were significantly reduced (Table 6.1), suggesting that NO generated had an effect on alleviating stunning during reperfusion of β1/β2-PC. Thus, inhibition of NO formation with NOS inhibitors, led to a further depression in functional recovery. This is in contrast with ischaemic preconditioning where activation of the bradykinin and opioid receptors via NO and PKG, mediates opening of the K_{ATP} channel [Cohen and Downey, 2006]. The absence of overt effects of NOS inhibition on β1/β2-PC is also surprising in view of the fact that isoproterenol activates eNOS via Giα, causing an increase in cGMP [Balligand et al., 1999]. However, the contribution of NOS activation and NO generation in β1/β2-PC appears unlikely in view of the putative harmful effects of NO generated by β3-AR signaling [Gauthier et al., 1998; Moens, 2010].

6.3.2 The role of mitochondrial K_{ATP} ($mitoK_{ATP}$) channel in β1/β2-PC

The role of the $mitoK_{ATP}$ channel in cardioprotection has been intensely investigated and was first illustrated in a canine model when the $mitoK_{ATP}$ blocker, glyburide (glybenclamide) abolished IPC [Gross and Auchampach, 1992]. It was also found that the structurally distinct blocker 5-HD (5-hydroxydecanoic acid) abolished IPC in a similar model [Auchampach et al., 1992]. The ability of K_{ATP} blockers to abolish IPC in rats has been variable and may be model dependent. However, it is accepted by most that the $mitoK_{ATP}$ channel is involved in ischaemic preconditioning and it has been demonstrated that the critical time for opening of the $mitoK_{ATP}$ channel is during the brief preconditioning protocol. It has also been shown that transient opening of this channel prior to ischaemia mimics ischaemic preconditioning and the protection could be blocked by a free radical scavenger [Pain et al., 2000].

In this study of β1/β2-PC, it was illustrated that the application of 5-HD in β1/β2-PC had no effect on the beneficial effects of this cardioprontection (Table 6.2 and Fig. 6.3), showing that the $mitoK_{ATP}$ channel does not play a role in the signaling pathways of β1/β2-PC, which is in contrast to its important role in ischaemic preconditioning. This significant observation lends support to the major role of adenosine in β1/β2-PC, since adenosine triggered protection is known not to be $mitoK_{ATP}$ channel dependent as previously outlined in the introduction of this chapter.

6.3.3 The role of Reactive Oxygen Species (ROS) in the Cardioprotective effects of β1/β2-PC

Oxygen free radicals / reactive oxygen species (ROS) and their metabolites have received much attention because they are known to play an important role in many biological reactions which maintain normal cell function. Biological systems have substantial ability to tolerate ROS under normal conditions. It is well established in the setting of ischaemia / reperfusion that the generation of ROS becomes deleterious and may contribute to cell damage [Hess and Manson, 1984; Park and Lucchesi 1999; Zughaib et al., 1993; Opie, 1991; Ambrosio and Tritto, 1999; Zweier et al., 1987].

Despite the notion that ROS are produced primarily with the reintroduction of oxygen following ischaemia, several investigators observed ROS generation also during ischaemia [Hess and Manson, 1984; Nohl and Jordan1986; Kevin et al., 2003] and studies in cardiomyocytes showed that the mitochondria may be the major source of these substances [Vanden Hoek et al., 1998]. This concept is of major importance because ischaemia-generated ROS play an important signaling role [Carmody and Cotter, 2001], which may contribute to direct cellular oxidant damage. It is likely, that this is the same source of ROS that has been reported to trigger the cardioprotection of ischaemic preconditioning (IPC) [Vanden Hoek et al., 1998].

It is well established that chronic activation of the β-AR pathway can lead to increased ROS production. This effect is most probably mediated via downregulation of antioxidants such as copper-zinc-superoxide dismutase [Srivastava et al., 2007]. In another study of chronic β-AR stimulation, it was shown that it may induce oxidative damage through reactive intermediates resulting from auto-oxidation, irrespective of their interaction with adrenergic receptors, thus representing an important aspect in the pathogenesis of catecholamine-induced cardiotoxicity [Yogeeta et al., 2006]. In the acute phase of isoproterenol infusion (intravenous infusion for 30 min), ROS are important activators of cardiac MAPKinase cascade; while in the chronic phase (intravenous infusion for 10 days) ROS may participate in cardiac remodeling, especially in respect to wall stiffness, based on fibrogenesis [Zhang et al., 2005]. Shorter episodes of β-AR stimulation also lead to ROS formation: It has been demonstrated that stimulation of the β_2-AR with 10 μM isoproterenol for 10 min leads to ROS formation [Moniri and Daaka, 2007]. In view of the above, it is possible that during acute administration of isoproterenol for 5 min, generation of ROS may occur, which in turn participates in signaling processes.

In this study of β-adrenergic preconditioning it was shown that the free radical scavenger NAC, (N-acetyl cysteine) applied prior to RI (trigger phase) or at the onset of reperfusion (mediatory phase) had no significant effect on any of the haemodynamic parameters at the end reperfusion (Table 6.3). However, when using infarct size as a endpoint, a role of ROS in β1/β2-PC could be clearly illustrated since the ROS scavenger, NAC when applied prior to or at the onset of reperfusion, significantly increased infarct size (25.97±2.41%, $p<0.05$ and 33.99±2.80%, $p<0.001$, respectively), thereby attenuating the protective effect of β1/β2-PC (Fig. 6.4).

Interestingly, although the application of the free radical scavenger, NAC prior to and more so, at the start of reperfusion increased the IS, the haemodynamic parameters during reperfusion remained unaffected and not different from those of β1/β2-PC hearts.

Changes in infarct size is not often associated with the expected changes in mechanical function during reperfusion due to concomitant stunning [Ovize et al., 1992; Lochner, Genade and Moolman, 2003; Cohen, Yang and Downey, 1999]. Since infarct size is generally regarded as the golden index of cell injury, the results obtained in this study therefore suggest that ROS generation during both the trigger and mediator phases of β1/β2-PC contribute to its anti-necrosis effects, while having no effect on stunning. In summary, β1/β2-PC can be triggered by adenosine and ROS generation, but not by NO production or opening of the mitochondrial K_{ATP} channel.

Summary and Conclusions

In our early studies we showed that transient β-AR activation with ligands such as isoproterenol and forskolin mimicked ischaemic preconditioning and consequently elicited protection against a subsequent period of sustained ischaemia. The necessary component in both ischaemic and pharmacological preconditioning is the washout period applied before the onset of sustained ischaemia. If washout or reperfusion is not performed in either cardioprotective method, the technique would be termed pretreatment and not preconditioning. Consequently, the brief washout/reperfusion episode prior to sustained ischaemia is essential to these cardioprotective strategies, since crucial signal-transduction pathways could be activated and maintained during this period and this may affect the subsequent response of the heart to ischaemia. For example, cAMP is generated [Lochner et al., 1999], p38MAPK [Marais et al., 2001] and CREB are activated [Marais, Genade, and Lochner, 2008] during a β-PC protocol, to name but a few. In the present study it was shown that also significant activation of ERK and PKB/Akt occurred during washout after isoproterenol administration. This phenomenon is of particular importance since inhibition of these kinases during the triggering phase, completely inhibited β1/β2-PC.

At the onset of the present study it was not clear which of the three β-AR receptors (β_1-, β_2- or β_3-AR) present in heart muscle, was involved in β-PC. Therefore the first aim was to determine which of the three receptor subtypes are involved in this process. Throughout this study the isolated working rat heart, subjected to 35 min coronary ligation followed by reperfusion, was used as model with functional recovery during reperfusion and infarct size as endpoints. The results showed that transient β-AR stimulation with isoproterenol (β_1/β_2-AR agonist) (β1/β2-PC) effectively induced cardioprotection as indicated by a significant improvement in functional recovery and reduction in infarct size. Similar cardioprotection was achieved with formoterol hemifumarate, a β_2-AR agonist, (β2-PC) but not with BRL 37344, a β_3-AR agonist (β3-PC) (Fig.6.5).

The relative importance of adrenergic stimulation and demand ischaemia as important preconditioning stimuli remains unclarified. In this study, it was demonstrated that β_1/β_2-AR stimulation by isoproterenol caused an increased workload with possibly demand ischaemia and thus concomitant adenosine production. The role of adenosine in eliciting the cardioprotection of IPC is well-established.

The data obtained in this study showed that, as in the case in IPC, adenosine generation during β1/β2-PC is involved in eliciting cardioprotection. Using appropriate antagonists, it was shown that the A_1-AdoR was not involved in β1/β2-PC, nor did it have any effect on ERK p44/p42 or PKB activation. The data obtained suggest that the triggering of isoproterenol-induced protection (β1/β2-PC) is mainly dependent on endogenous adenosine acting on the A_3-AdoR; while the mediatory phase is dependent on the activation of the A_3-AdoR in conjunction with mainly the A_{2A}-AdoR, but also the A_{2B}-AdoR during this phase. It should be noted that the A_{2A}-AdoR are located on smooth muscle and endothelial cells of blood vessels mediating the vascular effects of adenosine. Also, it was shown to regulate inflammatory tissue damage and remodeling associated with ischaemia and reperfusion and the possibility that the A_{2A}-AdoRs may have a more prominent role in cardioprotection at reperfusion is more likely. On the other hand, the low sensitivity A_{2B}-AdoR is responsive to increased adenosine levels only, such as occurs during sustained ischaemia or at the onset of reperfusion. Thus, as expected this receptor type was shown to be involved, largely at reperfusion following sustained ischaemia in β1/β2-PC (Fig.6.5).

However, the role of demand ischaemia and adenosine in eliciting β-PC needs to be further evaluated, since β_2-AR preconditioning with formoterol did not affect mechanical performance during the triggering phase.

IPC-induced cardioprotection is now known to be associated with activation of the RISK pathway during reperfusion. It is has been shown that pharmacological manipulation and up-regulation of these kinases as an adjunct to reperfusion may protect the myocardium from lethal reperfusion–induced cell death and provide a novel approach to salvage viable myocardium and limit infarct size. Similar to IPC, β1/β2-PC and β2-PC were also associated with significant activation of the prosurvival kinases in the early mediatory phase following sustained ischemia. The latter phenomenon appears to be of particular significance since inhibition of these kinases during the triggering phase, completely inhibited subsequent cardioprotection (Fig.6.5).

The involvement of established role players in IPC such as the Gi/o protein, PKA, mitoK_{ATP} channel, reactive oxygen species (ROS) and nitric oxide (NO) in the mechanism of β-adrenergic induced cardioprotection were also evaluated. In contrast to IPC, inhibition of the mitoK_{ATP} channel by using 5-hydroxy-decanoate during the triggering phase had no effect on IS limiting capabilities

of β1/β2-PC – indicating that it had no part in this cardioprotective phenomenon. Cardioprotection of β1/β2-PC was abolished by ROS inhibition in the triggering phase as well as at the start of reperfusion after sustained ischaemia, indicating its importance in both phases of the experimental protocol. In addition, it appears that NO is also involved in β1/β2-PC. The Gi protein may play a role in β2-PC, since prior treatment with pertussis toxin had no effect on the reduction in infarct size induced by either isoproterenol or formoterol, but reduced functional recovery after β2-PC.

Using appropriate inhibitors (Rp-cAMP and wortmannin respectively) PKA as well as PI3-K activation were illustrated to be essential components of cardioprotection prior to sustained ischaemia (trigger phase) and at the onset of reperfusion (mediatory phase) of β1/β2-PC. However the mechanism whereby PKA activation mediates cardioprotection as well as the downstream events involved remains to be established (Fig.6.5).

The results obtained in this study again demonstrate how application of one of the triggers released during an IPC protocol, when administered alone, can elicit cardioprotection similar to that of IPC. Other similarities are involvement of the adenosine A_3, A_{2A} and A_{2B} receptors, ROS, NO and to a lesser extent, the Gi protein (Fig.6.5).

Finally, the results obtained in this study stress the significance of the endpoints used in the interpretation of data. Infarct size is by many workers regarded as the "gold standard" for evaluation of cardioprotection and in many studies, it is the only endpoint used in the interpretation of results. Reperfusion function is more complex and may be influenced by amongst others, stunning apoptosis and arrhythmias. By using two different endpoints, we expected a reduction in infarct size (i.e. less necrosis) to be associated with increased functional recovery and vise versa. Indeed, this was observed in the case of β1/β2-PC, β2-PC as well as in the studies where inhibitors of the A_{2A}-, A_{2B}- and A_3-AdoR subtypes were used. However, in a number of studies a dichotomy was observed, where (i) studies an increase in infarct size was not associated with changes in functional recovery (for example in the studies with adenosine deaminase, PD and NAC) or (ii) an unchanged infarct size was associated with a reduction in functional recovery (for example Pertussis toxin pretreatment and formoterol; L-NAME and L-NNA), suggest an effect on stunning. These observations stress the potential danger of using one endpoint only in the interpretation of data in studies such as the present.

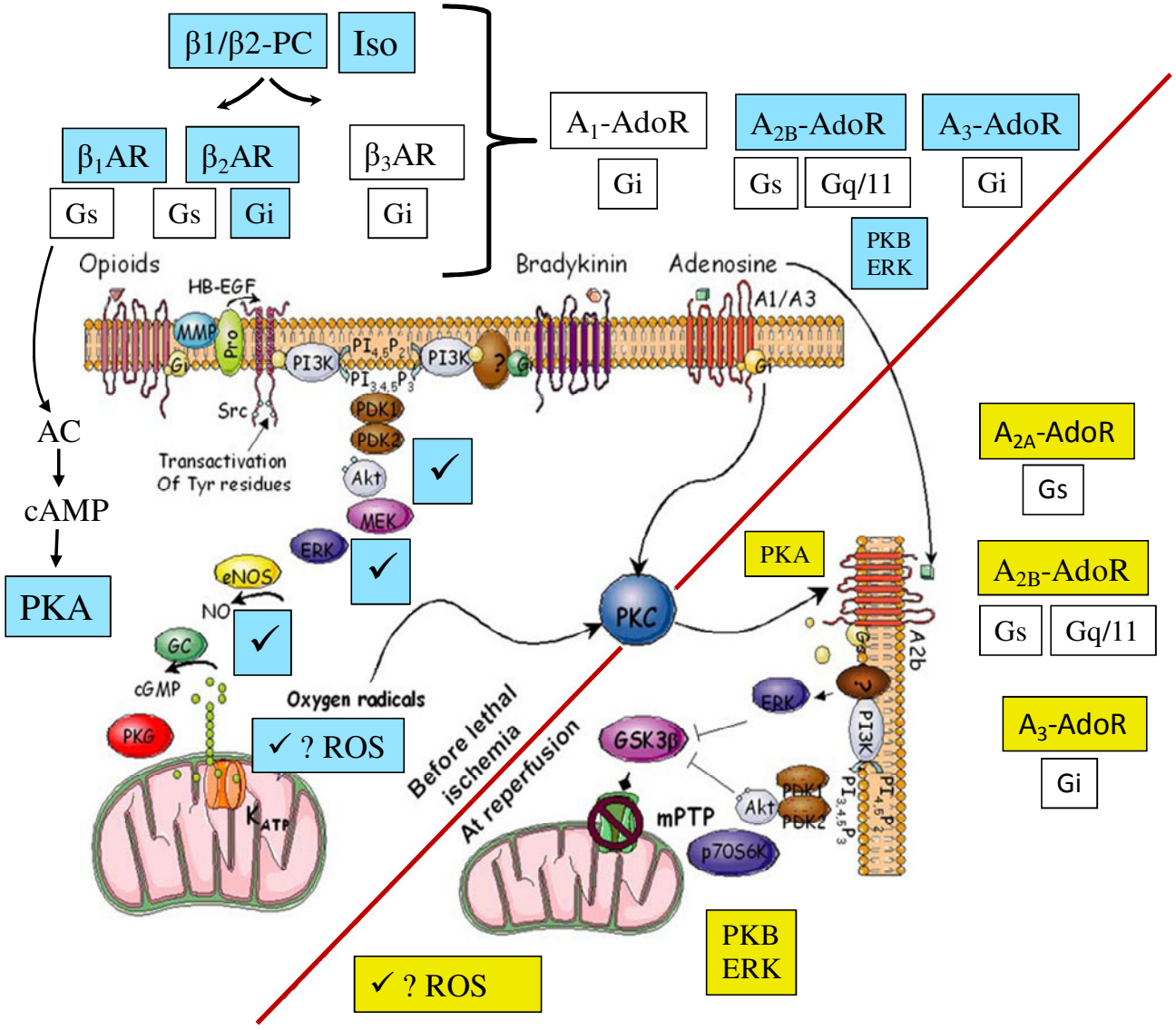


Fig. 6.5: Cartoon showing the sequence of signaling events involved in triggering the preconditioned state prior to the ischemic insult (events above the dividing line) and those that mediate protection in the first minutes of reperfusion (events below the dividing line), refer to text, section 1.3, page 5 [Tissier R, Cohen, and Downey, 2007; Downey, Krieg, Cohen, 2008; Lohse, Engelhardt and Eschenhagen, 2003; Zheng et al., 2005]. Also included in this cartoon are the receptors and signaling cascades that were shown to be involved in the cardioprotective strategy of β-PC prior to the ischemic insult in blue and those that mediated protection at the onset of reperfusion in yellow.

Future directions:

One of the most interesting observations made during the course of this study, was the fact that transient activation of the β_2-AR with formoterol was the most effective trigger of cardioprotection. It is envisaged that use of this agonist may shed more light on the mechanism whereby β-AR stimulation triggers the cardioprotective process, since formoterol per se had no effect on mechanical activity of the isolated rat heart. As yet, no information is available regarding its mechanism of action. For clinical application in the future, it is essential that its effects are also evaluated in an in vivo model.

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