The Role of the Beta3-Adrenergic Receptor (β3-AR) in Cardioprotection

By

Dr Aisha Khlani Hassan Alsalhin

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Supervisor: Dr Ruduwaan Salie

Co-supervisors: Prof Amanda Lochner

Dr Erna Marais

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Declaration

I, Dr Aisha.K.H.Alsalhin, 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.

Dr Aisha.K.H.Alsalhin

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Abstract

It is well-established that transient activation of the β-adrenergic signalling pathway with ligands such as isoproterenol, formoterol and dobutamine, elicits cardioprotection against subsequent long periods of ischaemia. Initially the focus was on the β1- and β2-adrenergic receptors (β1-AR, β2-AR), but recently the β3-AR also emerged as a potential target in the treatment of heart disease. In heart failure, β1- and β2-AR are typically known to be down-regulated while β3-ARs, on the other hand, are up-regulated (Moniotte et al., 2001). Thus, it has become important to examine the significance of the β3-AR and its downstream signalling under similar states of stress. It has been shown that β3-AR stimulation is resistant to short term agonist-promoted desensitization in vitro and in vivo (Liggett et al., 1993) and after being activated, this receptor is able to convey continual intracellular signals (Lafontan et al., 1994). Thus, it could be an ideal target for therapeutic intervention, also in ischaemic heart disease. We **hypothesized** that selective β3-AR stimulation during ischaemia / reperfusion may be cardioprotective, whereas selective inhibition of this receptor may prove useful in the end stages of sustained ischaemia and early reperfusion. **Methods**: The isolated working rat heart, subjected to 35 min of regional ischaemia (RI) and 60 min reperfusion was used as model. The β3-AR agonist (BRL37344) (1 µM) or antagonist (SR59230A) (0.1 µM) were applied as follows: (i) before 35 min RI **(PT)**, (ii) during the last 10 min of RI **(PerT)** and /or (iii) at the onset of reperfusion **(PostT)** and (iv) administration of BRL37344 during the last 10 min of RI **BRL37344 (PerT)** was followed by SR59230A during first 10 min of reperfusion **SR59230A (Post).** The contribution of nitric oxide synthase (NOS) in β3-AR was assessed, using the non-specific NOS inhibitor, L-NAME (50 µM). Endpoints were functional recovery and infarct size. In another set of experiments BRL37344 and SR59230A were applied according to the same protocols, but the left ventricle was dissected from the heart and freeze clamped at 10 min reperfusion for Western blot analysis of extracellular signal-regulated kinase **(ERK p44/p42)**, protein kinase B **(PKB/Akt)**, glycogen synthase kinase-3β **(GSK-3β)**, and endothelial nitric oxide synthase **(eNOS).** Data were analyzed with one or two-way analysis of variance (ANOVA). **Results:** Administration of the selective β3-AR agonist (BRL37344) (1µM) before 35 min RI **(BRL37344 (PT)**, significantly reduced infarct size when compared to the non-pretreatment group **(NPT)** (21.43±2.52 vs 43.17±1.20, $p < 0.001$). BRL37344 had similar effects on infarct size when

applied during the last 10 min of regional ischaemia **BRL37344 (PerT)** (14.94±2.34, vs NPT, p < 0.001) or at the onset of reperfusion **BRL37344 (PostT)** (19.06±1.81, vs NPT, p < 0.001). When BRL37344 was applied as a **(PerT+PostT)** strategy, infarct size was once again significantly reduced (20.55±2.01 vs 43.17±1.20, p <0.001). In contrast, administration of the β3-antagonist SR59230A according to the same protocol did not reduce infarct size and values similar to those of untreated hearts **(NPT)** were obtained. Surprisingly, when BRL37344 was applied during the last 10 min of regional ischaemia followed by the administration of the β3-AR antagonist (SR59230A) at the onset of reperfusion, **[BRL37344 (PerT) & SR59230A (PostT)]**, infarct size was significantly reduced to 20.78±3.02 (p <0.001 vs **NPT** and **SR59230A (PerT + PostT**). Involvement of nitric oxide (NO) was shown since the reduction in infarct size elicited by BRL37344 was totally abolished by, L-NAME, when administered in combination with BRL37344 for 10 minutes prior to RI or at the onset of reperfusion for 10 minutes (% infarct size: 41.48±3.18 and 35.75±3.54, p <0.001 vs **BRL37344 (PT)** and **BRL37344 (PostT),** respectively. Western blot results show that PKB/Akt is activated by BRL37344 regardless of the time of administration. The intervention **BRL37344 (PerT+PostT),** exhibited the most significant phosphorylation of PKB/Akt (fold increase: 14.2±3.71, p<0.01 vs **NPT** and p<0.05 vs **BRL37344 (PostT).** In addition, **BRL37344 (PT), (PerT), (PostT)** and **[BRL37344 (PerT) +SR59230A (PostT)]** showed significant activation of this kinase (2.92±0.22, 5.54±0.43, 4.73±0.47, and 6.60±0.78, respectively). ERKp44/p42 however, was not significantly activated by any of the treatments. Phosphorylation of eNOS and GSK-3β was significant only in the **BRL37344 (PerT+PostT)** and **[BRL37344 (PerT) + SR59230A (PostT)]** groups. The activation of eNOS-S-1177 in the **BRL37344 (PerT+PostT)** group was (2.82±0.46, p<0.01 and 0.05 vs NPT and BRL37344 (PostT), respectively) and in the **[BRL37344 (PerT) + SR59230A (PostT)]** group was (2.26±0.48, p<0.05 vs NPT). A very significant increased phosphorylation of GSK-3β was seen in the same two groups (68.8±7.73, p<0.001 vs NPT and 25.5±5.42 vs NPT, p<0.05, respectively). **Conclusion**: β3-AR has potent cardioprotective effects when administered either before, during and after ischaemia during early reperfusion as indicated by the reduction in infarct size as well as activation of PKB, GSK-3β and eNOS. These beneficial effects can be linked to NO production through activation of eNOS.

Abstrak

Dit is bekend dat verbygaande aktivering van die β-adrenerge seinpad, met ligande soos isoproterenol, formoterol en dobutamien, die hart teen daaropvolgende lang periodes van iskemie beskerm. Aanvanklik was die fokus op die β1- en β2-adrenerge reseptore (β1-AR, β2-AR); maar onlangs is ook die β3-AR as 'n potensiële teiken in die behandeling van hartsiektes ge-eien. In hartversaking, is dit bekend dat β1- en β2-AR afreguleer word, terwyl β3-ARs, aan die ander kant, opreguleer word (Moniotte et al., 2001). Dit het dus belangrik geword om die belang van die β3-AR en sy stroomaf seinpad onder soortgelyke strestoestande te ondersoek. Dit is bewys dat β3-AR stimulasie teen korttermyn agonis geïnduseerde desensitisering in vitro en in vivo bestand is (Liggett et al., 1993) en wanneer geaktiveer, is hierdie reseptor in staat om intrasellulêre seine voortdurend oor te dra (Granneman, 1995). Dit kan dus 'n ideale teiken vir terapeutiese intervensie wees, ook in iskemiese hartsiekte. Ons **hipotetiseer** dat selektiewe β3-AR stimulasie tydens iskemie / reperfusie kardiobeskermende mag wees, terwyl selektiewe inhibisie van hierdie reseptor effektief kan wees in die eindstadia van volgehoue iskemie en vroeë herperfusie. **Metodes**: Die geïsoleerde werkende rothart, onderwerp aan 35 min van streeksiskemie (SI) en 60 min herperfusie, is as model gebruik. Die β3-AR agonis (BRL37344) (1µM) of antagonis (SR59230A) (0.1 µM), is as volg toegedien: (i) voor 35 min SI **(PT)**, (ii) gedurende die laaste 10 min van SI **(PerT)** en / of (iii) tydens die aanvang van herperfusie **(PostT)** en (iv) gedurende die laaste 10 min van SI is BRL toediening **BRL37344 (PerT)** gevolg deur SR59230A tydens die eerste 10 min van herperfusie **SR59230A (Post)**. Die rol van stikstofoksiedsintase (NOS) in β3-AR is met behulp van die nie-spesifieke NOS inhibitor, L-NAME (50 µM) ondersoek. Eindpunte was funksionele herstel tydens herperfusie en infarktgrootte. In 'n ander reeks eksperimente is BRL37344 en SR59230A volgens dieselfde protokolle toegedien, maar die linker ventrikel is uit die hart gedissekteer na 10 min herperfusie en gevriesklamp vir Western klad analise van ekstrasellulêre-sein gereguleerde kinase **(ERK p44/p42)**, proteïen kinase B **(PKB/Akt)**, glikogeen sintase kinase-3β **(GSK-3β)**, en endoteel stikstofoksied- sintase **(eNOS)**. Data is met een of twee-rigting variansie analise (ANOVA) ontleed. **Resultate:** Administrasie van die selektiewe β3-AR agonis (BRL37344) (1µM) voor 35 min SI **BRL37344 (PT)**, het die infarktgrootte beduidend verminder vergeleke met die nie-behandelde groep **(NPT)** (21.43±2.52 vs 43.17±1.20, $p<0.001$). BRL37344 het 'n

soortgelyke effek op infarktgrootte wanneer dit gedurende die laaste 10 min van streeksiskemie **BRL37344 (PerT)** (14.94±2.34, vs NPT, p<0.001) of by die aanvang van herperfusie (BRL37344 (PostT) (19.06±1.81, vs NPT, p<0.001) toegedien word. Wanneer BRL37344 as 'n **(PerT+PostT)** strategie toegedien is, was infarktgrootte weereens beduidend verlaag (20.55±2.01 vs 43.17±1.20, p<0.001). In teenstelling hiermee, het administrasie van die β3-antagonis SR59230A volgens dieselfde protokol, nie infarktgrootte verminder nie en waardes soortgelyk aan dié van onbehandelde harte **(NPT)** is verkry. Interessant, wanneer BRL37344 gedurende die laaste 10 min van streeksiskemie toegedien is, gevolg deur die administrasie van die β3-AR antagonis (SR59230A) by die aanvang van herperfusie, **[BRL37344(PerT) & SR59230A(PostT)]**, was infarktgrootte aansienlik verminder tot 20.78±3.02 (p<0.001 vs **NPT** en **SR59230A (PerT+PostT)**. Die betrokkenheid van stikstofoksied (NO) is waargeneem deurdat die vermindering in infarktgrootte ontlok deur **BRL37344**, heeltemal deur L-NAME opgehef is, wanneer dit in kombinasie met **BRL37344** vir 10 minute voor SI of by die aanvang van herperfusie vir 10 minute toegedien is (% infarktgrootte: 41.48±3.18 en 35.75±3.54, p<0.001 vs **BRL37344 (PT)** en **BRL37344 (PostT)** onderskeidelik). Western kladresultate toon dat PKB/Akt deur BRL37344 geaktiveer word ongeag die tyd van die administrasie. Die intervensie **BRL37344 (PerT+PostT)**, toon die mees beduidende fosforilering van PKB/Akt (voudige toename: 14.2±3.71, p<0.01 vs **NPT** en p<0.05 vs **BRL37344 (PostT)**. Daarbenewens het **BRL37344 (PT), (PerT), (PostT)** en **[BRL37344 (PerT) + SR59230A (PostT)]** ook beduidende aktivering van hierdie kinase tot gevolg gehad (2.92±0.22, 5.54±0.43, 4.73±0.47 en 6.60±0.78, onderskeidelik). ERKp44/p42 is egter nie deur enige van die behandelings geaktiveer nie. Fosforilering van eNOS en GSK-3β was net beduidend in die **BRL37344 (PerT+PostT)** en **[BRL37344 (PerT) + SR59230A (PostT)]** groepe. Die aktivering van eNOS-S-1177 was beduidend in die **BRL37344 (PerT+PostT)** en **[BRL37344 (PerT) + SR59230A (PostT)]** groepe. 'n Baie beduidende toename in fosforilering van GSK-3β is in dieselfde twee groepe (68.8±7.73, p<0.001 en 25.5±5.42, p<0.05 vs NPT onderskeidelik) waargeneem. **Gevolgtrekking:** β3-AR het kragtige kardiobeskermende effekte wanneer dit, hetsy voor, tydens en na iskemie gedurende vroeë herperfusie toegedien word, soos deur die vermindering in infarktgrootte sowel as die aktivering van PKB, GSK-3β en eNOS aangedui is. Hierdie voordelige effekte kan aan NO produksie deur aktivering van eNOS gekoppel word.

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Chemicals, drugs and reagents

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

β3-AR agonist (BRL37344) ((±)-(R,R)-[4-[2-[2-(3-Chlorophenyl)-2-hydroxyethyl] amino] propyl] phenoxy] acetic acid sodium);

β3-AR antagonist (SR59230A) (3-(2-Ethylphenoxy)-1-[[(IS)-1,2,3,4-tetrahydronapth-1-yl] amino]-(2S)-2-propanol oxalate salt;

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

Antibodies were purchased from **cell signalling technology (Boston, MA, USA)** and all other routine chemicals were **MERCK** (analar grade).

Abbreviations list

Units of measurements

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

Chemical compounds

Ca^{2}	Calcium
CO_2	Calcium chloride
$MgSO_4$	Magnesium sulphate
O_2	Oxygen
$\cdot O_2^-$	Superoxide anion radical
H_2O_2	hydrogen peroxide
K+	Potassium
KCL	Potassium
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate

Tris	tris (hydroxymethyl) aminomethane hydrochloride
H_2O	Water

Other abbreviation

ACS	Acute Coronary Syndrome
AMI	Acute Myocardial Infarction
BAD	Bcl-2-associated death promoter
B-PC	β-preconditioning
cAMP	Cyclic adenosine monophosphate
Caspase	Cysteine-ASPartic acid protease
cGMP	Cyclic guanosine monophosphate
EGFR	Epidermal Growth Factor Receptors
eNOS	endothelial Nitric Oxide Synthase
ERK	Extracellular Signal-Regulated kinases
FOXOs	Forkhead box O proteins
GSK-3B	Glycogen synthase kinase-3 beta
Gs protein	Stimulatory Guanine nucleotide-binding protein
HNO	Hydrogen Nitrate or Bi Nitrate
IGF-1	Insulin-like Growth Factor-1
IHD	Ischaemic heart disease
iNOS	Inducible nitric oxide synthase
IPC	Ischaemic preconditioning
IPerC	Ischaemic perconditioning

IPostC	Ischaemic postconditioning
IRI	Ischaemia/reperfusion injury
IRS	Insulin Receptor Substrate
JNK	c-Jun amino-terminal kinases
LAD	left anterior descending coronary artery
MAPK	Mitogen-activated protein kinases
MEK	Mitogen/Extracellular Signal-regulated Kinase
mitoK$_{ATP}$	mitochondrial K$_{ATP}$ channels
mPTP	mitochondrial permeability transition pore
NPC	non preconditioning
NPT	non pretreatment
nNOS	neuronal nitric oxide synthase
NO	Nitric Oxide
OMM	Outer Mitochondrial Membrane
$ONOO^-$	Peroxynitrite anion
PCI	Percutaneous Coronary Intervention
PDK	phosphatidylinositide-dependent kinase
PDGFR	Platelet-Derived Growth Factor Receptors
PI3-K	phosphoinisotide 3-kinase
PIP2	phosphatidylinositide-4,5-biphosphate
PIP3	phosphatidylinositide-3,4,5-triphosphate
p70s6K	S6 ribosomal protein Kinase

PKA	Protein kinase A
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
p38MAPK	p38 Mitogen-Activated Protein Kinase
PTEN	Phosphatase and tensin homolog deleted on chromosome TEN
RIC	Remote Ischemic conditioning
RIPC	Remote Ischaemic Preconditioning
RPerC	Remote Ischaemic Perconditioning
RIPostC	Remote Ischaemic Postconditioning
RISK	Reperfusion Induced Salvage Kinases
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SWOP	Second Window Of Protection
SAFE	Survival Activation Factor Enhancement
STAT-3	Signal Transducer and Activator of Transcription 3
TNFα	Tumour Necrosis Factor alpha
VDAC	Voltage-Dependent Anion Channel

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Chapter 1

Introduction

Ischaemic heart disease (IHD) remains a leading cause of mortality and morbidity world-wide, and approximately more than 3 million men and women die of the disease annually (Murray and Lopez, 1997). In acute coronary syndrome (ACS), such as acute myocardial infarction (AMI), the coronary artery or one of its smaller branches is totally occluded by small migrated emboli from plaque rupture site while in chronic IHD, such as stable angina pectoris, the coronary artery is partially occluded by a previously formed intracoronary atherosclerotic plaque (Agewall S., 2008). Although the pathophysiological background for both conditions are similar, chronic IHD have better prognosis than ACS (Agewall S., 2008). Therefore, more studies about myocardial protection should be focused on utilizing pathophysiological models of AMI.

Following coronary occlusion, deprivation of oxygen and nutrient metabolites is the primary cause of damage to the myocardium. It is well-established that myocardial ischaemia results in numerous intracellular changes for example loss of ATP, a fall in pH, accumulation of lactate, oxidative stress, inorganic phosphate and calcium overload (for a review see Ong et al., 2015). Survival of the ischaemic myocardium is determined by the severity and duration of ischaemia. Many of these changes can be remedied by early reperfusion. However, it has become clear that reperfusion, although essential for reversal of the ischaemic damage, can also induce additional myocardial injury, a phenomenon called reperfusion injury. Reperfusion injury characteristically includes myocardial stunning, no-reflow phenomena, reperfusion arrhythmias and cell death (Hausenloy et al., 2007). Myocardial stunning is a condition when a portion of the myocardium, corresponding to the zone of a major coronary occlusion, contracts abnormally. This might be continuing for a period of time and is caused by reactive oxygen species (ROS) production and calcium Ca^{2+} accumulation (Bolli and Marban, 1999). Clinically, it does not appear to be a serious condition and disappears spontaneously. Similarly, reperfusion arrhythmias although it is potentially harmful, it can be treated easily using alpha blockers, high potassium, high magnesium,

calcium antagonists and free radical scavengers (Manning and Hearse, 1984, Yellon and Hausenloy, 2007). The no-reflow phenomenon occurs when the myocardial ischaemic area fails to be reperfused after reopening the previously occluded coronary artery, due to the resistance of microvascular blood flow, resulting from endothelial swelling, cardiomyocyte oedema and neutrophil infiltration (Ito et al., 2006, Krug et al., 1966).

It is well-established that the cell death due to ischaemia/ reperfusion injury (IRI) is caused primarily by both necrosis and apoptosis. Necrosis refers to cell death by swelling (oncosis), hypercontracture, due to calcium overload, ATP depletion and subsequent membrane rupture. The dead cells attract inflammatory cells including macrophages and fibroblasts with subsequent scar formation. Apoptotic cell death on the other hand, refers to cell shrinkage and accompanying biochemical degradation of intracellular contents with preservation of the cell membrane. This is followed by the separation of the nucleus into small portions, which will be engulfed by phagocytes (phagocytosis). This process is not associated with fibrosis and scar formation as in necrotic cell death, the apoptotic parts are cleared by the adjacent cells, leaving no trace behind (Opie L.H., 2004).

Although irreversible ischaemic damage is a consequence of either apoptosis or necrosis, it is still open to debate as to what specific mechanisms are involved by which reversible ischaemic damage regresses into irreversible damage. It is known that the development of such damage in the necrotic area causes loss of considerable amounts of ATP, sarcolemmal damage, release of free radicals, calcium overload, and sodium pump inhibition. However, although reperfusion injury could lead to cell death, early reperfusion has become obligatory for all conditioning strategies aimed at reduction of infarct size (Ovize et al., 2010). This allows an opportunity to intervene with cardioprotective strategies during reperfusion to delay the expected injury. For this reason intense research into the development of novel maneuvers and drugs that will limit infarct size is required. Consequently, cardioprotection should be aimed at attenuation of the dangerous effects of myocardial (IRI), thereby reducing mortality and morbidity in patients with IHD.

Numerous researches examined the ability of different strategies to reduce infarct size and improve functional recovery of the heart during reperfusion, some of which include mechanical interventions such as ischaemic preconditioning (IPC) (Murry et al., 1986), and ischaemic postconditioning (IPostC) (Cour et al., 2010), while others include pharmacological interventions using pharmacological agents such as calcium channel blockers (Vander Heide et al., 1994), adenosine (Liu et al., 1991), alpha1-adrenergic agonists (Bankwala et al.,1994), and beta-adrenergic antagonists and agonists (Zhang et al.,2010; Salie et al.,2011;2012).

Subsequently, a major number of drugs have been illustrated to be cardioprotective if administered at the time of reperfusion, e.g. activators of kinases (Hausenloy et al, 2004), inhibitors of protein kinase C (PKC-δ) (Inagaki et al., 2003), inhibitors of opening the mitochondrial permeability transition pore (MPTP) (Hausenloy et al., 2014) (see the review of Ong et al., 2014) and inhibition of glycogen synthase kinase-3β (GSK-3β) (Gross et al., 2004). However, until recently, translation of these laboratory trials into a successful clinical therapy to the pateints presented with IHD, has been largely disappointing (Sharma et a.l, 2012; Heusch et al., 2013; Perricone and Vander Heide, 2014).

Conditioning is a term that has been used to describe a series of short cycles of non-lethal ischaemia interrupted by short cycles of reperfusion applied during ischaemia/reperfusion protocol. Over the past three decades, researchers have designed several strategies to minimise IRI. Depending on the timing of conditioning stimulus application and the windows of therapeutic opportunity, conditioning strategies have been classified into three interventions. In ischaemic preconditioning **(IPC)**, the conditioning stimulus is applied before the subsequent sustained ischaemia, while in ischaemic perconditioning **(IPerC)** and postconditioning **(IPostC)**, the stimulus is applied during and after the major ischaemic event, respectively (Vinten-Johansen and Shi, 2011). Furthermore, remote ischemic conditioning **(RIC)** is another cardioprotective strategy in which the conditioning stimulus applied to an organ or tissue distant from the heart (Zhu et al., 2013; Vinten-Johansen and Shi, 2011). In addition, pharmacological conditioning is achieved by application of pharmacological agents to protect the heart against IRI (Hausenloy and Yellon,

2008). Elucidation of the mechanisms involved in these strategies may lead to the development of new drugs to elicit cardioprotection.

1.1 Ischaemic preconditioning (IPC)

It was demonstrated that exposure of the heart to short periods of ischaemia/reperfusion, significantly protects the heart against a following prolonged period of ischaemia, phenomena called ischaemic preconditioning (IPC) (Murry et al., 1986). This phenomenon has been investigated in several mammalian species, including sheep, monkeys, rabbits, rats, pigs and dogs and it has been illustrated to improve the cardiac contractility during reperfusion (Murry et al., 1990). Furthermore, IPC was shown to attenuate reperfusion arrhythmias and limit infarct size extention (Shiki and Hearse, 1987). Consequently, it has been recognized as the most effective cardioprotective intervention for many years (Yellon and Downey, 2003) (for a review see Zhu et al., 2013). It has been shown that IPC has two distinct stages of protection. The first stage, termed classical/early preconditioning, is elicited within minutes and lasts for 2-3 hours (Murray et al., 1986) and the second stage is called the second window of protection (SWOP) /delayed or late preconditioning, which develops within 12-24h and lasts for 2-3 days (Marber et al., 1993; Kuzuya et al., 1993) (for a review see Heusch et al., 2015). It has been shown that IPC successfully translated clinically to human. However, it is limited to elective clinical settings such as percutaneous coronary intervention (PCI) and coronary bypass grafting, but not in patients presenting with acute myocardial infarction (AMI) (Heusch et al., 2013). This has led to intensive research to develope new strategies which could be used in patients with AMI.

1.2 Ischaemic postconditioning (IPostC)

A large number of studies have established the ability of cardioprotective strategies, administered at the onset of reperfusion to reduce infarct size. Vinten-Johansen and colleagues, first noted that interrupting myocardial reperfusion with three cycles of 30 seconds coronary artery re-occlusions had significant beneficial effects including a reduction in infarct size, less myocardial oedema, reduced necrotic and apoptotic cell death and improved endothelial function (Zhao et al., 2003) and termed this ischaemic postconditioning (IPostC). Theoretically, this intervention should be clinically applicable since it is performed immediately at the beginning of reperfusion.

Subsequently, IPostC was used in patients with AMI undergoing PCI, and resulted in a reduction of myocardial infarct size by 36% and improved myocardial reperfusion (Staat et al., 2005). The mechanism of IPostC induced cardioprotection is not yet fully understood. However, emerging evidence suggests that IPC and IPostC have, at least in part, similar signalling pathways at the time of reperfusion. These include cell membrane receptors, protein kinase cascades such as the Reperfusion Injury Salvage Kinases (RISK) pathway as well as the Survival Activation Factor Enhancement (SAFE) pathway, redox signalling, and the MPTP (Hausenloy et al., 2005; Sun et al., 2005) (discussed later).

1.3 Remote ischaemic conditioning (RIC)

Remote ischaemic conditioning was initially demonstrated by Przyklenk and colleagues (1993) who found that 4 cycles of 5 minutes ischaemia and 5 minutes reperfusion of left circumflex coronary artery followed by 60 minutes ischaemia and reperfusion of the left anterior descending (LAD) coronary artery, resulted in a reduction in infarct size. Subsequently, myocardial protection was elicited by short ischaemia/reperfusion episodes in some organs distant from the heart such as brain, intestine, kidney, and skeletal muscle (Przyklenk et al., 2003). Afterwards it was illustrated that a brief renal ischaemia/reperfusion at the end of sustained myocardial ishaemia significantly reduced infarct size (Kerendi et al., 2005). This phenomenon which is termed **remote ischaemic preconditioning (RIPC)** might replace invasive interventions such as IPC and IpostC which have to be applied to the heart itself to bring about cardioprotection (Zhu et al., 2013). Another study using transient limb ischaemia, a more convenient stimulus, applied after myocardial infarction, termed **remote ischaemic postconditioning (RIPostC),** also showed a powerful myocardial protection (Andreka et al., 2007). Furthermore, it was demonstrated that transient intermittent limb ischaemia in a pig model, applied during the sustained myocardial ischaemia, **remote ischaemic perconditioning (RPerC)**, also resulted in a reduction of myocardial infarction and improved cardiac function (Schmidt et al., 2007). Subsequently, it was successfully applied clinically, by Botker et al and collaborators (2010) who reported that remote perconditioning induced by blood pressure cuff inflation on the arms of patients (during the attack of AMI) while being transported to the hospital (before application of PCI), resulted in infarct size reduction.

Remote conditioning phenomena have been shown to protect other organs as well as the vascular system (Heusch et al., 2013; also see the review of Heusch et al., 2015).

1.4 Signalling events of ischaemic conditioning

Previous studies have investigated the mechanism of IPC-induced cardioprotection to recognize the numerous triggers, mediators and effectors which could be involved in this phenomenon (for a review see Perricone and Vander Heide, 2014). **Triggers** are molecules released from some cells such as cardiomyocytes, endothelial cells, and leukocytes (Heusch et al., 2012), and stimulated during the preconditioning cycles. The cardioprotecton of IPC will be abolished if a trigger is inhibited. Triggers include adenosine (Liu et al., 1991; Thoronton et al., 1992), bradykinin (Goto et al., 1995), opioids (Genade et al., 2001) and catecholamines (Bankwala et al., 1994).

Nitric oxide (NO) (Lochner et al; 2002), reactive oxygen species (ROS), for example; $\cdot O_2^-$ and H_2O_2 and / or reactive nitrogen species (RNS), for example; $NO^{\cdot}$, Hydrogen Nitrate or Bi Nitrate (HNO) and Peroxynitrite anion ($ONOO^-$), have also been shown to act as triggers but without involvement of a receptor (Penna et al., 2009). **Mediators** are molecules which mediate the mechanisms of cardioprotection during the sustained ischaemia as well as at the beginning of reperfusion and they are downstream substrates of triggers. Mediators include the mitochondrial K_{ATP} channels, PKC (Miao et al., 2011) and PKA (Lochner et al., 1999) among others. Similarly, inhibition of these mediators leads to attenuation of IPC cardioprotection. **Effectors** are subcellular molecules or organelles which have an important role in salvaging the endangered cardiomyocytes and preventing them from cell death. Mitochondria are potential effectors and essential organelles in the cell signalling pathway (Heusch et al., 2008). Furthermore, attenuation of calcium accumulation and inhibition of proteases (Heusch et al., 2012) are also suggested to be effectors of cardioprotection.

1.4.1 Signalling during reperfusion: RISK and SAFE pathways

It is well-known that the cardioprotection of IPC is crucially induced by activation of the **Reperfusion Injury Salvage Kinase (RISK)** and the **Survival Activation Factor Enhancement (SAFE)**

pathways at the onset of myocardial reperfusion (Hausenloy et al., 2004, 2011) and there is substantial evidence linking these two pathways to MPTP inhibition (Hausenloy et al., 2005; 2009).

It was well-established that IPC produce its cardioprotective effects by activation of PI3-K/PKB and Mitogen-activated protein kinases /extracellular signal-regulated kinases (MEK1/2ERK1/2) during reperfusion (Hausenloy et al., 2005), the so-called RISK pathway. Activation of the RISK pathway, through triggering of membrane receptors, by autacoids, has also been suggested to be involved in the mechanism of postconditioning (Hausenloy et al., 2005). Stimulation of the receptors of endogenous adenosine, bradykinin, and opioid has been illustrated to be able to activate and phosphorylate the prosurvival kinases PI3K-Akt and MEK1/2-ERK1/2 in IPostC (Bell and Yellon, 2003; Penna et al., 2007; Gross et al., 2004). Thus, pharmacological inhibition of either of these cascades early in reperfusion abolishes the cardioprotective effects of IPC (Hausenloy et al., 2005) as well as IPostC (Tsang et al., 2004). It was therefore concluded that both IPC and IPostC actually exert their protection early in reperfusion.

It has been shown that under certain conditions another signalling pathway, the so-called SAFE pathway is activated at the onset of myocardial reperfusion (Hausenloy and Yellon, 2004; 2011). This pathway, which includes signal transducer and activator of transcription 3 (STAT-3), tumour necrosis factor alpha (TNFα) and its receptor subtype 2, has been shown to be involved in cardioprotection elicited by both IPC and IPostC at the time of reperfusion (Deuchar et al., 2007; Lecour et al., 2009; Lacerda et al., 2009). Activation of the RISK and SAFE pathways has been suggested to converge on the mitochondria and to prevent opening of the MPTP during reperfusion, resulting in cardioprotection (Heusch et al., 2015; Boengler et al., 2010). However, the exact mechanism still needs to be elucidated.

Regarding the signalling of RIC phenomena, some researchers have suggested that transmission of the cardioprotective signals from the stimulus to the heart is a result of either neuronal or hormonal transmission, as the signal might be transmitted by blood between species (Przyklenk et al., 2011) (see a review by Heusch et al., 2013). Local ischemia/reperfusion injury in an organ distant from the heart stimulates the sensory fibres in that organ and subsequently activates the cardiac innervation via the central nerve system (Heusch et al., 2015). In the heart the

cardioprotective signalling of RIC appears to be similar to those elicited by IPC and IPostC, especially the involvement of the local triggers such as bradykinin (Jones et al.,2009); opioids (Patel et al., 2002) and adenosine (Leung et al., 2014). However, it has been noticed that PKC (Jones et al., 2009), the RISK pathway (Hausenloy et al., 2012) and eNOS (Rassaf et al., 2014) play a more important role in RIC than in IPC and IPostC (Heusch et al., 2015). Furthermore, mitochondria (Tsang et al., 2004) and the K_{ATP} channels (Schmidt et al., 2007) are also involved as effectors in RIC.

1.4.2 The mitochondrial permeability transition pore (MPTP) and cardioprotection

The MPTP, a non-selective channel of the inner mitochondrial membrane (Griffiths et al., 1995), has been demonstrated to be a critical determinant of cardiomyocyte death. It was illustrated that this channel remains closed during prolonged ischaemia and only starts opening during the first few minutes of reperfusion (Griffiths and Halestrap, 1995; Halestrap and Richardson, 2014). Interestingly, it has been found that lactate accumulation during ischaemia produced a powerful inhibitory effect on the MPTP (Bernardi et al., 1992, see a review Ong et al., 2015). On the other hand, rapid correction of physiological pH during the first few minutes of reperfusion, due to washout of lactic acid as well as stimulation of the Na^+–H^+ and Na^+–HCO_3 transporters, causes the MPTP to open at this time. MPTP opening collapses the mitochondrial membrane potential and uncouples oxidative phosphorylation that result in ATP depletion. In addition, the outer mitochondrial membrane rupture and subsequent release of cytochrome C which stimulate apoptosis and the proteolytic processes that lead to cell death. This has been confirmed by a number of researchers using different methods (Di Lisa et al., 2001; Murata et al., 2001; Matsumoto-Ida et al., 2006; Heusch et al., 2010).

Inhibition of MPTP during reperfusion is the main aim for all cardioprotective manoeuvres to harness IRI. Although the exact mechanism by which the RISK and SAFE pathways inhibits the MPTP is unclear, it has been suggested to produce this effect via phosphorylation of downstream targets of PKB/Akt and ERKp44/p42 such as PKG, GSK-3β (for a review see Ong et al., 2014) and PKCε (Hausenloy et al., 2005).

1.5 The contribution of PKB/Akt, glycogen synthase3-β and the mitogen activated protein kinases (MAPK) in cardioprotection

1.5.1 PI-3K and PKB/Akt and cardioprotection:

Phosphoinositide 3-kinase (PI-3K) is a group of enzymes which act as lipid and protein kinases. Based on the specificity of their downstream substrate, mode of action and molecular structure, they have been classified into three classes (I, II, and III) (Oudit et al., 2004). Class I PI3-K has been subdivided into two subclasses, Class IA PI3-K and Class IB PI3-K, both of which are expressed in cardiomyocytes. 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). Class IB PI3-K consists of a 110γ catalytic subunit and a p101 regulatory subunit (Naga Prasad et al., 2002). Class IA PI3-Ks are activated in response to receptor tyrosine kinase pathways (Luo et al., 2005), whereas Class IB PI3-K is coupled to G protein-coupled receptors (GPCR) and has been shown to play an important role in the regulation of cardiac contractility (Nienaber et al., 2003).

It has been recognized that PKB/Akt is activated during ischaemia/reperfusion, and subsequently, it was suggested that activation of this kinase could be an important target for cardioprotective interventions to attenuate IRI (Matsui et al., 2001). For example, it has been clearly illustrated that brief activation of the β-AR with isoproterenol resulted in enhanced tyrosine kinase-associated PI3-K activity and phosphorylation of PKB/Akt and p70S6K in cardiomyocytes (Yano et al., 2008). Furthermore, PKB/Akt has an important role in manipulation of some processes such as hypertrophy and cardiomyocyte apoptosis (Oudit et al., 2004).

Although it has been shown that acute stimulation of this signalling pathway has beneficial effects, prolonged stimulation has potentially harmful effects (Franke et al., 2003). PKB/Akt could be dephosphorylated by PTEN (phosphatase and tensin homolog deleted on chromosome ten) (Leslie and Downes, 2004), a protein which is responsible for protecting normal cells against chronic stimulation of PI3-K/Akt pathway.

A number of pharmacological agents for example insulin (Jonassen et al., 2001), urocortin (Brar et al., 2001), atorvastatin and bradykinin (Bell and Yellon, 2003), have been demonstrated to be

involved in the PI3-K/Akt signalling pathway, and they have also been shown to be cardioprotective aganist IRI. Subsequently, these observations suggest that pharmacological activation of this pro-survival kinase is able to protect the heart from IRI and subsequent cell death (Hausenloy and Yellon, 2004).

Insulin or insulin-like growth factor-1 (IGF-1) signalling is mediated through tyrosine kinase phosphorylation of insulin receptor substrate -1 and -2 (IRS-1; IRS-2) which subsequently activate PI3-K. Furthermore, the platelet-derived growth factor receptors (PDGFR) and the epidermal growth factor receptors (EGFR) are also involved in activation of PI3-K (Kamohara et al., 1995). Activated PI3-Ks convert phosphatidylinositide-4,5-biphosphate (PIP2) to phosphatidylinositide-3,4,5-triphosphate (PIP3), which acts as a second messenger enhancing different downstream effectors, such as phosphatidylinositide-dependent kinase-1 (PDK1/2) which subsequently activates a variety of kinases including protein kinase B (PKB/Akt) (Burgering and Coffer, 1995). The serine / threonine kinase PKB/Akt plays an important role in the modulation of cellular growth, survival and metabolism. Its isoforms, Akt1,-2,-3, can be stimulated by activated beta-adrenergic receptors (β-ARs), adenosine, and a number of hormones and growth factors, including insulin, insulin growth factor-1, and VEGF. Activated PKB/Akt may lead to phosphorylation and negative regulation of its proapoptotic downstream targets such as glycogen synthase kinas-3-beta (GSK-3β) (Nishihara et al., 2006), the Bcl-2-associated death promoter (BAD) (Jonassen et al., 2001), Cysteine-ASPartic acid protease 9 (Caspase 9) and Forkhead box O proteins (FOXOs) (Yu et al., 2015). Furthermore, activated PKB/Akt might also lead to phosphorylation and activation of its antiapoptotic targets such as S6 ribosomal protein kinase (p70s6K) (Jonassen et al., 2001) and endothelial nitric oxide synthase (eNOS) (Bell and Yellon., 2003; Yu et al., 2015) which lead to cell survival (Fig.1.1). In addition, it is well known that activation of PKB/Akt leads to stimulation of the eNOS-NO-cGMP-PKG signalling pathway, which subsequently inhibits MPTP opening (Garlid et al., 2004) (see a recent review by Heusch et al., 2015).

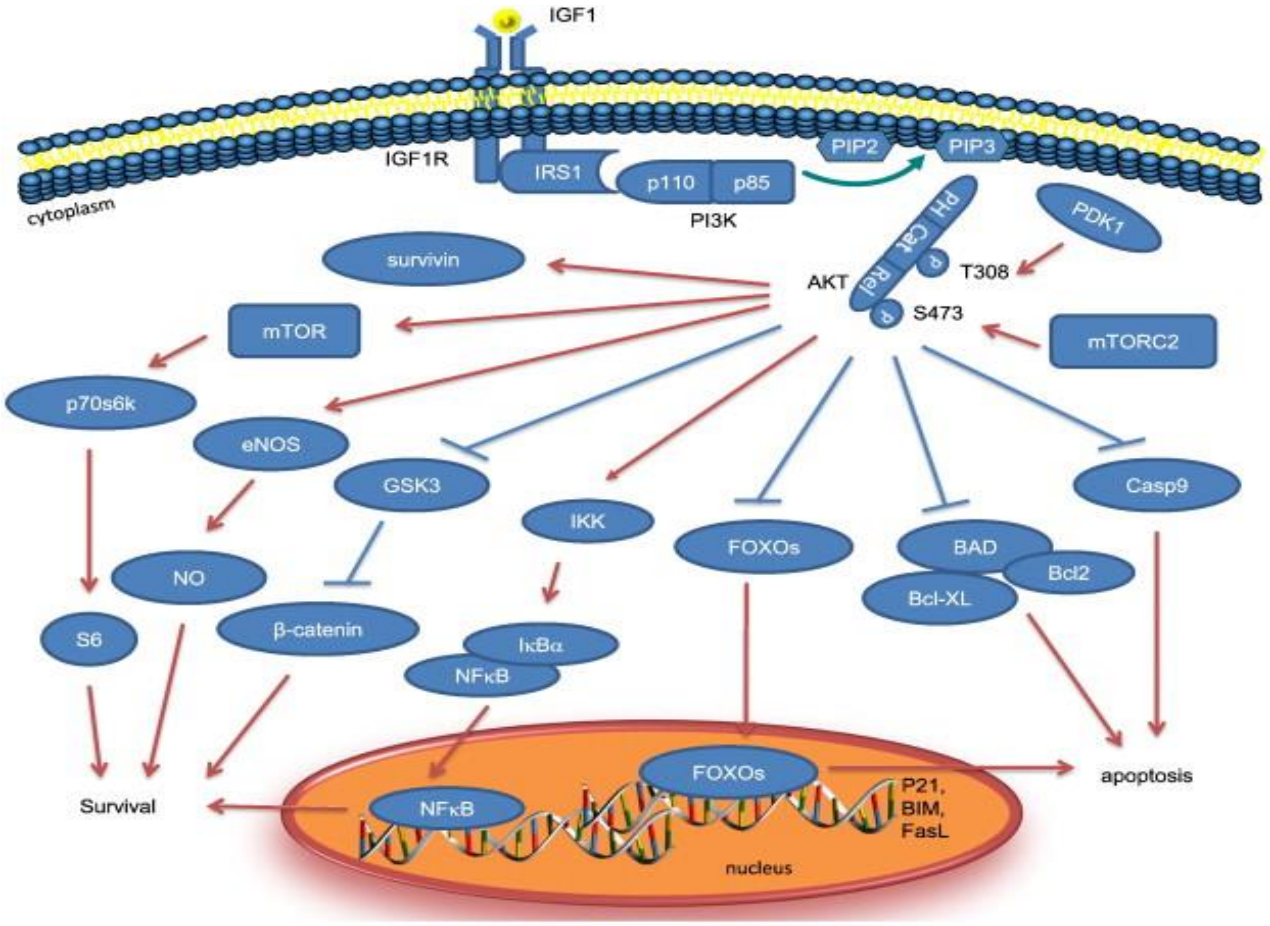


Fig. 1.1 Akt-mediated survival signalling (Yu et al., 2015)

1.5.2 Glycogen synthase kinase-3β (GSK-3β)

It is well-known that glycogen synthase kinase-3β (GSK-3β) is a downstream target of PI3-K-PKB/Akt and a host of other kinases (Juhaszova, et al, 2009; Ong et al., 2014) and it was suggested that phosphorylation and inhibition of this kinase can be linked to a diversity of cardioprotective signalling pathways (Ong et al., 2015). This has been supported by a study where IPC caused an increase in GSK-3β phosphorylation, and this increase was blocked by wortmannin, a specific PI3-kinase inhibitor (Tonget al., 2002). Therefore, GSK-3β has been recognized as an important role player in mediating the downstream signalling which converges on the MPTP (Juhaszova et al., 2004. The convergence of these pathways via inhibition of GSK-3β, on the end effector, MPTP, to

limit permeability transition, is currently regarded as the general mechanism of cardiomyocyte protection (Juhaszova et al., 2004; Hausenloy et al., 2005; see review of Heusch et al., 2015). However, the specific roles of this kinase in cardioprotection still not clear. Moreover, GSK-3β is an important target of the RISK pathway and has been linked to cardioprotection elicited by IPC as well as IpostC (Gomez et al., 2008). This was explained by the fact that phosphorylation and inactivation of this kinase allows the dephosphorylation of the outer mitochondrial membrane (OMM) and voltage-dependent anion channel (VDAC), which may enable mitochondrial depolarization, reduction of calcium accumulation and generation of ROS. This in turn, leads to inhibition of the MPTP at the time of reperfusion (Heusch et al., 2010) and prevention of cell death. Similarly, hexokinase II, a glycolytic enzyme, another mediator of PKB/Akt, has been thought to be cardioprotective by maintaining the outer mitochondrial membrane (OMM) and attenuating free radicals generation (Nederlof et al., 2014).

1.5.3 Mitogen activated protein kinases (MAPKs)

MAPKs are groups of kinases which have been identified in different species, and include: ERKs (1, 2, 3, 4 and 5), c-Jun amino-terminal kinases (JNK 1, 2 and 3) as well as p38 MAPK isoforms (α, β, γ and δ) (Chen et al., 1992; Kyriakis and Avruch., 2001).

1.5.3.1 ERK p44/p42 MAPK

ERKp44/p42 MAPK (ERK1/2) are protein-serine/threonine kinases that bind to docking sites on their targets and phosphorylate them at Ser/Thr-Pro motifs (Yang et al., 2003). They have been considered to be downstream in the Ras-Raf-MEK-ERK signal transduction pathway (Roskoski et al., 2012). This pathway has a crucial role in regulating a substantial number of processes including cell survival, metabolism, proliferation, and transcription. ERKp44/p42 are expressed in various tissues and have 83% amino acid homology (Chen at al., 1992). In addition, intense activation of these two kinases has been observed in response to osmotic stress, growth factors, cytokines, as well as some ligands of the heterotrimeric GPCRs (Lewis et al., 1998). Cell surface receptors such as tyrosine kinases and GPCRs transmit activating signalling to the Raf/MEK/ERK cascade via different isoforms of the small GTP-binding protein Ras (Neufeld et al., 2000). Upstream MEK1/2 stimulates the phosphorylation of human ERKp44/p42 at Tyr204/187 and then Thr202/185

(Roskoski et al., 2012). The phosphorylation of both tyrosine and threonine residues are essentially required for activation of ERKp44/p42.

It has been shown that ERKp44/p42 could be phosphorylated in response to the short cycles of ischaemia / reperfusion before a sustained ischaemic insult (Fryer et al., 2001). Furthermore, it was suggested that mediator factors such as adenosine, ROS, or protein kinase C (PKC) act to mediate ERKp44/p42 activation at the time of reperfusion (Hausenloy et al., 2005; Samavati et al., 2002). Activation of ERKp44/p42 was illustrated to form complexes with mitochondrial PKCε which subsequently inhibits the MPTP and elicit cardioprotection (Baines et al., 2002; 2003) (see a review of Hausenloy et al., 2005)

1.5.3.2 P38 MAPK

P38 MAPK is a class of mitogen-activated protein kinases which are responsive to stress stimuli, such as cytokines, ultraviolent irradiation, heat shock, and osmotic shock (Han and Lee, 1994). Four p38 MAP kinases have been identified, p38-α (MAPK14), β (MAPK11), γ (MAPK12 / ERK6), and δ (MAPK13 / SAPK4) (Kariakis and Avruch, 2003). A number of studies have shown that p38 MAPK activation during ischaemia exacerbates myocardial damage (Barankic et al., 2000). Its activation also aggravates cardiovascular pathologies such as cardiomyocyte remodelling, arrhythmias and cardiac failure (Wang et al., 2012; Dejong et al., 2013; Engel et al., 2006). Therefore, it was suggested that inhibition of this kinase should be beneficial and confers cardioprotection (Pompo et al., 1994; Martin et al., 2001, Marais et al., 2001). Although p38 is stimulated by and involved in lethal ischaemic injury, it has also been identified to be a crucial role player in triggering the protection of IPC, since inhibition of its activation during this phase attenuates protection (Marais et al 2001). It has been suggested that transient p38 activation during short preconditioning cycles of ischaemia might be responsible for attenuated p38 activation during subsequent lethal prolonged ischaemia (Nagarkatti and Sha'afi, 1998; Marais et al., 2001; Sanada et al., 2001).

1.5.3.3 C-Jun amino-terminal kinases (JNK)

This kinase has three different isoforms (JNK1, JNK2, and JNK3, also known as stress-activated protein kinases, SAPKα, SAPKβ, and SAPKγ respectively). The JNKs are strongly activated in response to stress stimuli such as UV irradiation, osmotic shock, and cytokines (Enslen and Davis et al., 2001). JNK kinase signaling pathways are activated to a lesser extent or not activated during ischaemia. However, they could be activated strongly during reperfusion (Barankcik et al., 1997; Knight and Buxton, 1996). Furthermore, JNK/SAPK stimulation has been demonstrated to be an important element during an IPC protocol (Ping et al., 1999; Fryer et al., 2001). This was recognized when IPC increased JNKs activities (Ping et al., 1999). Similarly, pharmacological preconditioning was also shown to activate JNKs (Barankcik et al., 1999).

1.6 Pharmacological preconditioning

Over the past two decades the increasing interest in the mechanism of cardioprotective pathways involved in ischaemic preconditioning has led to the identification of a number of pharmacological targets, capable of cardioprotection against ischaemia-reperfusion injury. Pharmacological agents that have been investigated in this regard include adenosine (Toombs et al., 1992), statins (Leferet al., 1999), erythropoietin (Wright et al., 2004), P-selectin antagonists (Kumar et al., 1999), cyclosporine (Massoudyet al., 1997) and metoprolol (Ibanez et al., 2007). Conditioning the heart with such drugs, i.e. **pharmacological preconditioning** can easily be applied clinically and would prevent the need for an invasive IPC protocol.

It has been well recognized that accumulation of the second messenger, cyclic adenosine monophosphate (cAMP), during sustained ischaemia plays an important role in mediating cardiomyocyte damage (Marks et al, 2003). Conversely, cAMP has also been shown to act as a trigger of IPC, since it was demonstrated to be significantly increased at the end of each preconditioning episode suggesting a role for the Beta-adrenergic receptor (β-AR) signalling pathways in this process (Lochner et al., 1998, 2000). Therefore, it has been suggested that pharmacological activation of this pathway might be able to provide 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 subsequent long period of ischaemia. This phenomenon was called β-preconditioning (Lochner et al 1999; Miyawaki and Ashraf., 1997; Nasa et al., 1997; Salie et al., 2011). Furthermore, it was illustrated that transient Beta 2-adrenergic receptor stimulation with formoterol (1 nM) (β2PC), followed by a 5 min washout before index ischaemia elicits cardioprotection similar to ischaemic preconditioning (Salie et al., unpublished data). Investigation and elucidation of the mechanism of β-preconditioning, opened new avenues into the specific characters of β-AR subtypes and their role in cardioprotection.

1.7 The role of the Beta-adrenergic signalling pathway in cardioprotection

1.7.1 Beta-adrenergic receptors (β-ARs)

It is now well known that cardiovascular function is regulated by three different β-adrenergic receptor subtypes, β1- , β2- and β3-AR. A fourth beta adrenergic receptor (β4-AR) has been suggested and described in human atrial and adipose tissue (Kaumann et al., 1997), (for review see Gauthier et al., 2000). Stimulation of sympathetic β-adrenergic receptors regulates a diversity of physiological processes such as cardiac contractility, carbohydrate and fat metabolism as well as cell survival and death (Zheng et al., 2005). β-adrenergic receptor blockers have been shown to play a key role in the management of cardiovascular disease by improvement of coronary circulation, protection against apoptosis and reduction of infarction (Reiken et al., 2003). Although these β -blockers mainly target the β 1- and β 2-receptors, the β3-AR has recently emerged as a potential target in the treatment of heart disease.

These receptors belong to the superfamily of GPCRs and comprise of a single 408 amino acid residue peptide chain (Fig. 1.2). It possesses seven transmembrane (TM) segments, linked with three intracellular and three extracellular loops (formed by hydrophobic stretches containing about 21–28 residues). Furthermore, it has an extracellular amino acid (N)-terminal and carboxyl (C)-terminal loop located intracellulary. In 1967, Lands et al, classified β-ARs into β1-AR and β2-AR, as cardiac/lipolytic and vascular/bronchial subtypes respectively. Subsequently, the gene encoding

the β2-AR was successfully cloned (Dixon et al., 1986). In the next several years, many GPCRs were cloned, including human β1-AR (Feielle et al., 1987) and human β3-AR, using pharmacological and cloning methods (Emorine et al., 1989).

β1- and β2-ARs functionally coexist in cardiomycytes of many mammalian species including humans (Brodde et al., 1991), with striking qualitative and quantitative differences in their functions and signalling mechanisms (Xiao et al., 2001). In the human heart the β1-AR is the predominate receptor and it is equally distributed in all parts of the heart (Brodde et al., 1991; Myslivecek et al., 2006). β1- and β2-ARS are expressed at a ratio of about 70-80 %: 30-20 % in the ventricles (Brodde et al., 1991) and 60-70 %: 40-30 % in the atria, both of which have positive inotropic and chronotropic effects. However, the β3-AR shares about 40-50 % amino acid sequence similarity with the β1-and β2-ARs.

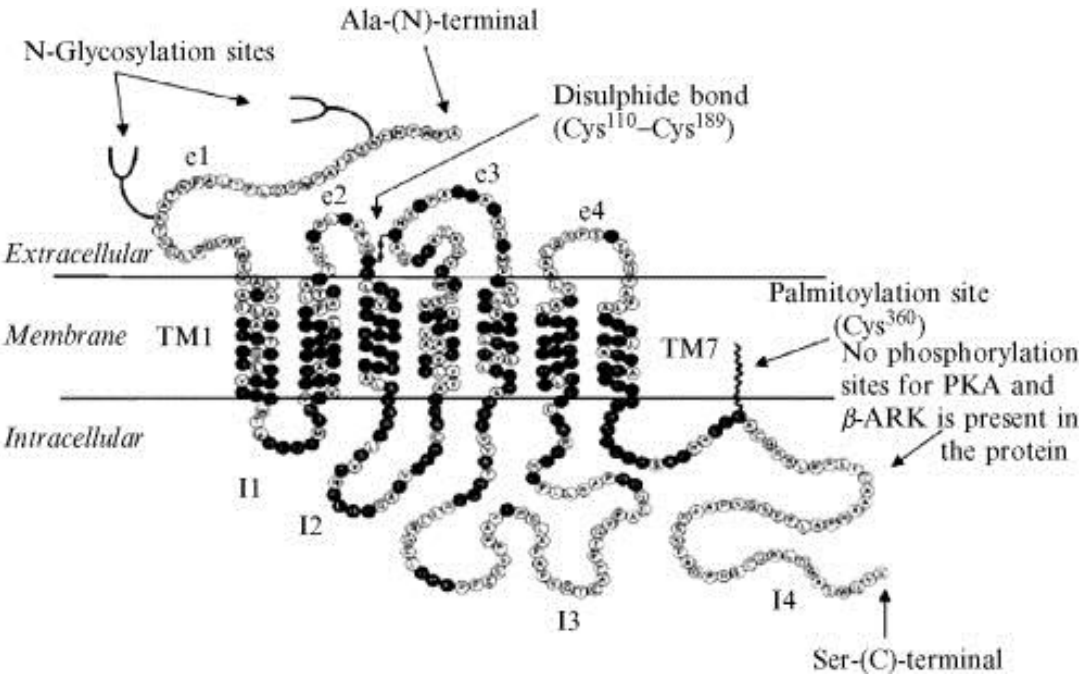


Fig.1.2 Structure of human β3-AR (Strosberg et al., 2000a)

Furthermore, the gene that encodes the β3-AR in humans has intron while the classical β1- and β2-ARs are intronless (for reviews see Perrone and Scilimati, 2010; Gauthier et al., 1996).

The β3-AR differs structurally and pharmacologically from the other two subtypes (Bylund et al., 1994). Both β1-and β2-ARs have serine and threonine residues in the intracellular C-terminus tail that act as substrate for G protein-coupled receptor kinases and for phosphorylation by the cAMP-dependent protein kinase PKA (Gauthier et al., 1996; 1998). In contrast, the β3-AR lacks a PKA phosphorylation site and has fewer serine and threonine residues in the C-terminus tail which may explain the resistance of these receptors to short term agonist–promoted desensitizationin vitro and in vivo (Strosberg et al., 1993; Liggett et al., 1993). Desensitization is a process in which the cell desensitizes itself from the continuous stimulus of catecholamines and the process includes **(i)** uncoupling of the receptors from the stimulatory guanine nucleotide-binding protein (Gs); **(ii)** sequestration of the receptors away from the cell surface; **(iii)** downregulation of cellular receptor complement. In addition, protein kinase A (PKA) activates the β-adrenergic receptor kinase (βARK), a specific protein that phosphorylates only the active, agonist-occupied form of several G-protein-coupled receptors (Benovic et al., 1988), which subsequently promotes the binding of another protein, called β-arrestin, which was suggested to be the cause of uncoupling of receptors from the Gs protein (Lohse et al., 1990).

Despite the fact that the β3-ARs have some structural differences between species, it has been shown that there is a high degree of sequence homology: approximately 80–90% between human, bovine, rodent, and canine β3-AR. Furthermore, the human, monkey, and bovine β3-ARs are more similar to each other than to the rodent, especially in the first TM segment (Perrone et al., 2010). Since the gene encoding human β3-AR was cloned in 1989 (Emorine et al., 1989), it has been subsequently identified in several species, including rat, mouse (Strosberg and Pietri-Rouxel et al., 1996), bovine, monkey, dog (Strosberg et al., 1997), sheep and goat (Forrest and Hickford, 2000). Moreover, the β3-AR subtype was recognized in a variety of tissues, including the coronary vascular bed (Strosberg et al., 1997), mammalian heart (Gauthier et al., 1998; Kaumann et al., 1997), bladder, uterus, colon, pancreas, and central nervous system (Emorine et al., 1994; Grazia and Scilimati, 2010). In addition, it was also recognized in adipose tissue and skeletal muscle, where its activation promotes lipolysis and energy expenditure (Arch J R., 2002; Emorine et al., 1994). The β3-AR subtype can be considered a drug target for the treatment of different pathologies such as obesity, type 2 diabetes, cachexia, metabolic syndrome, heart failure, anxiety

and depressive disorders, preterm labor, overactive bladder, control of colon motility, and as coadjuvants in colon cancer therapy (Perrone and Scilimati, 2010). Despite the fact that several agonists and antagonists have been used in the study of the β3-AR, the investigation of its precise function and regulation has been hampered by the lack of highly specific agonists/antagonists (Vrydag et al., 2007).

The β3-AR is pharmacologically characterized by the following set of criteria: **(i)** it is potentially and selectively stimulated by agonists such as BRL37344 and SR58611A, which have a low affinity for other β1- and β2-ARs (Arch and Kaumann, 1993; Emorine et al., 1994); **(ii)** it is partially activated by several β1- and/or β2-AR antagonists, such as cyanopindolol, bucindolol and CGP 12177A (Blin et al., 1993); **(iii)** it is weakly antagonized by conventional β-AR antagonists such as propranolol and nadolol and **(iv)** the β3-AR is selectively antagonized by nonselective β-AR antagonists such as bupranolol (Langin et al., 1991) and the selective β3-AR antagonist SR 59230A (Kaumann and Molenaar, 1996; Arch, 2002).

1.7.1.1 β3-AR agonists

Two different classes of β3-AR agonists have been described, the first class includes the **phenyl-ethanolamines**, such as BRL37344, SR58611A, and CL316243; however, BRL37344 and SR58611A also have a low affinity for β1- and β2-AR stimulation. The second class consists of the **aryloxypropanolamines**, including CGP12177A and cyanopindolol, in addition to L-755507, which activate cloned human and rhesus monkey β3-ARs much more potently than the other two classical β1- and β2-ARs (Ursino et al., 2009). Interestingly, a study by Gauthier et al., 1996, using human endomyocardial biopsies obtained from the right interventricular septum of cardiac transplant and open-heart surgery patients, demonstrated that β3-AR stimulation by some selective agonists in the presence of nadolol, a β1- and β2- adrenergic receptor antagonist, resulted in negative inotropic effects with an order of potency: BRL37344> SR58611A≈ CL316243>CGP12177A. Notably, BRL37344 induced a dose-dependent negative inotropic effect at concentrations ranging from 0.1 nM to 1 mM (Gauthier et al., 1996); similarly, SR58611A was also observed to have a dose-dependent negative inotropic effect. However, a previous in vivo study using dogs, clearly demonstrated that β3-AR agonists, BRL37344 and CGP12177A, when

administered intravenously, produced a positive chronotropic effect (Tavernier et al, 1992). Since these effects were not observed in denervated animals (Shen et al., 1994), it was concluded that the positive chronotropic effect was a result of a baroreceptor reflex in response to the sudden drop in blood pressure mediated by the vasodilatory effects of β3-AR agonists (Weeldon et al., 1993 and 1994).

Currently, a substantial number of scientific papers have been published, describing nebivolol, not only as a third generation selective antagonist of β1-ARs, but also as a β3-AR agonist, in the heart as well as some vasculature systems (Ferro et al., 2006; Quanget al., 2009 and Aragon et al., 2011). Nebivolol has been considered to be a highly cardioselective β1-AR blocker, and it has no antagonistic activity for alpha-adrenergic receptors (α-ARs) (Janssen et al., 1989). Furthermore, it possesses vasodilation effects mediated by NO dependent pathways. In 1994, Bowman et al was the first to provide strong evidence that the vasodilatory effect of nebivolol was mediated by NO. Infusion of nebivolol into the dorsal hand veins of healthy men previously injected with phenylephrine, produced vasorelaxation of veins and this effect was completely abolished by L-NMMA, a NO-synthase inhibitor. Since then, a substantial number of studies confirmed Bowman's hypothesis (Cockcroft et al., 1995; Altwegg et al., 2000). Therefore, nebivolol was clinically licensed to be used in the treatment of essential hypertension (Ferro et al., 2006). In addition to its ability to cause peripheral vasodilation, nebivolol has recently been shown to improve cardiac dysfunction by enhancing the ejection fraction (EF %) after its administration during reperfusion for 4 weeks (Sorrentino et al., 2011). In addition, it has been shown that nebivolol, both in human myocardium and coronary microcirculation, induces NO-dependent negative inotropic effects and vasodilatation through activating β3-ARs (Dessy et al., 2005; Gauthier et al., 2011)

1.7.1.2 β3-AR antagonists

Although the β3-ARs could be blocked with very weak affinity by typical β-AR antagonists, they are particularly blocked by β3-AR antagonists such as SR59230A which is characteristically a selective antagonist of the rodent β3-AR; however, this compound has no selective antagonist activity against the human β3-AR (Gauthier et al., 2011). On the other hand, two more selective antagonists, L-748328 and L-748337of human cloned β3-AR have been described and it has been shown to have a low affinity for rodent β3-ARs (Candelore et al., 1999). Therefore, in the study of

β3 adrenergic receptors, the selectivity of antagonists and the types of animal models or the species should be taken into account.

1.7.2 The classical/ traditional view of β-AR signalling in the heart

The dual coupling to Gi and Gs proteins of β2-and β3-AR but not β1-AR

Despite many similarities, β1-AR and β2-AR couple to distinct signal transduction pathways to elicit different cellular responses with regard to the G protein. In other words, the β1-AR subtype couples exclusively to Gs protein, which activates the Gs–AC–cAMP–PKA pathway. This in turn, phosphorylates target proteins involved in metabolic regulation, growth control, muscle contraction as well as cell survival or death (Zheng et al., 2005). In contrast, β2-AR couples to both Gs and Gi proteins giving rise to functionally opposing pathways. Coupling of β2-ARs to Gi proteins activates the Gi–GβY–-PI3K-Akt pathway, which in turn, leads to functional localization and inhibition of the Gs-AC-cAMP-PKA signalling and protects the cardiomyocytes against apoptosis (Zheng et al., 2005). Subsequently, it was also shown that the β3-AR couples to both Gs and Gi proteins (Zheng et al., 2005; Sato et al., 2007) and possesses the same classical intracellular signalling pathways as β1- and β2-ARs (Fig.1.3).

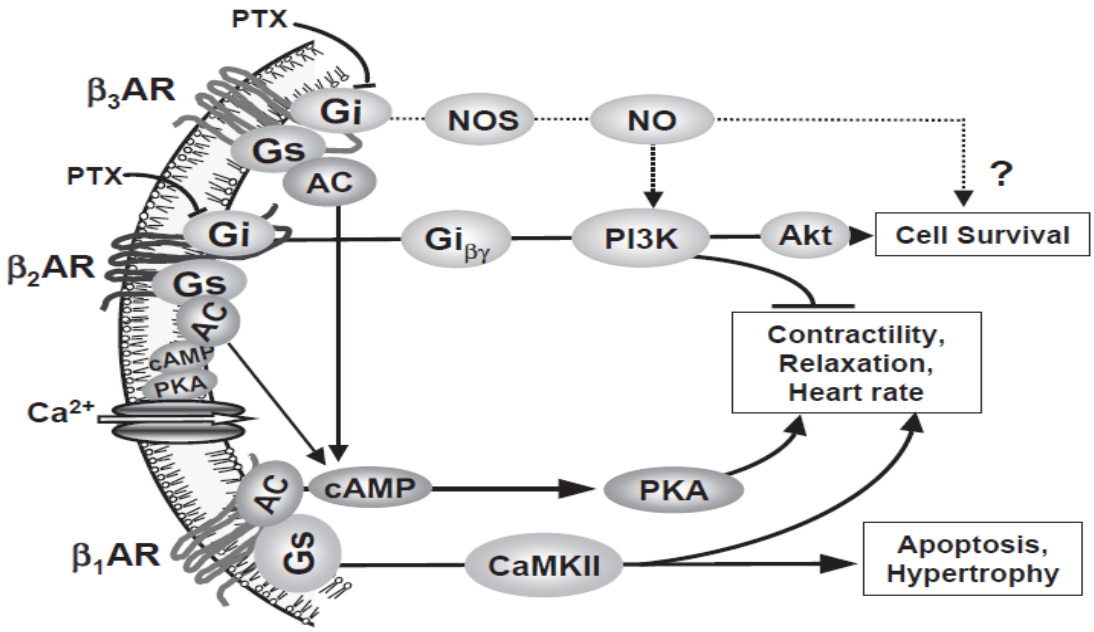


Fig.1.3 signalling pathways of cardiac β-ARs (Zheng et al., 2005)

In the heart, the beta adrenergic pathways are the primary means of increasing cardiac performance in response to acute or chronic stress. However, depending on the tissue type, β3-AR stimulation leads to either opposite or comparable functional effects to those produced by stimulation of β1- and β2-ARs. The β3-AR is activated at higher concentrations of catecholamines than the β1- and β2-adrenergic receptors and once activated; the receptor would deliver a more sustained intracellular signal (Lafontan et al., 1994). This may suggest that following prolonged activation by the sympathetic nervous system as in the case of prolonged ischaemia, the β3-AR-mediated response might be preserved, whereas the β1- and β2-adrenergic mediated responses are diminished (Gauthier et al.,2000).

1.7.3 Intracellular signalling pathways of β3-AR stimulation in cardiovascular tissue

β3-AR and its downstream signalling pathways have been recognized as novel modulators of heart function. Despite the fact that most of the available information about the β3-ARs signalling has been obtained in studies of adipocytes, recent studies revealed that these signalling pathways could be consistently generated in cardiac tissues (Gauthier et al., 2000). Therefore, increasing evidence puts the spotlight on the involvement of β3-ARs, via eNOS, in the production of cyclic 3,

5, guanosine monophosphate (cGMP) (Dessy and Balligand, 2010). A previous study by Gauthier et al., 2000 has concluded that concurrent activation of cAMP-dependent positive (β1- and β2-ARs) and NO-dependent negative (β3-ARs) inotropic pathways within the same cardiomyocyte would provide an integration-point between the adrenergic receptors-mediated stimulation of cardiac contraction. According to this hypothesis, the β3-ARs-mediated pathway would function as a countervailing 'rescue' mechanism preventing cardiomyocyte damage from excessive stimulation by β1- and β2-ARs (Gauthier et al., 2000). To summarize, β3-ARs are stimulated at high catecholamine concentrations and induce negative inotropic effects, serving as a "brake" to protect the heart from catecholamine overstimulation (Niu et al., 2012).

It has been demonstrated that overexpression of β3-AR in human cardiomyocytes is sufficient to stimulate the signalling transduction pathway that ultimately, via increased cGMP and NO production, induces negative inotropic effects (Tavernier et al., 2003). NO exerts many of its effects through its target molecule, soluble guanylyl cyclase (sGC). The activated form of (sGC) converts the guanosine triphosphate (GTP) to cyclic 3, 5, guanosine monophosphate (cGMP), the common downstream second messenger of natriuretic peptides and nitric oxide. cGMP, in turn, exerts its physiological effect in cardiomyocytes, through the activation of protein kinase G (PKG) signalling, and the activation and/ or inhibition of phosphodiesterases (PDEs) (Boris M., 2012).
It was suggested that the eNOS-NO-GC-cGMP-PKG-dependent signalling pathway plays an important role in attenuating hypertrophic remodelling, through the inhibition of the Ca^{2+} dependent signalling pathways (Hammond and Balligand, 2012). Thefore, cGMP signals would be a promising target for manipulating cardiovascular pathologies. Furthermore, it was also illustrated that an increase in NO formation through modulation of eNOS activity after the application of BRL37344 is specifically coupled to stimulation of the cardiac β_3-AR. In contrast, activation of eNOS, after stimulation of β3-AR in $\beta3^{-/-}$ mice with the selective agonist BRL37344, was completely absent (Gauthier et al., 1998).

In human atria, β3-AR stimulation has been illustrated to produce positive inotropic effects by the phosphorylation of calcium channels and increase of intracellular Ca^{2+} (Skeberdis et al., 2008), suggesting that the β_3-AR may be coupled to the stimulatory Gs protein as well. However, this

receptor is also capable of exerting negative inotropic effects in human ventricles by coupling to the inhibitory Gi protein and stimulating NO dependent signalling pathways (Gauthier et al., 1996; 1998).

Recently, a study by Aragon et al (2011) has showed that administration of nebivolol or BRL37344 at the time of reperfusion caused a significant reduction in infarct size of mice subjected to 45 min of myocardial ischaemia. They also demonstrated that these protective effects were mediated by rapid activation of eNOS and nNOS and increased production of NO and its metabolites. Subsequently, a more recent study investigated the mechanisms involved in the cardioprotective effects of β3-AR in mice as well as pigs and found that pre-reperfusion administration of BRL37344 decreased infarct size and improved long-term LV contractile function in both animal models (Garcia-Prieto et al., 2014). Using isolated cardiomyocytes exposed to hypoxia/reoxygenation, these workers also showed that selective β3-AR stimulation is associated with an increase in PKB/Akt phosphorylation and NO formation, thus exerting an Akt-eNOS-NO dependent effect in cardiomyocytes which ultimately induced a delay in the opening of the MPTP and thus enhanced cell viability. Therefore, it could be concluded that activation of cardiac β3-AR elicits protection via inhibition of MPTP, through a PKB/Akt-NOS-NO-cGMP-PKG dependent signalling pathway.

1.8 Nitric oxide

Intensive research over several years identified the so-called endothelium-derived relaxing factor (EDRF) indeed as nitric oxide (NO). It was subsequently recognized as an essential signalling molecule in the cardiovascular and nervous system. In the cardiovascular system endothelial cells are the main source of NO production, with the platelets contributing to a lesser extent. Its main function is to cause vasodilatation, inhibit platelet aggregation as well as vascular smooth muscle cell (VSMC) proliferation. In mammalian cells it is synthesized from the amino acid L-arginine by oxidation of one of the terminal guanidine nitrogen atoms to produce L-citrulline and NO (Palmer et al., 1988). This reaction is catalyzed by the enzyme NO synthase (NOS). Three main isoforms of this enzyme have been identified thus far. **Endothelial nitric oxide synthase (eNOS)** was first purified and cloned from vascular endothelial cells (Lamas et al., 1992) and platelets (Mehta et al., 1995). **Neuronal nitric oxide synthase** (**nNOS)** although first identified in both central and

peripheral nervous tissues (Papapetropoulos et al., 1999), it has been detected also outside the nervous system. **Inducible nitric oxide synthase (iNOS)** is an inducible form of the enzyme, and found in vascular smooth muscle cells (VSMCs) (Beasley et al., 1991). The latter isoform binds to calmodulin even in the presence of low concentrations of Ca2+ (Lincoln et al., 1997) and its activation is independent of intracellular Ca2+ levels. Both eNOS and nNOS are constitutively activated, and their activation is dependent upon the binding of calmodulin via increased intracellular Ca2+ levels (Forstermann et al., 1991., Lincoln et al., 1997). However, eNOS can also be activated independently of Ca2+ elevation, by phosphorylation of various serine residues of some of protein kinases (Dimmeler et al., 1999) (for review see Queen and Ferro, 2006). The activity of eNOS is modulated by phosphorylation of the following sites: serine 1177 (phospho-eNOS Ser1177), threonine 497 (phospho-eNOS Thr497), serine 633 (phospho-eNOS Ser633) and serine 114 (phospho-eNOS Ser114) (Kolluru et al., 2010). Phosphorylation at Ser 1177 activates eNOS, whereas phosphorylation at Ser 114 and Thr 497 inhibits its activity (Fleming et al., 2003; Mount et al., 2007).

Convincing evidence exists regarding a link between activation of β3-ARs and NO generation (Gauthier et al., 1996; Tavenier et al., 2003; Queen et al., 2006; Niu et al., 2012; Aragon et al., 2011). For example, it has been shown that β3-AR in human hearts stimulated the production of NO through the activation of eNOS present in ventricular myocytes as well as in vascular endothelial cells (Gauthier et al., 1998; Brunner et al., 2001).

β3-ARs-eNOS coupling was first illustrated by Pott et al (2003) who tested the effect of the β3-AR agonist (BRL37344) on human atria. A subsequent study by the same group revealed that β3-ARs-eNOS coupling was mediated by the PI3-K/Akt pathway and modulated by phosphorylation at the serine 1177 and serine 114 residues (Brixius et al., 2004). Recently, studies have shown that stimulation of β_3-AR, leads to production of NO also via nNOS in diabetic and aged rat hearts (Birenbaum et al., 2008). These studies suggest that although β3-AR's functional significance may not be apparent in healthy subjects, it has the capability to signal through nNOS and change the contractile response. However it should be borne in mind that the β3-AR-agonist BRL37344 is

selective for rodents, therefore, extrapolating these findings to humans was restricted (Queen et al., 2006).

Over the past decade, it has been shown that the plasma level of NO metabolites, nitrite and nitrosothiols, increase during exercise in both rodents and humans (Brown et al., 2005; Zhang et al., 2006). Therefore, some studies have emerged suggesting that exercise training elicits sustainable cardioprotection against IRI in animal as well as after acute myocardial infarction in humans (Calvert et al., 2011; Lennon et al., 2004; Brown et al., 2005). Subsequently, it was shown that voluntary exercise reduces myocardial injury in mice following a 4 week training period and that these protective effects can be sustained for at least 1 week after cessation of training. These cardioprotective effects of exercise were mediated by changes in the phosphorylation status of eNOS which, in turn, were mediated by β3-AR stimulation (Lefer et al., 2011).

The cardiac effects of β3-stimulated release of NO have been shown to be attenuated by the non-specific NOS inhibitor L-NAME (Nappet al. 2009). Similarly, the protective effects of nebivolol were significantly inhibited by the selective β3-AR blocker (SR59230A) and NOS inhibitor L-NAME (Zhang et al., 2014), indicating that cardioprotective effects of β3-AR are mediated through a NOS-NO dependent pathway. In addition, it was observed that nNOS was overexpressed in failing hearts after AMI, suggesting that nNOS might also be contributing to β3-AR induced cardiac regulation (Niu et al., 2012).

A recent in vivo study demonstrated that selective stimulation of β3-AR with BRL37344 prevented cardiac dysfunction, significantly attenuated the apoptotic effects of IRI, and inhibited fibrosis via activation of eNOS and nNOS, which contributed to the enhanced cardiac function after AMI (Niu et al., 2014). In this study, C57BL6/J mice were used and MI was performed by ligation of left anterior descending (LAD) coronary artery via left thoracotomy under anesthesia with 2% isoflurane. Animals were divided into four groups, sham group, MI group, and the last two groups were treated with BRL37344 (MI+BRL37344 group) or SR59230A (MI+SR59230A group) respectively at 0.1 mg/kg/hour for one day, and the mice were sacrificed 4 weeks after the operation. The sham group underwent the same operation but suture under left coronary artery was not tied. The expression of β3-AR was significantly increased in the MI group as well as

MI+BRL37344 treated group. Interestingly, no changes were noted in the expression of cardiac β1-AR and β2-AR (Niu et al., 2014). This study also illustrated that BRL37344 treatment increased the expression of phospho-eNOS Ser1177, and decreased the level of phospho-eNOS Thr497, indicating significant activation of eNOS. Moreover, it significantly increased expression of nNOS and phospho-nNOS, while having no effect on iNOS expression.

Similarly, a concurrent in vivo study provided supportive evidence that nebivolol decreased cardiomyocyte apoptosis, improved cardiac function after IR via activation of β3-AR-nNOS and eNOS dependent pathways and enhanced cardiac β3-AR expression after MI (Zhang et al., 2014). Interestingly, the protective effects of nebivolol were inhibited by β3-AR antagonist SR59230A and NOS inhibitor L-NAME. C57BL6/J mice were also used in this study and divided into 5 groups, sham, MI, MI+nebivolol, MI+nebivolol+SR59230A, and MI+nebivolol+L-NAME. All the above drugs were administered for 4 weeks after the MI procedure respectively. Moreover, the expression ratio of β3-AR to β-actin in nebivolol treated group (MI+nebivolol) was significantly increased although it was significantly decreased in the MI group compared with the sham group (Zhang et al., 2014). Taken together, these data demonstrated that the cardioprotective effects of BRL37344 as well as nebivolol are mediated by β3-AR stimulation, and that both eNOS and nNOS are involved in the release of NO after β3-AR activation (Birixius et al., 2006). The participation of nNOS in cardioprotection was further confirmed by Aragon and coworkers (2011) who reported that the nebivolol-induced cardioprotection was associated with increased expression of nNOS, while the drug failed to reduce infarct size in nNOS knockout mice. Confirmatory evidence is still needed to prove which NOS isoform is the principal factor in β3-AR mediated cardioprotection. Therefore further studies, revealing the specific signalling mechanisms by which β3-AR produces its effects in the myocardium are needed before clinical application in MI.

Hypothesis

Regarding the role of the β3-AR in MI, the exact physiological and pathophysiological roles of the β3-AR remain to be elucidated and have to be defined more accurately by selective manipulation of this receptor. While most recent studies (Aragon et al., 2011; Zhang et al., 2014; Niu et al., 2014; and Garcia-Prieto et al 2014) support a significant role for β3-AR in modulation of reperfusion injury after AMI, we **hypothesize** that selective β3-AR stimulation during ischaemia / reperfusion may be cardioprotective, whereas selective β3-AR inhibition may prove useful in the end stages of sustained ischaemia and early reperfusion. Therefore, in this study we intended to investigate the possible cardioprotective effects of β3-AR modulation prior to; at the end of sustained ischaemia and / or early reperfusion.

Research Aims

In view of the above, the aims of this study were to explore the mechanisms involved in the cardioprotective response of β3-AR modulation. The following aspects were investigated:

1. The cardioprotective effects of β3-AR activation before the onset of sustained ischaemia (pre-treatment) **(PT)** by application of a β3-AR agonist or antagonist before regional ischaemia followed by reperfusion. Functional recovery and infarct size were assessed.

2. The cardioprotective effects of β3-AR activation during ischaemia and early reperfusion. The β3-AR agonist or antagonist was administered during last 10 min regional ischaemia (per-treatment) **(PerT)** and /or during the first 10 minutes of reperfusion (post-treatment) **(PostT)**. Functional recovery and infarct size were assessed.

3. Investigation of the role of NOS in β3-AR mediated cardioprotection before regional ischaemia (pre-treatment) and during early reperfusion (post-treatment), using the NOS inhibitor, L-NAME. Functional recovery and infarct size were assessed.

4. Evaluation of the effects of β3-AR modulation on intracellular signalling pathways (i.e. extracellular signal-regulated kinase (ERKp44/p42), phosphoinositide-3-kinase (PI3K) /protein kinase B (PKB/Akt), glycogen synthase kinase-3β (GSK-3β) and endothelial nitric oxide synthase (eNOS) by Western Blot analysis.

2. Materials and methods

2.1 Animals

Male Wistar rats weighing 250 to 350 g were used in this study. The rats were obtained from the University of Stellenbosch Central Research Facility, and they had free access to food and water prior to experimentation. The project was approved by the Ethics committee of the University of Stellenbosch (Faculty of Health Sciences), ethical approval number: P08/05/009. The animals were treated according to the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

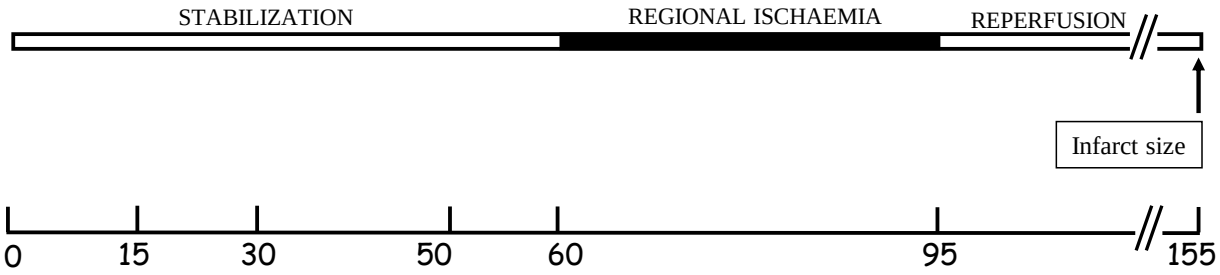
2.2 Perfusion Technique

The rats were euthanized (30 mg pentobarbital/rat) by intraperitoneal injection. The hearts were rapidly excised and arrested in ice-cold (4°C) Krebs-Henseleit buffer, containing in mM: NaCl 119; $NaHCO_3$ 25; KCl 4.75; KH_2PO_4 1.2; $MgSO_4$ 0.6; Na_2SO_4 0.6; $CaCl_2$ 1.25; glucose 10; pH 7.4. The buffer was gassed with 95 % O_2 and 5 % CO_2 prior to and during the perfusion protocol. After removal, the hearts were mounted on the aortic cannula of the Neely-Morgan perfusion system and perfused retrogradely in a non-recirculating manner, at 100 cm H_2O for 15 minutes. During this time the left atrium was cannulated to allow atrial perfusion at a preload of 15 cm H_2O. After 15 min of retrograde perfusion, the mode of perfusion was changed to the working heart mode for 15 minutes, the left ventricle ejecting against a hydrostatic pressure of 100 cm H_2O (afterload). Subsequent to this stabilization period of 30 minutes, the experiments were continued according to the protocols discussed later. The myocardial temperature was monitored by insertion of a temperature probe in the coronary sinus and maintained throughout the experiment at 37 °C. 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.

2.2.1 Regional ischaemia

Regional ischaemia was applied by inserting a silk suture underneath and around the proximal left anterior descending (LAD) coronary artery. 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 and the heart was surrounded by a water-jacketed chamber to maintain the myocardial temperature at 36.5^{o}C. Reperfusion was initiated by releasing the ligation and restoration of coronary flow to the ischaemic area. Hearts were reperfused retrogradely for 10 minutes, followed by perfusion in the working mode for 20 minutes during which time the functional recovery was measured twice. This was followed by retrograde perfusion for another 30 minutes, for measurement of infarct size (as described below).

Basic perfusion Protocol



2.2.2 End - points of ischaemic damage

2.2.2.1 Myocardial Function

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

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

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

Where

PAO = aortic pressure and CO = cardiac output (Qa+ Qe)

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

2.2.2.2 Determination of infarct size

At the completion of regional ischaemia and reperfusion, the silk suture around the LAD coronary artery was permanently tied and a 0.25% Evans blue solution infused into the heart to outline viable tissues. Hearts were removed, frozen, cut into 1-2 mm thick transverse tissue segments and incubated in 1 % triphenyltetrazolium chloride (TTC) in phosphate buffer, pH 7.4 for 10-15 minutes. Damaged tissues take on a deep red coloration, resulting from the reaction of cardiac lactate dehydrogenase enzymes with tetrazolium which was subsequently reduced to red TPF (1,3,5-triphenylformazan) to form a red formazan / crystalline deposit. Infarcted tissues (areas of necrosis) are not stained and have a white colour, since these enzymes have been either denatured or degraded. The reaction with TTC was stopped by placing the tissue segments in 10 % formalin. Tissue segments were then 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 Centre at San Antonio, Texas). The infarct size was expressed as a percentage of the area at risk (I/R %).

2.2.2.3 Western Blotting

The left ventricle of each heart was removed and snap-frozen at the time intervals indicated in the protocol section. Immunoblotting and detection of total as well as phosphorylated proteins, total ERK p44 / p42 MAP Kinase, phospho-ERKp44/p42 (T202/Y204); total PKB/Akt, phospho-PKB/Akt (Ser-473); total GSK-3β, phospho-GSK-3β (S9); and total eNOS, phospho-eNOS(Ser-1177), using appropriate antibodies from Cell Signalling Technology.

2.2.2.3.1 Preparation of lysate

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 phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml Leupeptin, 10 µg/ml Aprotinin, 1 % Triton. $NaVO_3$ was prepared weekly; PMSF 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 minutes, 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 with sample and lysis buffer after which samples were boiled for 5 min and stored at – 80^{o}C.

2.2.2.3.2 Western Immunoblot Analysis

Samples were subjected to electrophoresis on a 12 % or 7.5 % polyacrylamide gel (SDS –PAGE), depending on the size of the protein of interest, using the standard BIO-RAD Mini Protean III system. The separated proteins were transferred to an Immobilon membrane (Millipore) (Billerica, MA, USA: Polyvinylidenedifluoride (PVDF) membrane), using the Trans-Blot®Turbo™ Transfer system. 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 (5g) in a 100 ml TBS-Tween (Tris-buffered saline + 0.1 % Tween 20) for 1-2 hours at room temperature and incubated overnight with the primary antibodies (Cell Signalling Technology, Massachusetts, USA) that recognize total or phosphorylated proteins: PKB/Akt, ERKp44/p42, GSK-3β and eNOS. The membranes were washed with TBS-T (3x5 min) and then incubated with a diluted horseradish peroxidase-labelled secondary antibody (Amersham Life

Science, Buckinghamshire, UK). After thorough washing with TBS-T, membranes were covered with Enhanced chemiluminescence ECL detective reagent for one minute and briefly exposed with the Chemidoc MP Imager System with Image lab 5. Blots were normalized with the Ponceaued membranes using Bio-Rad Image lab.5 in Chemi Doc system, to determine equal loading. Data were standardized against the negative controls, which were freeze clamped hearts after 30 minutes retrograde perfusion, to obtain fold increases of kinase activation. Positive controls, which were cardiac microvascular endothelial cells treated with 500 nM Okadaic acid for 30 minutes, were used only with eNOS evaluation.

2. 3 Drugs

Chemicals used in this study were: selective β3-AR agonist, **BRL37344 (1 µM)**; selective β3-AR antagonist **SR59230A (0.1 µM)** and nitric oxide synthase inhibitor, Nitro-L-arginine methyl ester hydrochloride, **L-NAME (50µM).** BRL37344 and SR59230A were dissolved in dimethylsulfoxide **(DMSO)** and the concentration of the DMSO in KREBS buffer was 0.008% and 0.0008 % respectively.

2.4 Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). For multiple comparisons one or two-way analysis of variance (ANOVA) was utilised (Graph Pad PrismPlus Version 5.0). Post-hoc testing for differences between selected groups was done using Bonferroni's method. A p-value of < 0.05 was considered significant.

2.5 Experimental protocols (infarct size protocols)

2.5.1 Investigating the effect of pretreatment (PT) with the β3-AR agonist, BRL37344 or antagonist, SR 59230A on infarct size and functional recovery

Non-Pretreatment protocol (NPT): hearts were stabilized for 60 minutes and subjected to 35 minutes regional ischaemia (RI), followed by 60 minutes reperfusion (n=6/group) (Fig.2.1).

Pre-treatment (PT): hearts were stabilized for 50 minutes, thereafter pre-treated with either a β3-AR agonist BRL37344 or antagonist SR5923OA for 10 minutes after which the hearts were subjected to 35 minutes regional ischaemia RI and 60 minutes reperfusion (n=6 hearts for each drug) (Fig.2.2). Haemodynamic parameters were recorded at the end of the 15 minutes working heart mode, prior to regional ischaemia, and compared with haemodynamic parameters after 20 and 30 min reperfusion. Infarct size was assessed at the end of 1h reperfusion.

Fig. 2.1 Non-Pretreatment

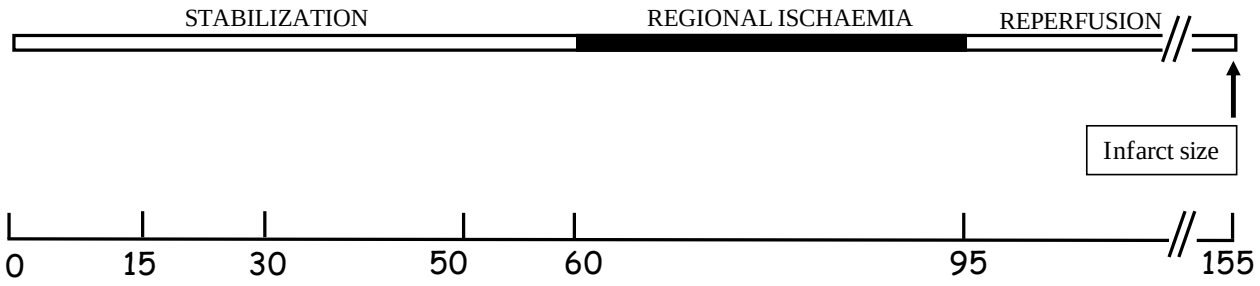
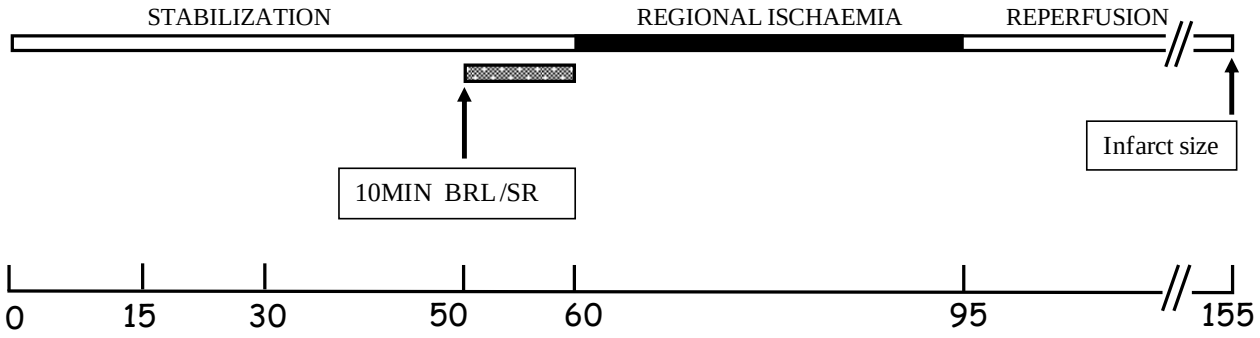


Fig. 2.2 BRL37344/SR59230APre-treatment



2.5.2 Investigation of the effect of β3-AR stimulation or inhibition during the last 10 minutes of regional ischaemia (Per-treatment/PerT) and the first 10 minutes of reperfusion (Post-treatment/PostT)

After the stabilization period, hearts were exposed to 35 minutes regional ischaemia. The β3-AR agonist BRL37344 or antagonist SR5923OA was administered during the last 10 minutes of regional ischaemia **(PerT)** (Fig.2.3). In another set of experiments hearts were exposed to BRL37344 or SR5923OA for 20 min, during the last 10 minutes of RI as well as the first 10 minutes of reperfusion **(PerT + PostT)**, (n=6 hearts for each drug) (Fig.2.4). Measurements were made as described above (2.5.1)

Fig. 2.3 BRL37344/SR59230A Per-treatment

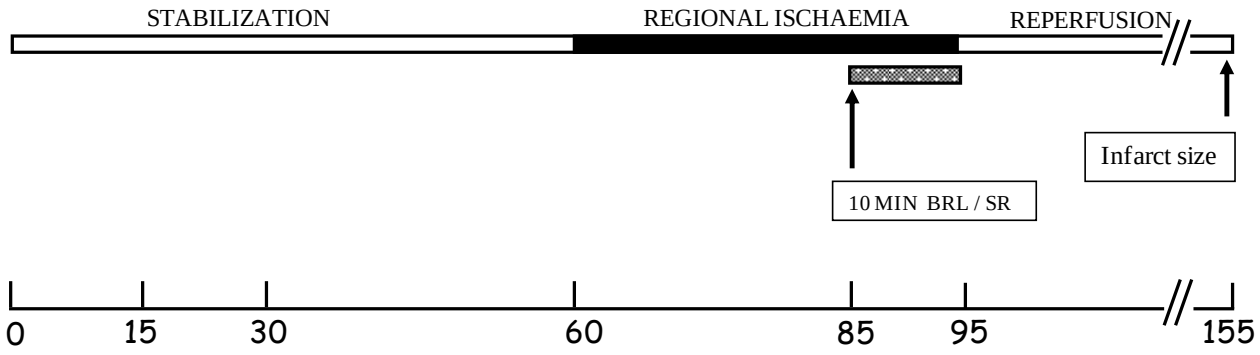
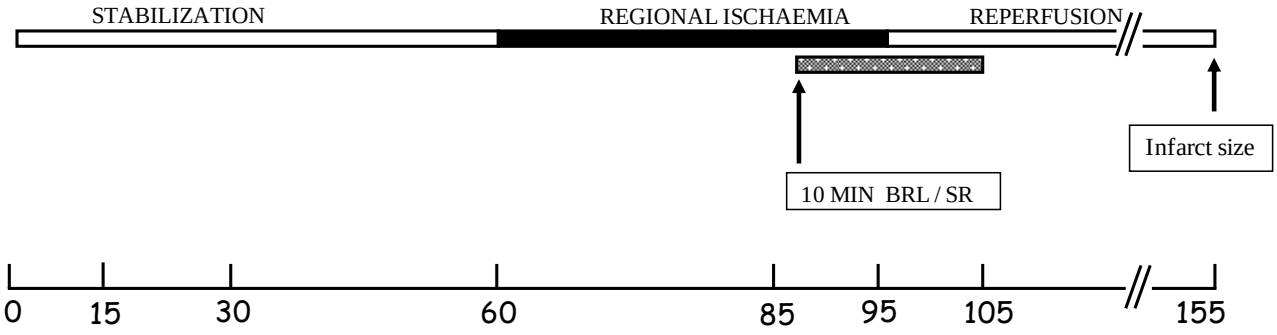


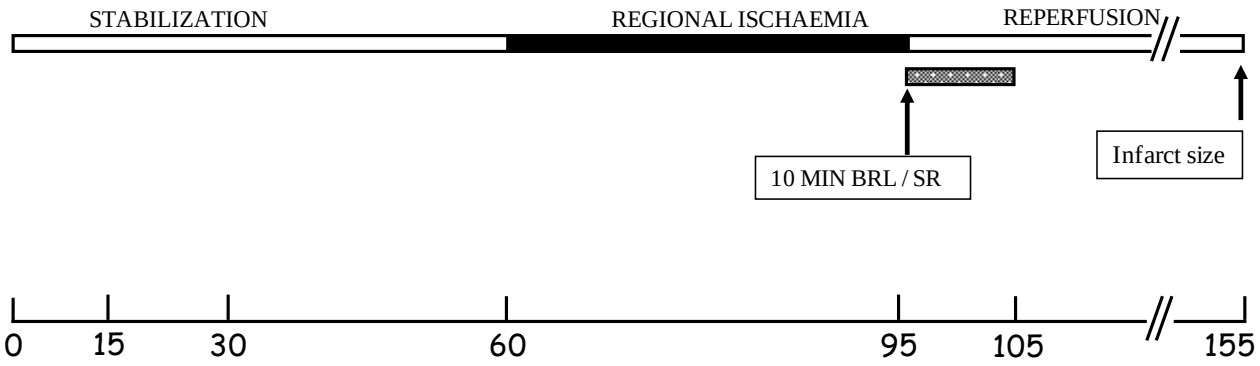
Fig. 2.4 BRL37344/SR 59230A Per-treatment and Post-treatment



2.5.3 Investigation of the effect of the β3-AR agonist, BRL37344 or antagonist, SR5923OA at the beginning of reperfusion (post-treatment)

Hearts in this group were stabilized for 60 minutes, subjected to 35minutes regional ischaemia (RI) and BRL37344 or SR5923OA administered during the first 10 minutes of reperfusion (n=6 hearts for each drug) (Fig.2.5). Measurements were made as described in 2.5.1.

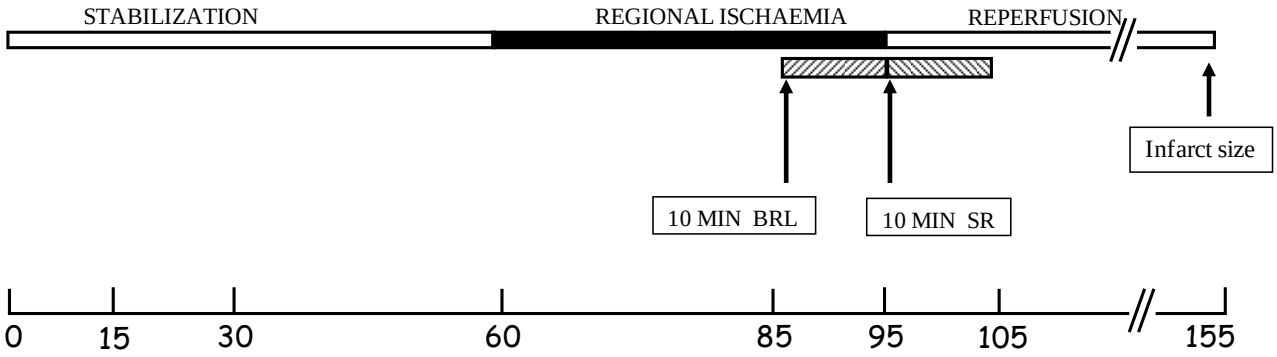
Fig. 2.5 BRL37344/SR59230A Post-treatment



2.5.4 Investigation of the effects β3-AR activation with the agonist BRL37344 during ischaemia and subsequent inhibition with the antagonist SR5923OA during reperfusion

The BRL37344 was administered during the last 10 minutes of regional ischaemia RI, and the SR59230A during the first 10 minutes of reperfusion (n=6 hearts) (Fig.2.6). Measurements were made as described in 2.5.1.

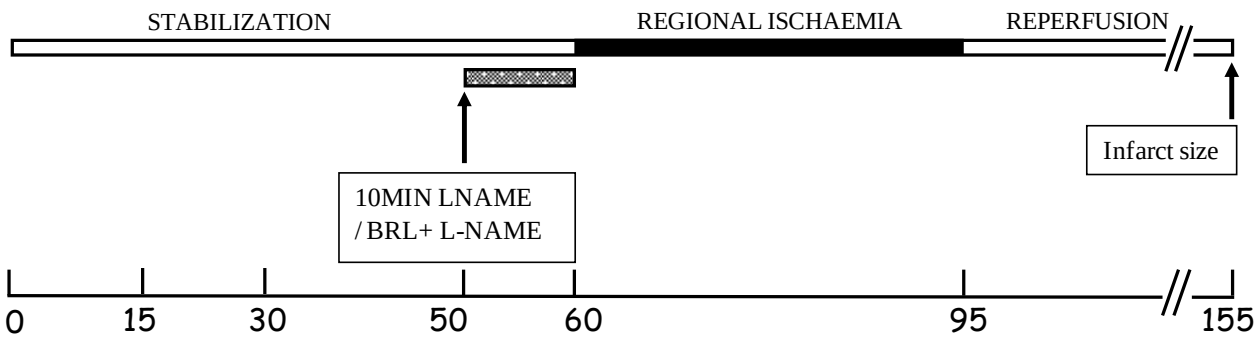
Fig. 2.6 BRL37344 Per-treatment and SR 59230A Post-treatment



2.5.5 Assessment of involvement of nitric oxide synthase (NOS) in β3-AR stimulation before regional ischaemia and during reperfusion, using nitric oxide synthase inhibitor (L-NAME)

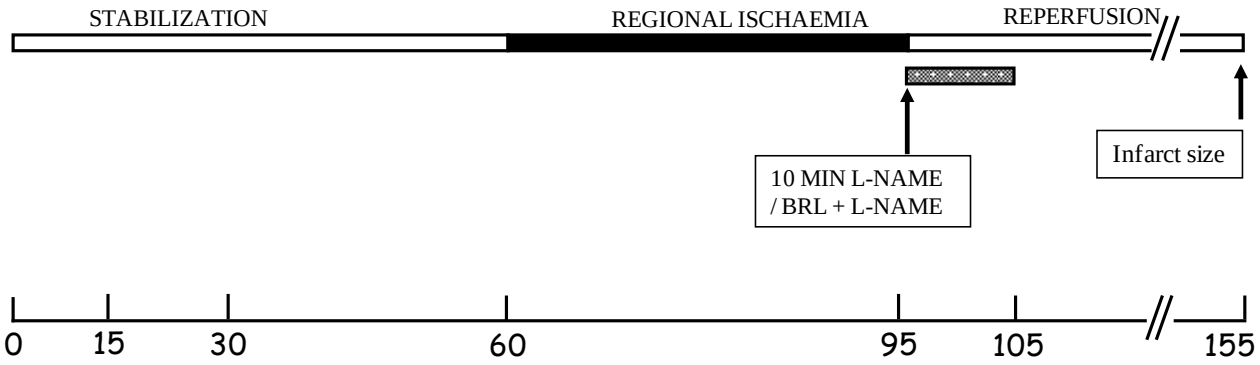
Hearts were pre-treated with the nitric oxide synthase inhibitor (L-NAME) alone or in combination with BRL37344, for 10 minutes after which the hearts were subjected to 35 minutes regional ischaemia RI and 60 minutes reperfusion (Fig.2.7A).

Fig. 2.7 (A) L-NAME / BRL37344 + L-NAME Pre-treatment



For the post-treatment protocols, hearts were stabilized for 60 minutes and then subjected to 35 minutes regional ischaemia (RI); L-NAME or the combination of BRL37344 and L-NAME was administered during the first 10 minutes of reperfusion (Fig.2.7B) (n=5-6 hearts). Measurements were made as described above.

Fig. 2.7 (B) L-NAME / BRL37344 + L-NAME Post-treatment



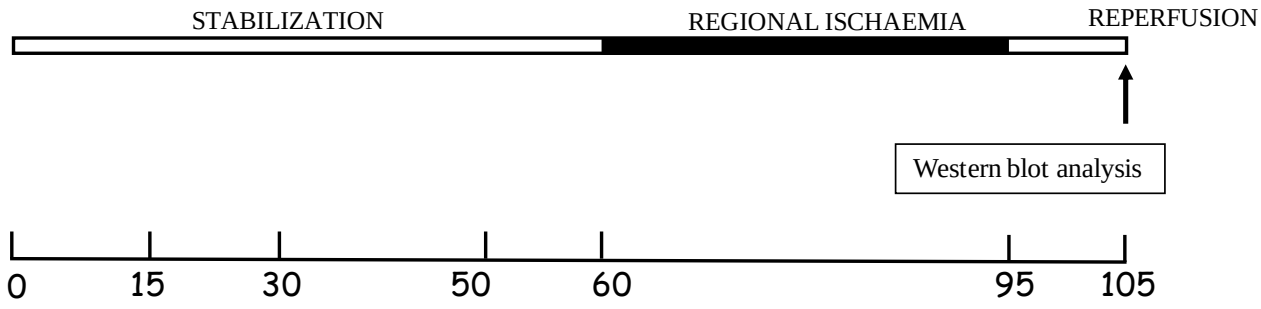
2.6 Evaluation of the effect of β3-AR modulation on signalling pathways (Western Blotting Protocols)

Hearts (left ventricles only) were cut and freeze-clamped at the indicated time intervals below, for subsequent measurements of phosphorylated and total ERKp44/p42, PKB/Akt, GSK-3B, and eNOS by Western blot analysis.

2.6.1 Non-pretreatment

Hearts were stabilized for 60 minutes, followed by 35 minutes RI and only left ventricles freeze-clamped immediately after 10 min reperfusion (n=3) (Fig.2.8).

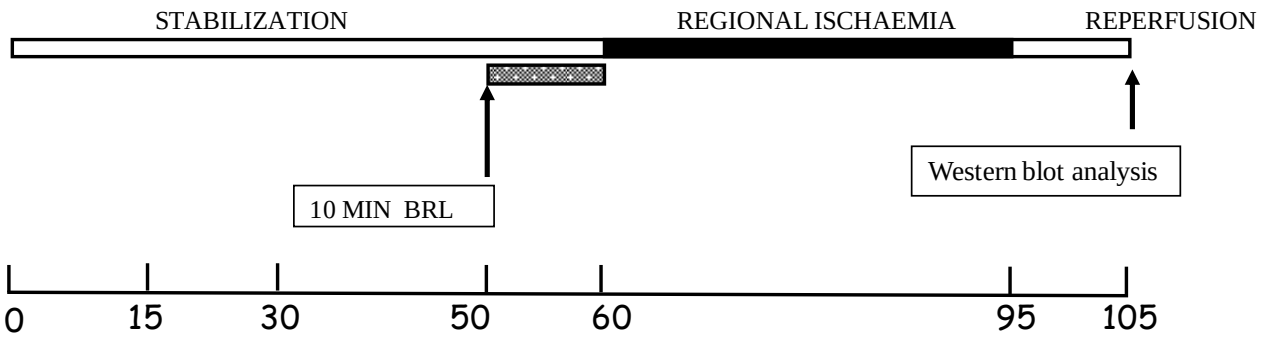
Fig. 2.8 Non-Pretreatment



2.6.2 β3-AR agonist BRL37344 Pre-treatment

Hearts were pre-treated with the β3-AR agonist BRL37344 prior to 35 min regional ischaemia and only left ventricles freeze-clamped immediately after 10 min reperfusion (n=3) (Fig.2.9).

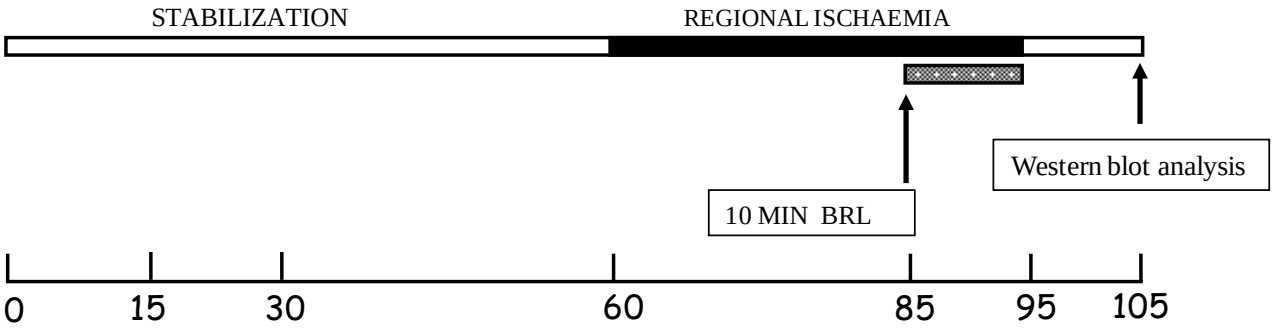
Fig. 2.9 BRL37344 Pre-treatment



2.6.3 β3-AR agonist BRL37344 Per-treatment

Hearts were stabilized for 60 minutes and subjected to 35 minutes regional ischaemia during which BRL37344 was applied at the last 10 min of regional ischaemia and left ventricles freeze-clamped at 10 min reperfusion (n=3)(Fig.2.10).

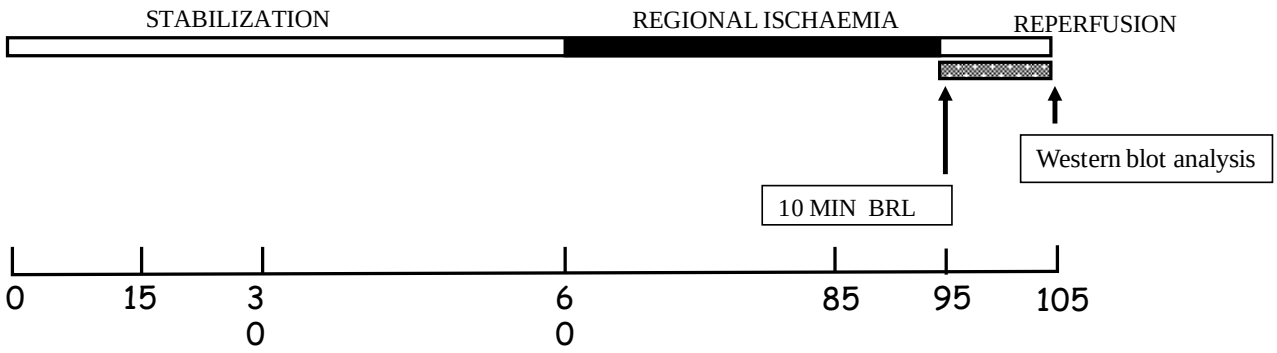
Fig. 2.10 BRL37344 Per-treatment



2.6.4 β3-AR agonist BRL37344 Post-treatment

In this group, hearts were stabilized for 60 minutes and subjected to 35 minutes regional ischaemia RI, followed by the administration of BRL37344 during the first 10 minutes of reperfusion and the left ventricles freeze-clamped at this point (n=3) (Fig.2.11).

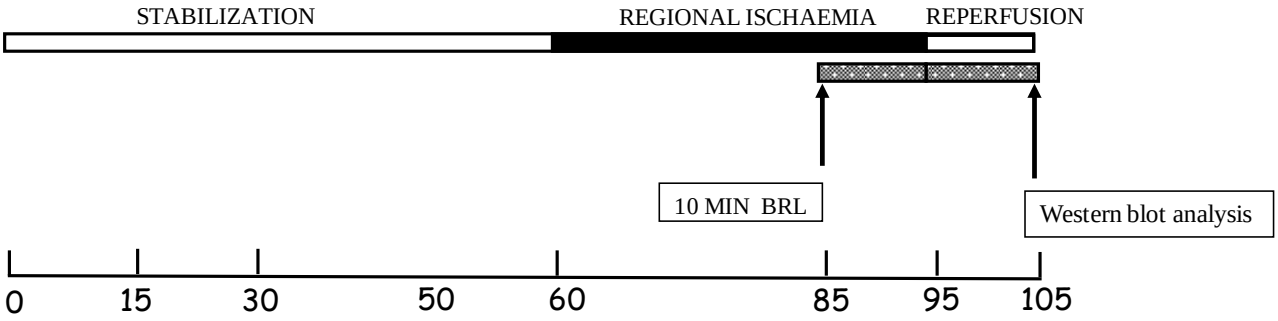
Fig. 2.11 BRL37344 Post-treatment



2.6.5 β3-AR agonist BRL37344 Per-treatment and Post-treatment

After stabilization for 60 minutes and 35 minutes regional ischaemia, BRL37344 was applied during the last 10 minutes of regional ischaemia as well as during the first 10 minutes of reperfusion, left ventricles freeze-clamped after 10 minutes reperfusion for Western blot analysis (n=3)(Fig.2.12).

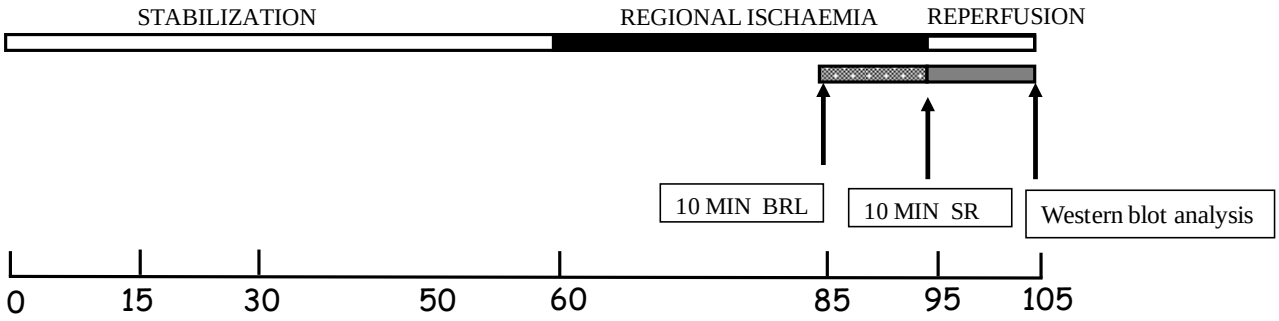
Fig. 2.12 BRL37344 Per + Post-treatment



2.6.6 β3-AR agonist BRL37344 Per-treatment and β3-AR antagonist SR59230A Post-treatment

After stabilization for 60 minutes and 35 minutes regional ischaemia, the β3-AR agonist BRL37344 was applied during the last 10 min of regional ischaemia, the antagonist SR59230A during the first 10 minutes of reperfusion and left ventricles freeze-clamped at this point (n=3) (Fig.2.13).

Fig. 2.13 BRL37344 Per-treatment SR59230A Post-treatment



3. Results

3. 1 Effect of β3-AR modulations (stimulation and inhibition) on infarct size

3.1.1 β3-AR modulation: Pre-treatment (PT)

Pre-treatment (PT) was elicited by administration of the selective β3-AR agonist **BRL37344**, for a period of 10 minutes before 35 minutes regional ischaemia (RI), significantly reduced infarct size (% infarct size per area at risk: 21.43±2.52, compared to non-pretreatment group **(NPT)** 43.17±1.20, p <0.001) (Fig.3.1). Pretreatment with the vehicle, dimethylsulfoxide **(DMSO)** and the

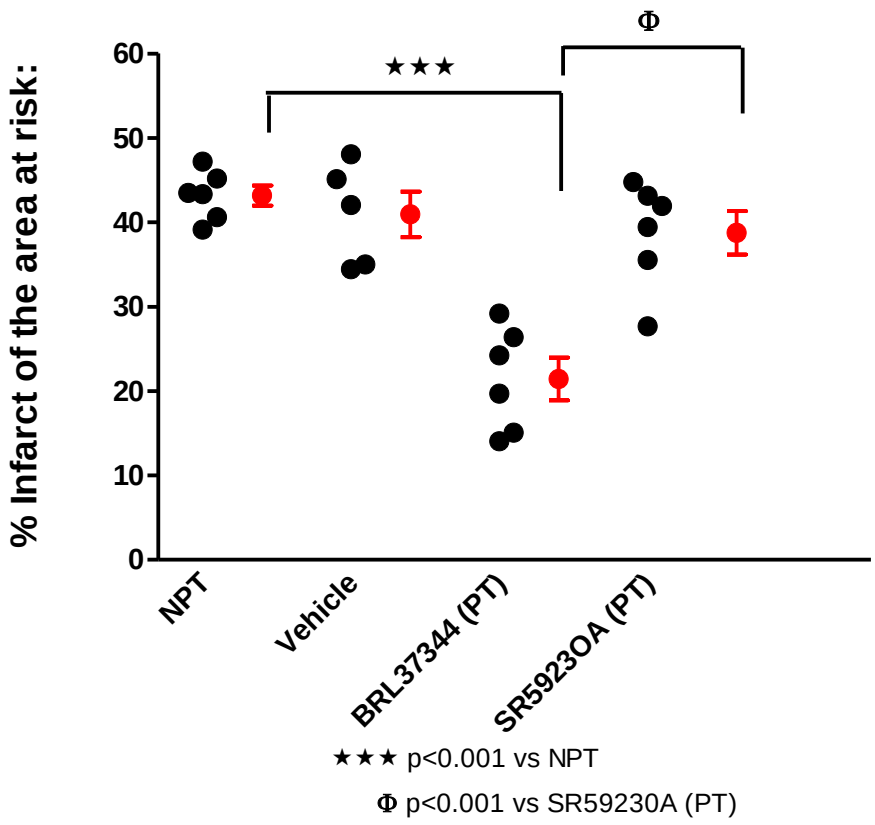


Fig.3.1: Effect of pre-treatment with vehicle, (DMSO), β3-AR agonist **BRL37344,** and β3-AR antagonist **SR59230A** on infarct size of heart after 35 min regional ischaemia and 60 min reperfusion (n = 6 / group).

β3-AR antagonist, **SR59230A** had no effect on infarct size (% infarct size per area at risk: 40.94±2.70 and 38.75±2.57 %, respectively compared to **NPT** group, 43.17±1. 20%).

3.1.2 β3-AR modulation: Per-treatment (PerT)

Interestingly, when the selective β3-AR agonist **BRL37344** was applied during the last 10 minutes of regional ischaemia, β3-Per-treatment (PerT), the infarct size was considerably reduced (% infarct size per area at risk: 14.94±2.34, compared to the **NPT** group 43.17±1.20, $p < 0.001$) (Fig.3.2). The application of β3-AR antagonist, **SR59230A** and **DMSO** had no effect on infarct size (% of infarct size per area at risk: 40.94±2.70 and 44.66±2.80%, respectively, compared to **NPT** group 43.17±1.20).

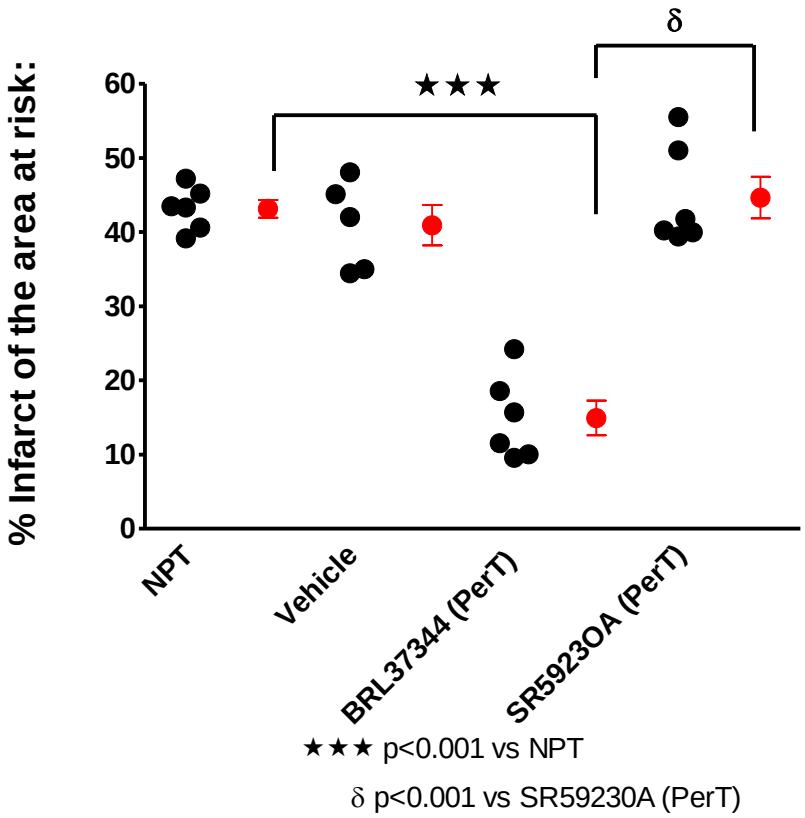


Fig.3.2: Effect of vehicle **DMSO**, β3-AR agonist **BRL37344**, and antagonist **SR59230A**, treated during last 10 min of RI on infarct size (n = 6 / group).

3.1.3 β3-AR modulation: Post-treatment (PostT)

Application of BRL37344 during the first 10 minutes of reperfusion, β3-post-treatment (PostT), caused a significant reduction in infarct size as well (% infarct size per area at risk: 19.06±1.81, compared to the **NPT** group 43.17±1.20, $p < 0.001$). Treatment with the β3-AR antagonist, **SR59230A (PostT)** had no effect on infarct size (% infarct size: 38.14±2.40 compared to **NPT** and **DMSO** groups, 43.17±1.20 and 38.75±2.57 %, respectively (Fig.3.3).

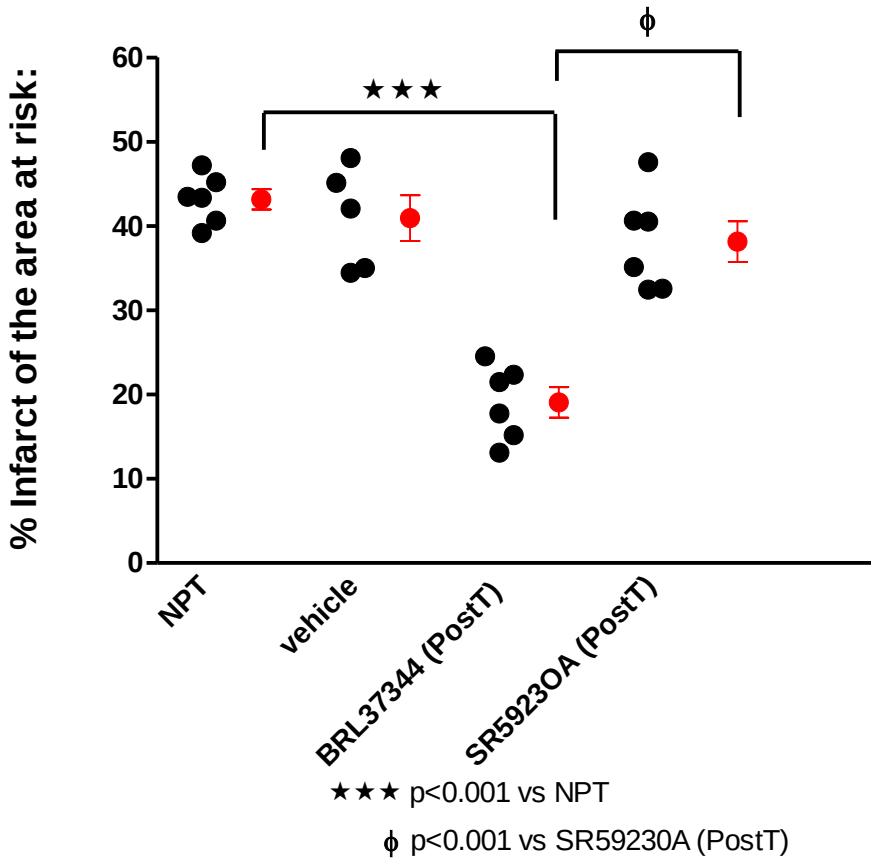


Fig.3.3: Effect of vehicle**, DMSO**, β3-AR agonist **BRL37344** and antagonist **SR59230A**, applied during first 10 min of reperfusion, on infarct size after 35 min regional ischaemia and 60 min reperfusion (n = 6 / group).

3.1.4 β3-AR modulation: Per-treatment (PerT) and Post-treatment (PostT)

Treatment of hearts with β3-AR agonist **BRL37344** during the last 10 minutes of regional ischaemia as well as during the first 10 minutes of reperfusion, β3-per-treatment and post-treatment **(PerT+PostT)**, resulted in a significant reduction in infarct size when compared to non-pretreatment **(NPT)** group (% infarct size per area at risk:20.55±2.01 vs 43.17±1.20, p <0.001) (Fig.3.4). The groups treated with the antagonist **SR59230A** and the vehicle **DMSO** had no effects on infarct size (% infarct size: 34.44±1.88 and 38.75±2.57 %, respectively, compared to **NPT** group 43.17±1.20%-

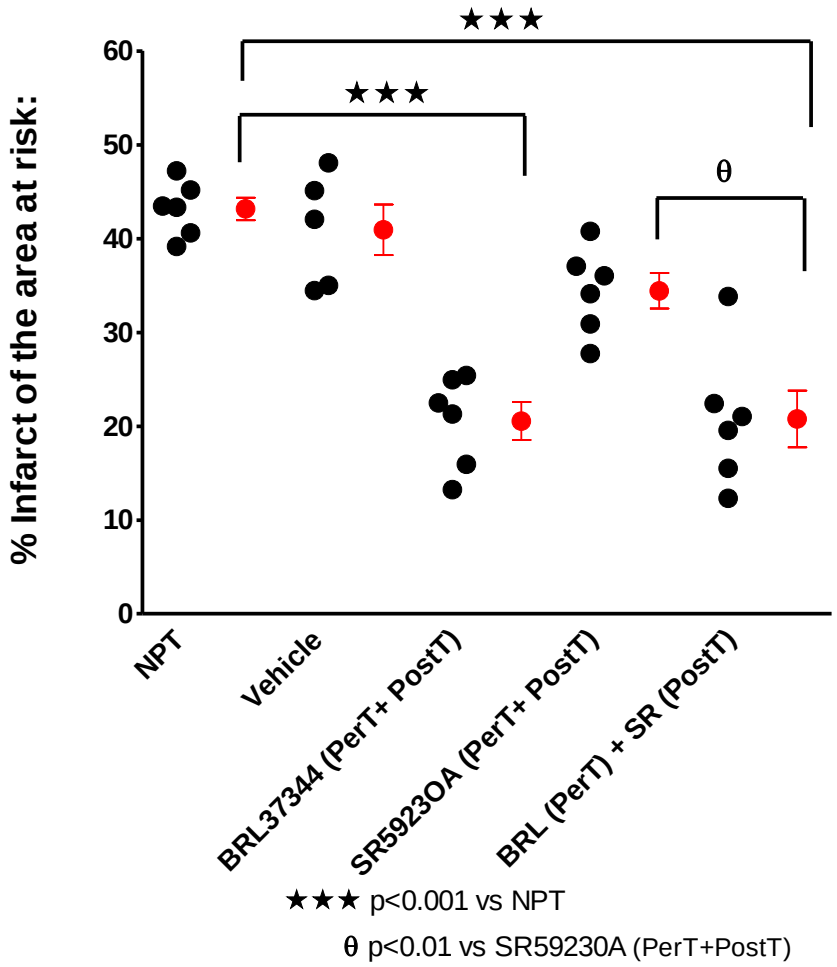


Fig.3.4: Effect of vehicle **DMSO**, β3-AR agonist **BRL37344** and **SR59230A**, applied during last 10 min of RI as well as first 10 min of reperfusion, and effect of **BRL37344** applied during RI and **SR59230A** during reperfusion on infarct size of heart exposed to 35 min regional ischaemia and 60 min reperfusion (n = 6 / group).

Surprisingly, administration of **BRL37344** during the last 10 min of regional ischaemia and the antagonist **SR59230A** at the onset of reperfusion, **[BRL (PerT) + SR (PostT)]**, significantly reduced infarct size (% infarct size per area at risk: 20.78±3.02 vs NPT, p <0.001) (Fig.3.4).

3.1.5 The effect of NOS inhibition with the nitric oxide synthase inhibitor L-NAME on the infarct size

The nitric oxide synthase inhibitor, Nitro-L-arginine methyl ester hydrochloride (L-NAME), administered alone, before regional ischaemia, **L-NAME (PT)** and during reperfusion, **L-NAME (PostT)**, had no effect on infarct size, (% infarct size per area at risk: 39.86±3.67 and 34.70±2.22, respectively). However, the reduction in infarct size elicited by the β3-AR agonist, BRL37344, administered 10 minutes before regional ischaemia and in another group at the first 10 min of reperfusion, was abolished by L-NAME, when administered in combination with BRL37344 for 10 minutes before regional ischaemia, **BRL37344+L-NAME (PT)** (% infarct size per area at risk: 41.48±3.18 vs **BRL37344 (PT)** 21.43±2.52, p <0.001) or during first 10 minutes of reperfusion, **BRL37344+L-NAME (PostT)** (% infarct size per area at risk: 35.75±3.54 vs **BRL37344 (PostT)** 19.06±1.81, p <0.001) (Fig.3.5) (A), (B) .

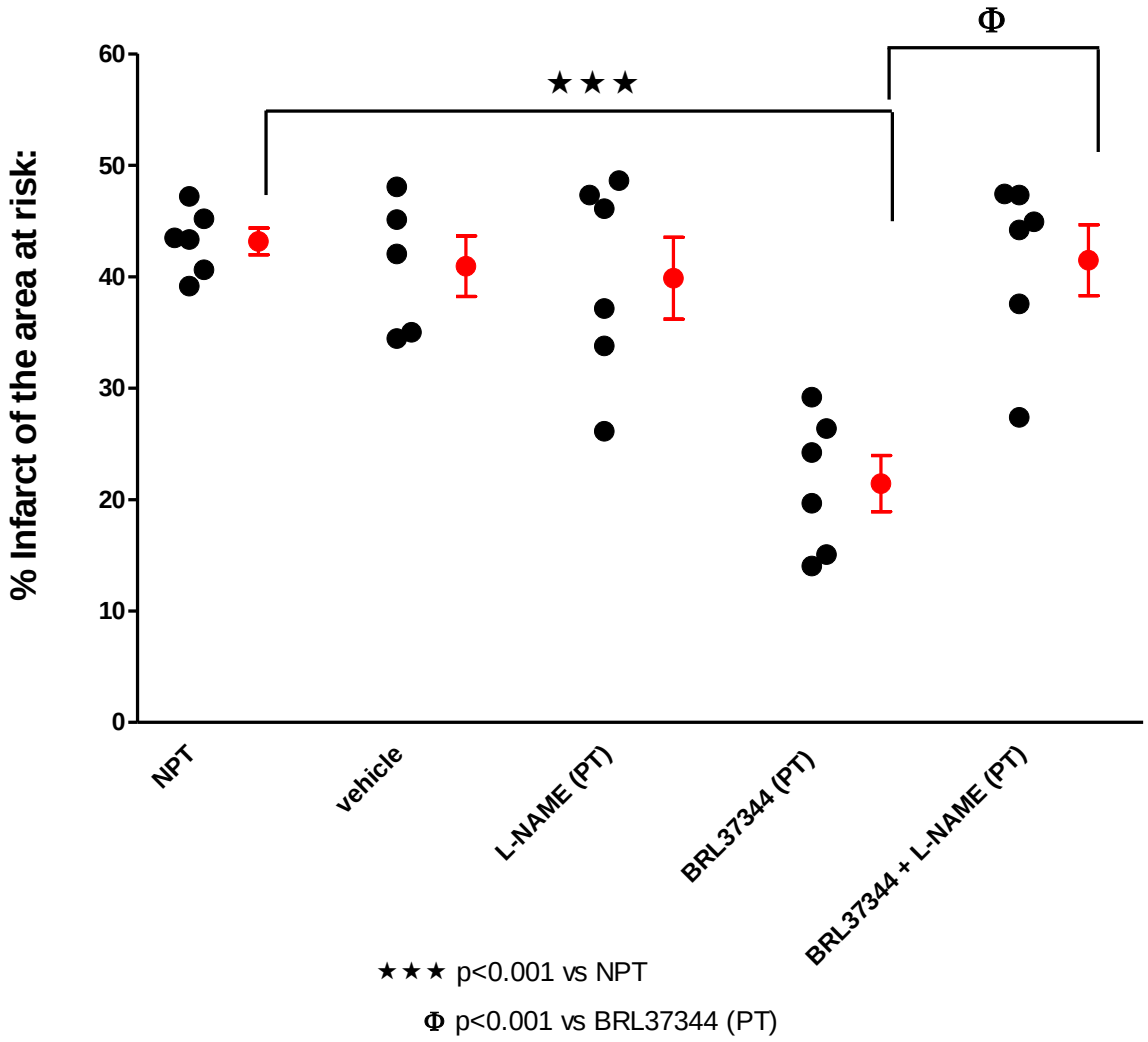


Fig.3.5A: Effects of **L-NAME**, β3-AR agonist **BRL37344**, and the combination of **L-NAME** and **BRL37344**, administered at 10 min before regional ischaemia, on infarct size after 35 min regional ischaemia and 60 min reperfusion.

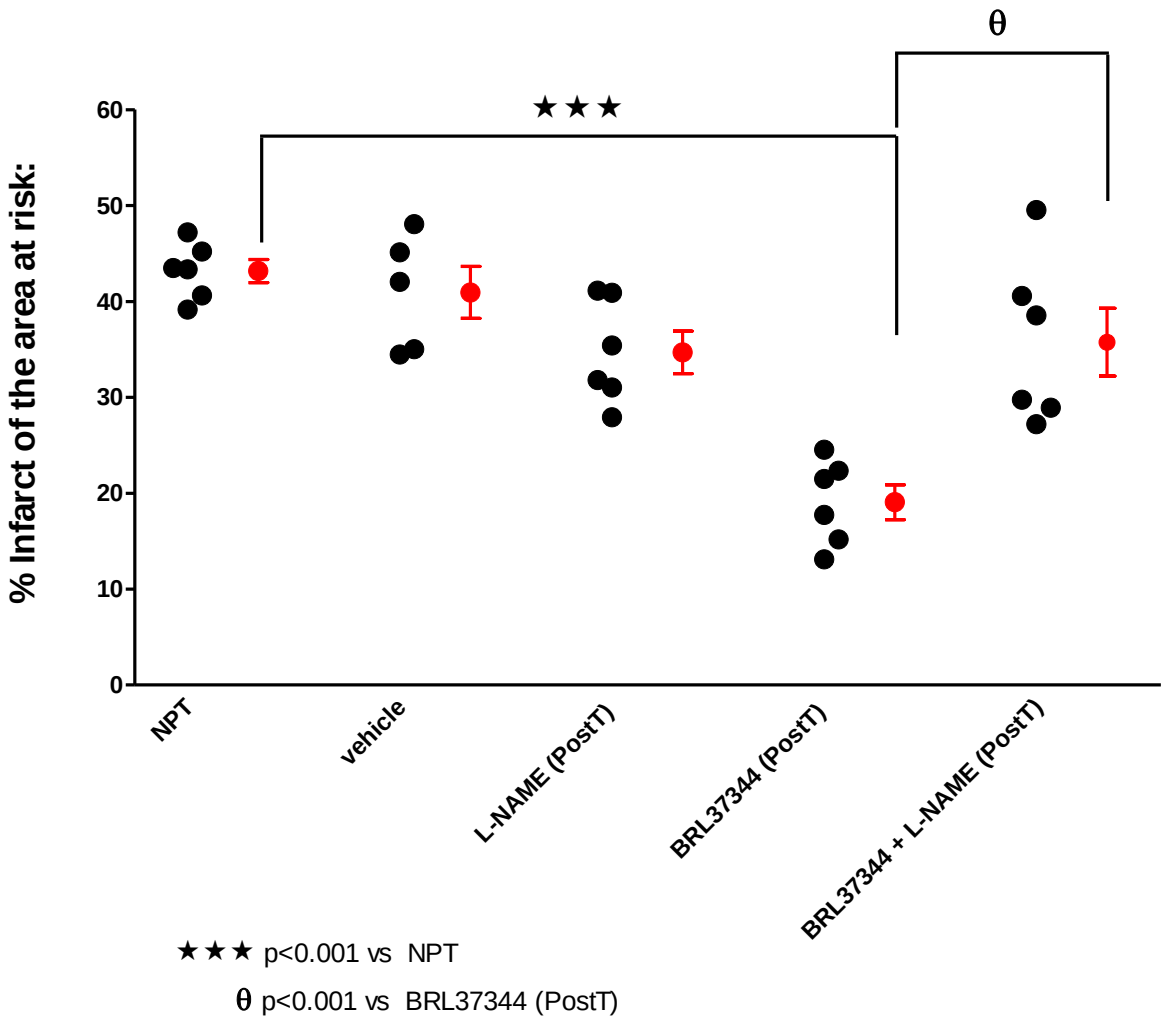


Fig.3.5B: Effects of **L-NAME**, β3-AR agonist **BRL37344**, and the combination of both **L-NAME** and **BRL37344**, applied at first 10 min of reperfusion, on infarct size after 35 min regional ischaemia and 60 min reperfusion. (n = 6 / group).

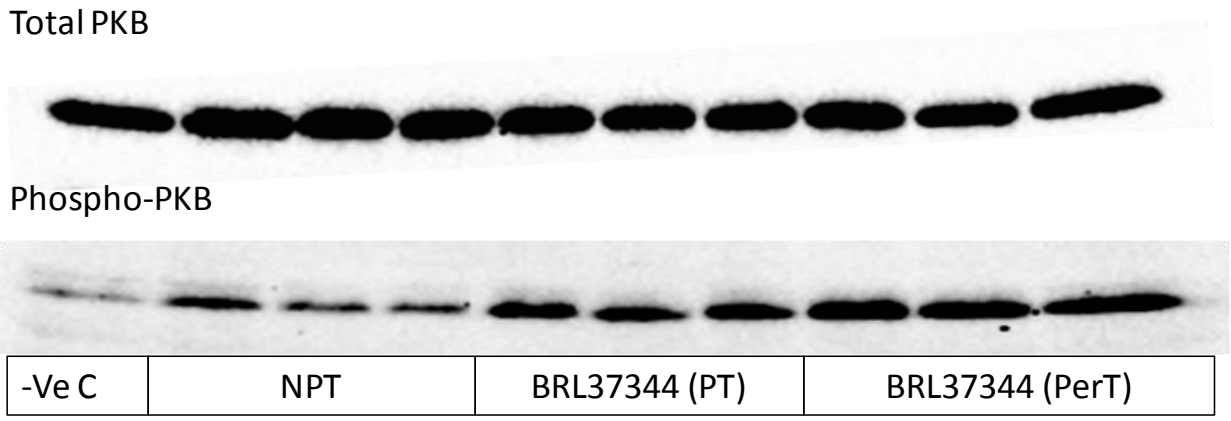
3.2 Western blot analysis of total and phosphorylated PKB/Akt, ERKp44 / p42 MAPKinase, GSK-3β and eNOS after treatment with β3-AR agonist BRL37344 or the antagonist SR59230A

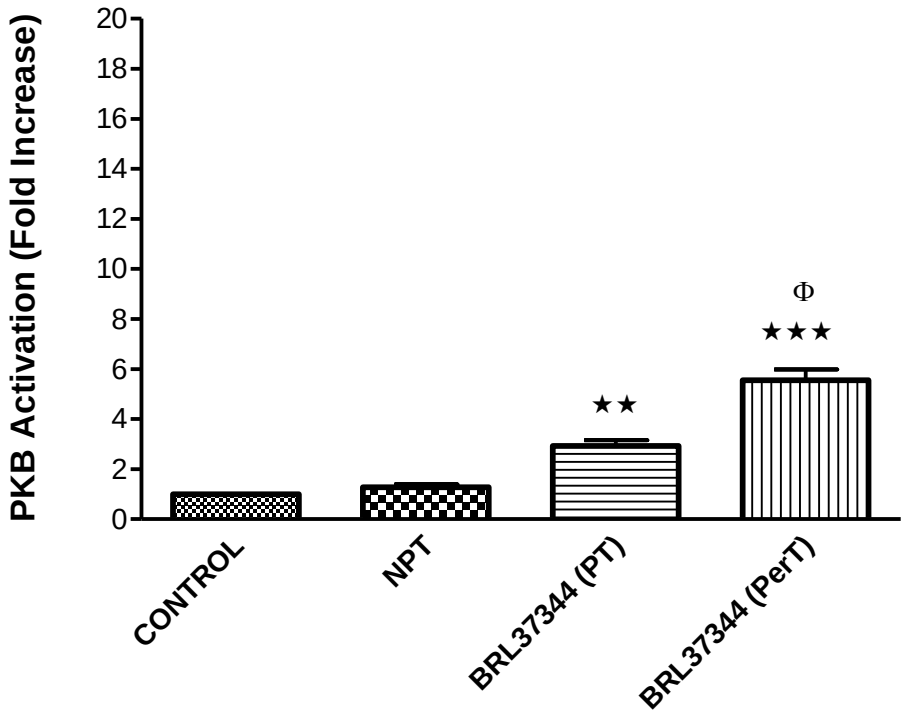
Since none of the protocols with the selective β3-AR antagonist SR59230A showed any kinase activation with western blotting, we excluded these data. The only protocol of significance was where the selective β3-AR agonist BRL37344 were applied during the last 10 min of RI and the selective β3-AR antagonist SR59230A during the first 10 minutes of reperfusion **[BRL37344 (PerT) & SR59230A (PostT)]**.

3.2.1 Activation of PKB/Akt

Total PKB/Akt showed no significant changes between the different groups and interventions. However, the administration of the selective β3-AR agonist BRL37344 before RI **BRL37344 (PT)**, significantly increased phospho-PKB/Akt (fold increase: 2.92±0.22, $p< 0.01$ vs NPT). Also, when the β3-AR agonist was applied during the last 10 minutes of regional ischaemia **BRL37344 (PerT)**, phospho-PKB/Akt was significantly increased (5.54±0.43, $p<0.01$ vs NPT and PT) (Fig.3.6a).

Activation of phospho-PKB/Akt when BRL37344 was applied during the first 10 minutes of reperfusion **BRL37344 (PostT)** was also higher than the NPT and control groups (fold increase: 4.73±0.47), however, this activation was found not to be significant. A significant increase in phospho-PKB/Akt was observed when BRL37344 was administered during the last 10 minutes of regional ischaemia as well as at the onset of reperfusion for 10 minutes, **BRL37344 (PerT+PostT)** (fold increase: 14.2±3.7, $p<0.01$ vs NPT and $p<0.05$ vs **BRL37344 (PostT)** (Fig.3.6b). Furthermore, when the β3-AR was stimulated with BRL37344 during late regional ischaemia and inhibited with SR59230A at the start of reperfusion, **[BRL37344 (PerT) & SR59230A (PostT)]**, increased the phospho-PKB/Akt: fold increase: 6.60±0.7. Even though, it was decreased when compared to the **BRL37344 (PerT+PostT)** group, this reduction was found not to be significant (Fig.3.6b).



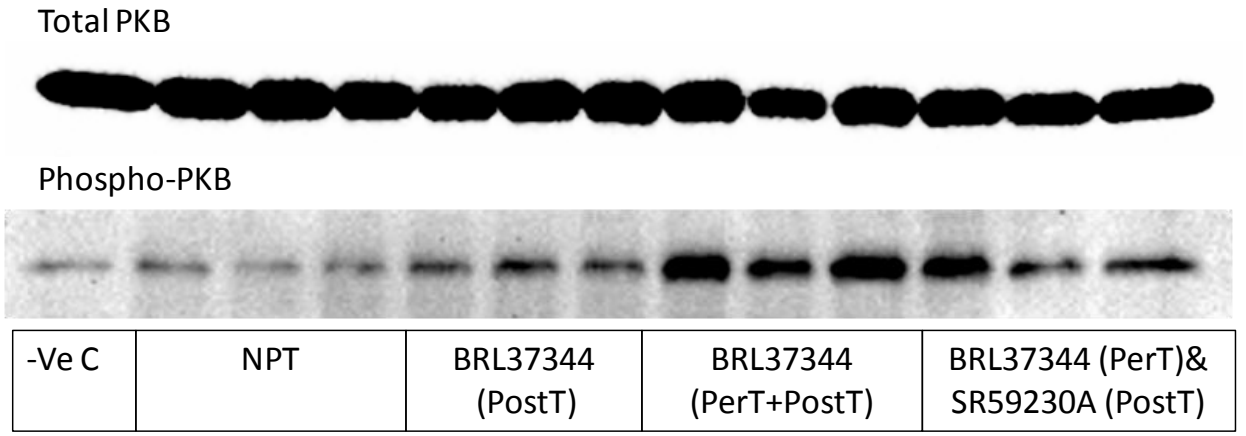


★★ p<0.01 vs NPT

★★★ p<0.001 vs NPT

Φ p<0.001 vs BRL37344 (PT)

Fig.3.6 (a): Representative blots for **PKB/Akt** activation after stimulation of the β3-AR with **BRL37344** prior to RI **BRL37344** (**PT**) or during last 10 min of RI **BRL37344** (**PerT**) (n=3/group).



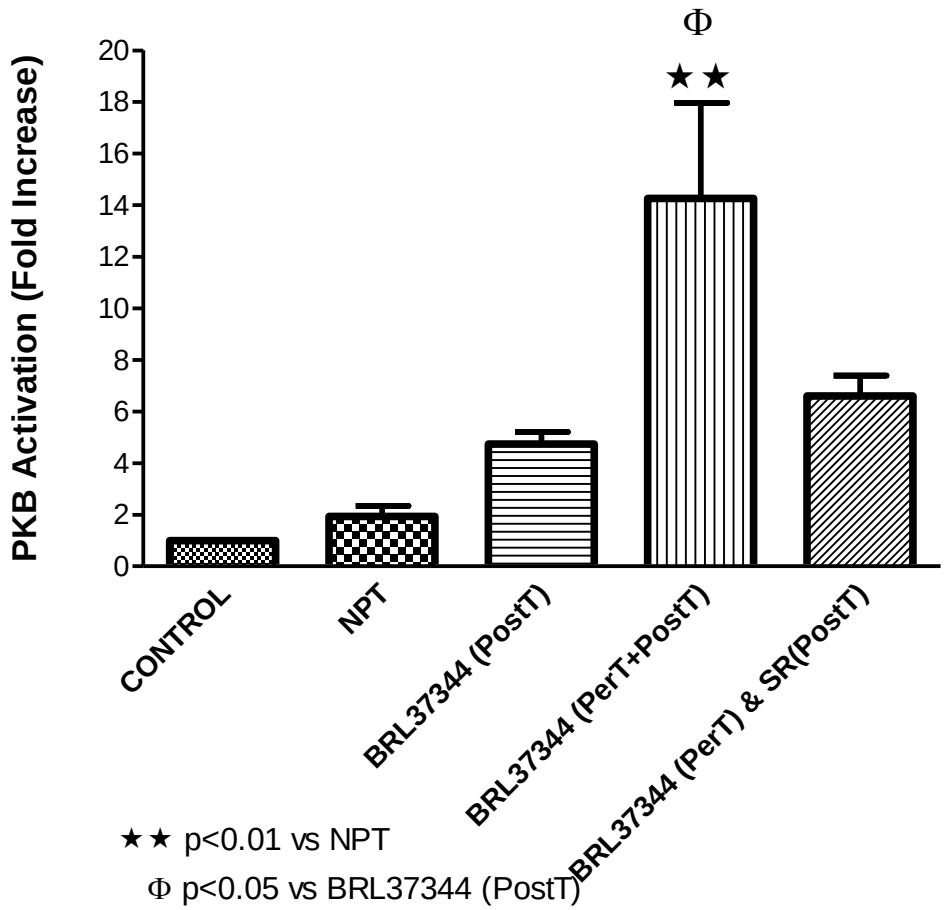


Fig.3.6 (b): Representative blots for **PKB/Akt** activation after stimulation of the β3-AR with **BRL37344** at beginning of reperfusion **BRL37344 (PostT)**; during last 10 min RI and the onset of reperfusion **BRL37344 (PertT+PostT)**; and lastly when **BRL37344** was-

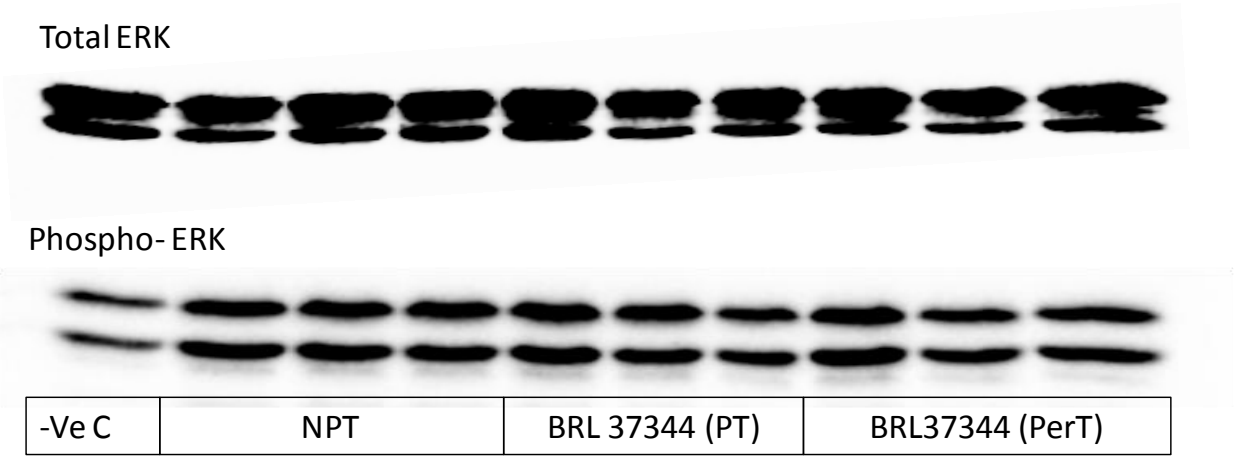
administered during the last 10 min of RI followed by the antagonist **SR59230A**during the first 10 min of reperfusion **[BRL37344 (PerT) &SR59230A (PostT)]** (n=3/group).

3.2.2 Activation of ERKp44/42

Total ERKp44/p42, did not show any significant changes between the different groups. Although phospho-ERKp44/p42 did not show differences between the different interventions when compared with the non-treated group, the activation obtained in the different groups was significantly higher than in the controls. An increase in ERKp44/p42 (fold increase: 1.82±0.06 and 1.83±0.08, respectively, $p<0.05$ vs control) was observed in hearts not receiving any treatment (NPT). An increase in ERKp44/p42 activation (fold increase: 1.87±0.16, $p<0.05$ vs control and 1.97±0.16 $p<0.01$, vs control, respectively) was observed in the group treated during regional ischaemia **BRL37344 (PerT)**. However the group treated before regional ischaemia did not show significant increase (Fig.3.7 a).

Similarly, BRL37344 administered during reperfusion, **BRL37344 (PostT)**, resulted in a 3.9±0.34 and 3.3±0.11 fold increase in ERKp44/p42, respectively ($p<0.01$ vs control and <0.05 vs control and **NPT** groups, respectively). Phospho-ERKp44/p42 also significantly increased when BRL37344 was applied during both regional ischaemia and reperfusion **BRL37344 (PerT+PostT)** (the fold increase of ERKp44/p42 were 4.24±0.80 and 4.02±0.43, $p<0.01$ vs control, respectively).

Furthermore, a 3.35± 0.56 and 3.88±0.78 fold increase in ERKp44/p42 activation was observed, when the β3-AR stimulated during regional ischaemia and inhibited during reperfusion **[BRL37344 (PerT) & SR59230A (PostT)]**, $p<0.05$ and 0.01 vs controls, respectively (Fig.3.7 b).



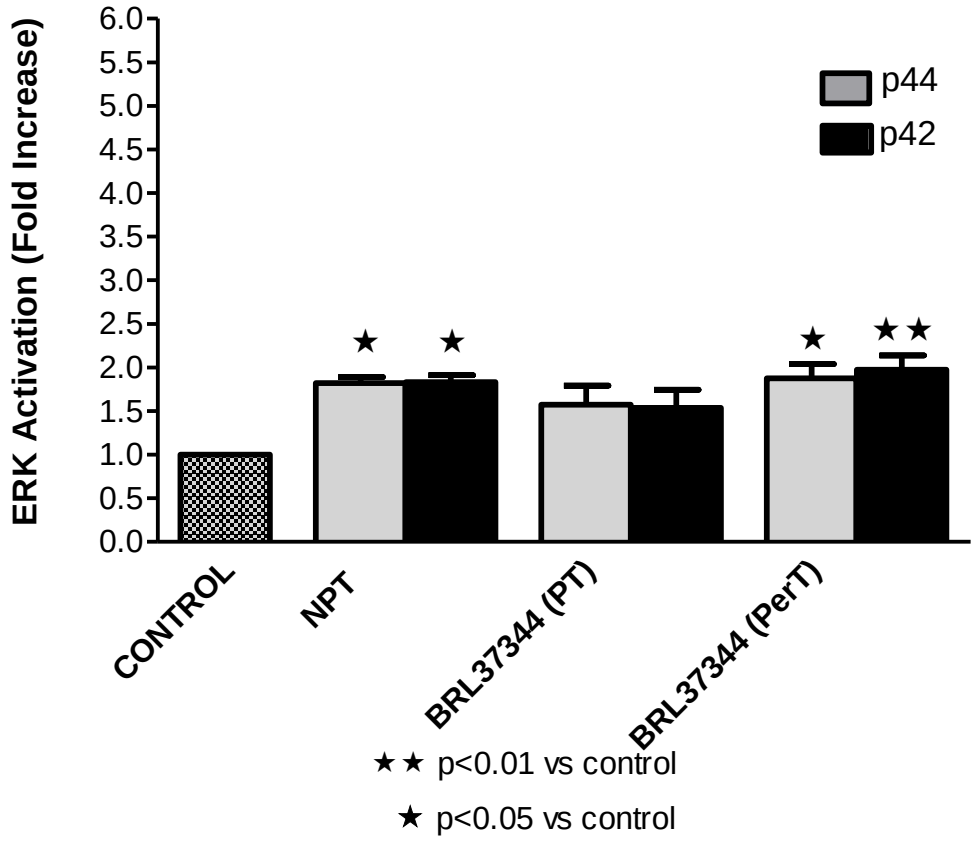
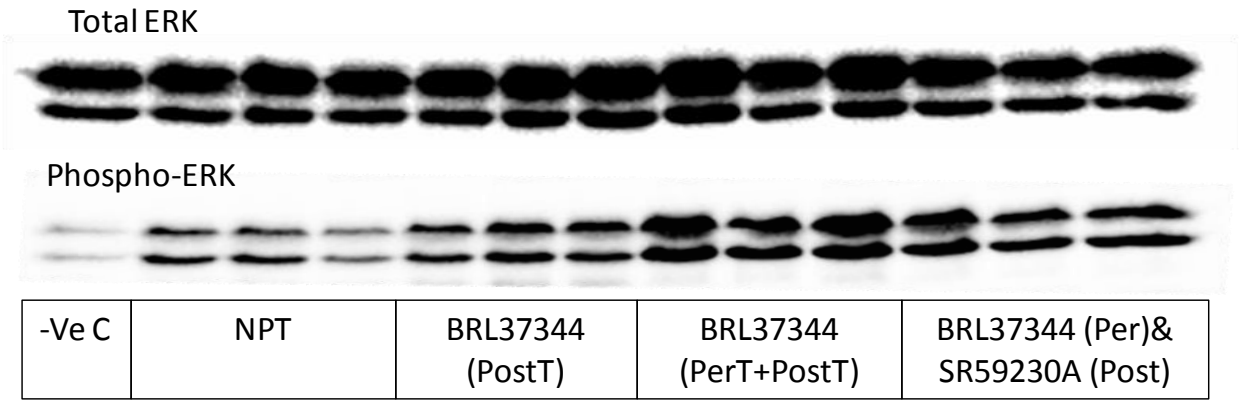


Fig.3.7 (a): Representative blots for ERKp44/p42 activation after stimulation of β3-AR with **(BRL37344)** before **BRL37344 (PT)** and during last 10 min **BRL37344 (PerT)** of regional ischaemia (n=3/group)



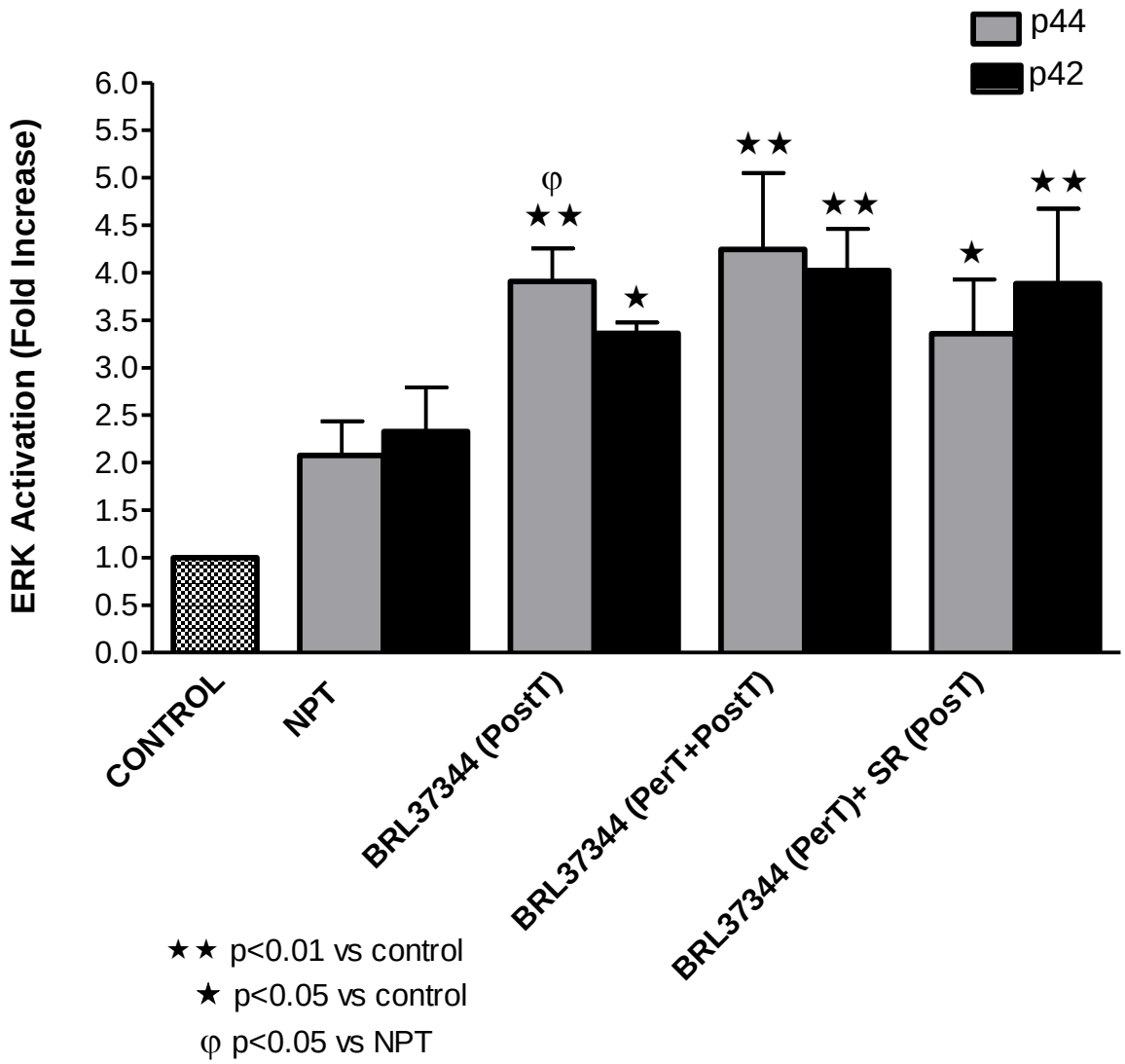


Fig.3.7 (b): Representative blots forERKp44/p42 activation after stimulation of β3-AR with the-

BRL37344 at beginning of reperfusion (**BRL37344 (PostT)**, during last 10 min RI as well as first 10 min reperfusion (**BRL37344 (PerT+PostT)**; and lastly when **BRL37344**-was applied during regional ischaemia and the antagonist **SR59230A** during reperfusion **[BRL37344 (PerT) & SR59230A (PostT)]** (n=3/group).

3.2.3 Activation of Glycogen synthase kinase-3β (GSK-3β)

Stimulation of β3-AR with β3-AR agonist BRL37344 during pre-treatment (PT), per-treatment (PerT), post-tratment (PostT) did not phosphorylate GSK-3β protein (Fig.3.8 a) and (Fig.3.8 b). However, a significant phosphorylation of GSK-3β was observed when β3-AR stimulation occurred during both regional ischaemia and reperfusion **BRL37344 (PerT+PostT)**: (68.85±7.73 vs non-pretreatment **NPT**: 1.32±0.41, $p<0.001$). Interestingly, activation of β3-AR with BRL37344 at the end of regional ischaemia and its inhibition with SR59230A at the beginning of reperfusion **[BRL37344 (PerT) & SR59230A (PostT)]** also resulted in a significant phosphorylation of GSK-3β, however, this phosphorylation was observed to be significantly reduced when compared to **BRL37344 (PerT+PostT)** (Fig.3.8 b).

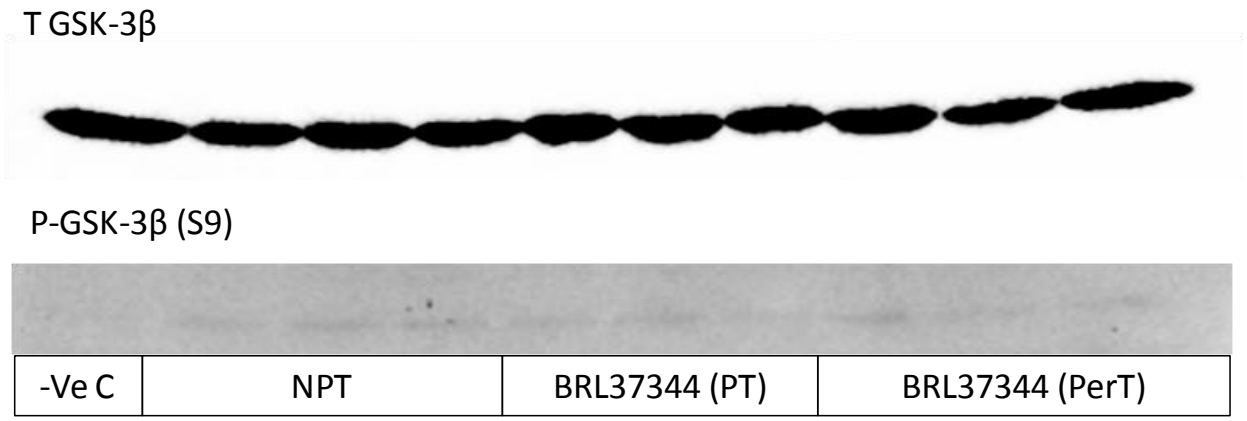
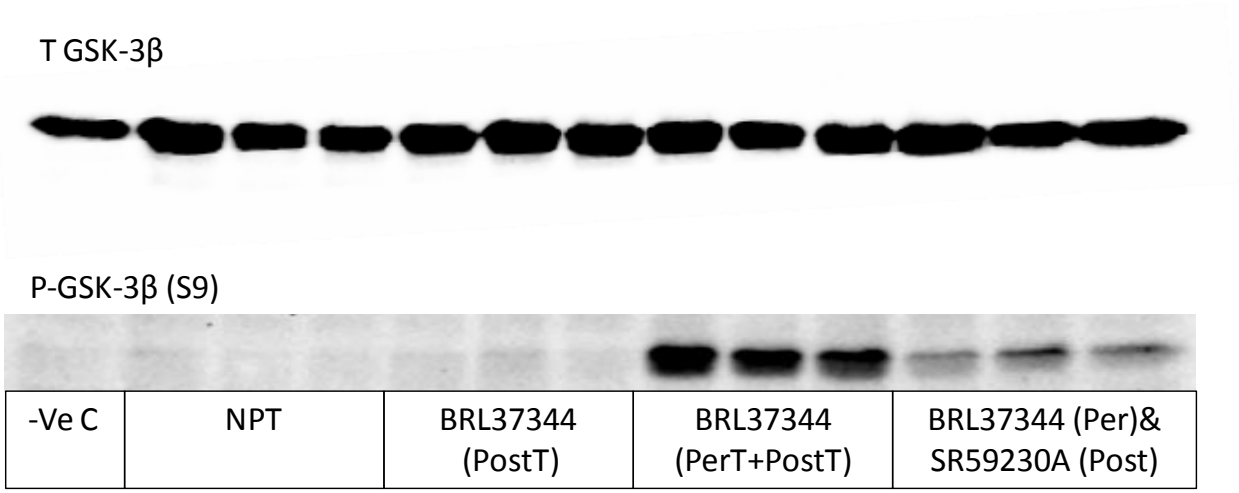
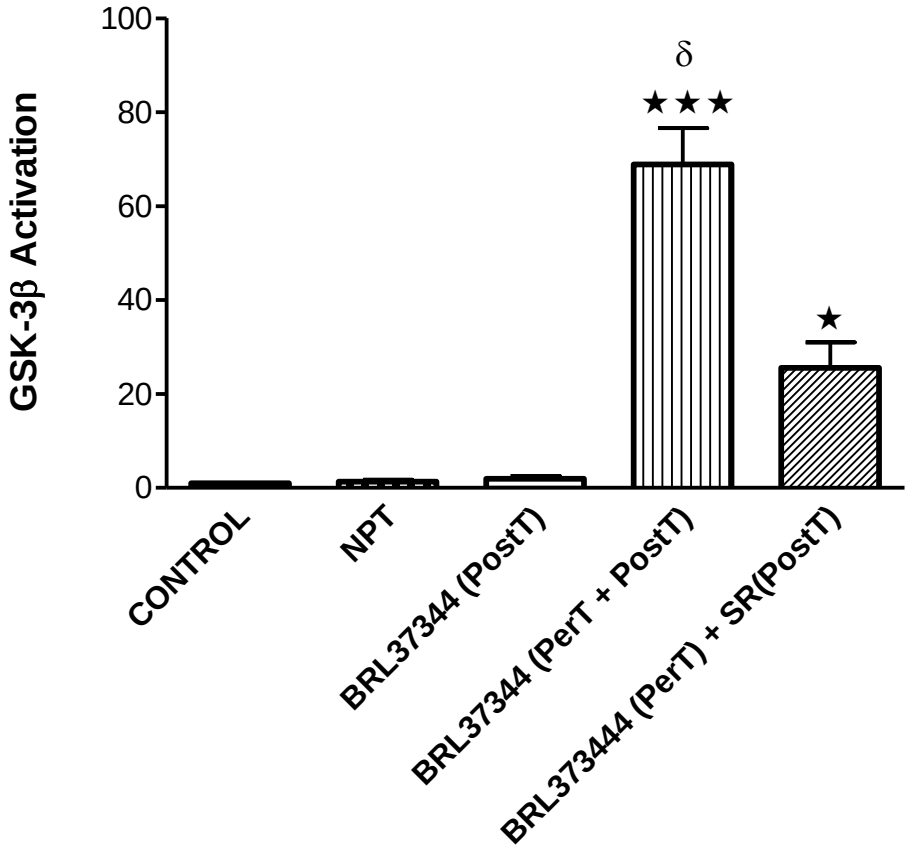


Fig.3.8 (a) Representative blots for **Glycogen synthase kinase-3β (GSK-3β)** phosphorylation after stimulation of β3-AR with **BRL37344** before regional ischaemia **BRL37344 (PT)**, and during the end of regional ischaemia **BRL37344 (PerT)**. No **GSK-3β** phosphorylation could be seen**.**





★★★ p<0.001 vs NPT & BRL37344 (PostT)

★ p<0.05 vs NPT & BRL37344 (PostT)

δ p<0.001 vs BRL37344 (PerT) + SR (PostT)

Fig.3.8 (b) Representative blots for **Glycogen synthase kinase-3β (GSK-3β)** phosphorylation after-

stimulation of β3-AR with **BRL37344** at beginning of reperfusion **BRL37344 (PostT)**; during last 10 min of RI as well as first 10 min reperfusion **BRL37344 (PerT+PostT)**; and lastly when **BRL37344** was applied during RI and the antagonist **SR59230A** during reperfusion **[BRL37344 (PerT) &SR59230A (PostT)]** (n=3/group).

3.2.4 Involvement of endothelial nitric oxide synthase eNOS in β3-AR stimulation

Interestingly, endothelial nitric oxide synthase (eNOS) activation followed the same pattern as GSK-3β phosphorylation. The application of the selective β3-AR agonist BRL37344 as a PT or PerT did not affect eNOS activation Fig.3.9 (a). However, BRL37344 administered during last 10 minutes of regional ischaemia as well as the first 10 minutes of reperfusion **BRL37344 (PerT + PostT)**, resulted in a 2.82±0.46 fold activation of eNOS-Ser-1177, compared to **(NPT)** and **BRL37344 (PostT)**, $p<0.01$ and 0.05 respectively. β3-AR stimulated with BRL37344 at the end of regional ischaemia and inhibited with SR59230A at the beginning of reperfusion **[BRL37344 (PerT) & SR59230A (PosT)]**, caused a 2.26±0.48 fold increase of eNOS-Ser-1177 vs **(NPT)** group, $p<0.05$) Fig.3.9 (b).

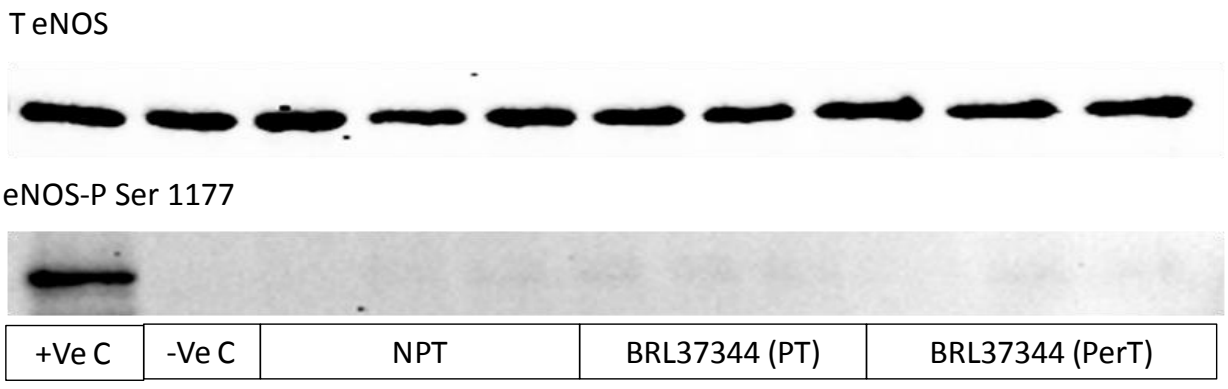
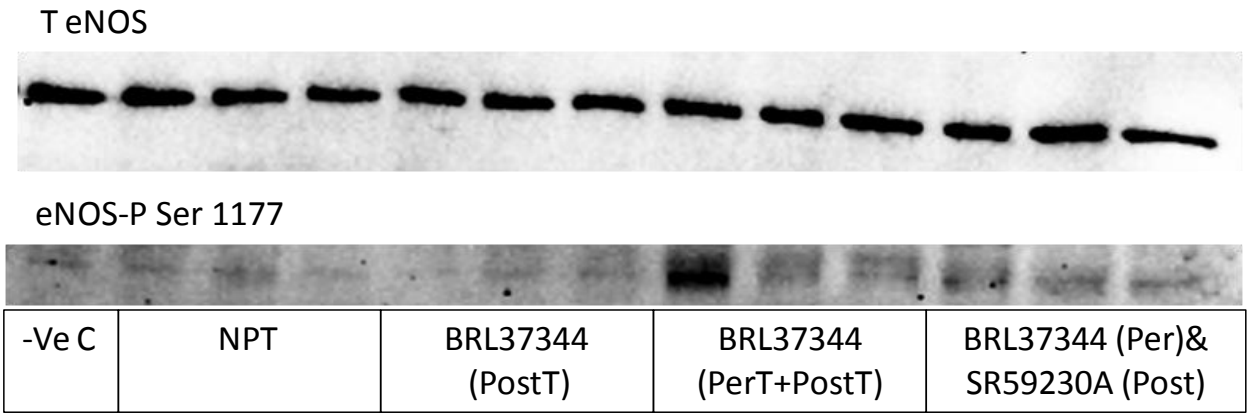


Fig.3.9 (a) Representative blots for **eNOS** activation after stimulation of β3-AR with **BRL37344** before regional ischaemia **BRL37344 (PT)**, and during the end of regional ischaemia **BRL37344 (PerT).**



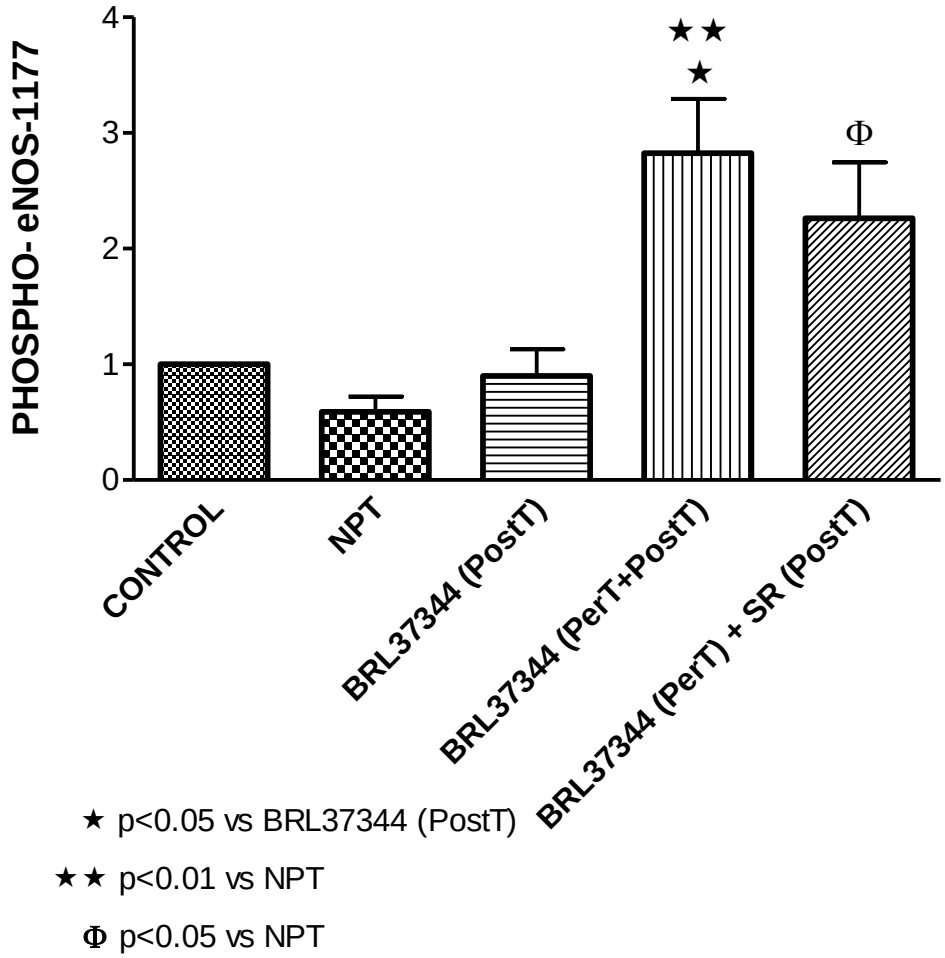


Fig.3.9 (b) Representative blots for **eNOS** activation after stimulation of β3-AR with BRL37344 at beginning of reperfusion **BRL37344 (PostT)**; during last 10 min of RI as well as first 10 min reperfusion **BRL37344 (PerT + PostT)**; and the application of **BRL37344** during -

regional ischaemia RI and the antagonist **SR59230A** during reperfusion **[BRL37344 (PerT) &SR59230A (PostT)]** (n=3/group).

3.3 Haemodynamic data results

3.3.1 Effects of BRL37344 on coronary flow

It was observed that coronary flow during retrograde perfusion of hearts treated with BRL37344 was significantly increased by 40-50 % when the drug was administered before RI and at the start of reperfusion (data not shown). However, administration of SR59230A had no effects on coronary flow.

3.3.2 Haemodynamic parameters and percentages of functional recovery

Functional performance of the 8 groups studied before the onset of RI was similar. Functional recovery after ischaemia, as reflected in cardiac output and total work, were significantly lower than those observed before induction of ischaemia in all groups (See tables 3.1 and 3.2). However, in 6 of the 8 groups their recovery was similar except in two groups, namely **BRL37344 (PT) & SR59230A (PostT)** and **SR59230A (PerT+PostT)** which showed a significant reduction when compared with the other groups. For example, compared with the **NPT** group, the % recovery in cardiac output was significantly less (post-ischaemic/pre-ischaemic of cardiac output recovery: 16.38±10.38% and 21.39±9.79%, respectively compared with NPT 64.00±6.32%, p-value <0.01). Similarly, the total work of these two groups reflected the same trends: (post-ischaemic/pre-ischaemic: 14.77±9.34% and 22.02±9.87%, respectively compared with NPT 58.39±6.20%, p-value <0.01).

Protocol Name	CF ml/min	AF ml/min	CO ml/min	HR Beat/min	PSP mmHg	WT mWatts
NPT	16.50±1.31	46.17±1.97	63.67±3.28	302±3.74	93.00±1.03	13.21±0.77
BRL37344 (PT)	13.00±1.00	39.67±2.65	58.67±6.10	266±17.21	89.83±1.55	10.53±0.77
BRL37344 (PerT)	14.67±0.98	44.67±3.88	59.67±5.04	295±11.66	90.50±1.70	12.15±1.20
BRL37344 (PostT)	16.00±1.86	44.67±2.95	61.17±3.93	290±10.82	92.83±0.79	12.62±0.87
BRL37344 (PerT+ PostT)	17.25±0.45	51.75±2.46	69.00±2.95	306±7.22	92.88±0.89	14.27±0.70
BRL37344(PerT) & SR59230A(PostT)	16.00±1.09	42.40±1.93	58.40±2.31	324±24.43	87.80±1.24	11.44±0.56
SR59230A (PT)	14.67±0.98	44.00±2.58	58.67±3.52	317±13.16	90.33±0.71	11.78±0.72
SR59230A (PerT)	16.00±1.15	45.67±3.02	61.67±3.73	306±18.02	91.33±0.55	12.50±0.75
SR59230A(PostT)	14.67±0.88	41.67±3.32	56.33±3.83	328±28.90	88.17±1.75	10.99±0.92
SR59230A (PerT+ PostT)	14.44±0.72	43.33±1.52	57.78±2.14	301±19.44	91.44±1.26	11.77±0.51
L-NAME (PT)	16.00±0.51	45.67±3.2	61.33±3.63	299±12.31	85.48±3.19	12.07±0.96
LNAME+BRL (PT)	18.33±0.80	50.33±1.5	68.67±2.10	319±14.04	83.83±4.79	13.46±0.61
L-NAME (PostT)	17.00±1.23	42.33±3.7	59.33±3.67	294±17.7	89.86±3.40	12.10±0.83
LNAME+BRL (PosttT)	17.67±1.74	45.67±0.6	63.33±1.52	344±33.15	88.67±2.24	12.59±0.42

Table 3.1 Haemodynamic parameters (mean ± standard error) of the different treated groups before regional ischaemia.

Protocol Name	CF ml/min	AF ml/min	CO ml/min	HR Beat/min	PSP mmHg	WT mWatts
NPT	16.00±1.03	24.67±2.9	40.33±3.73	311±10.05	84.83±0.98	7.578±0.66
BRL37344 (PT)	13.83±1.04	20.17±3.6	33.33±3.55	327±33.30	85.00±1.915	6.32±0.73
BRL37344 (PerT)	11.67±3.40	14.67±4.6	26.33±7.47	247±57.27	66.67±13.40	4.74±1.38
BRL37344 (PostT)	12.33±2.55	11.33±3.0	23.67±5.09	270±56.54	68.00±13.64	4.307±0.94
BRL37344 (PerT+ PostT)	15.00±0.75	15.50±2.7	30.50±2.99	318±7.61	81.25±0.97	5.543±0.59
BRL37344(PerT) & SR59230A(PostT)	3.20±3.20*	2.80±2.8*	6.00±6.00*	69±69.80*	16.20±16.20*	1.07±1.07*
SR59230A (PT)	12.33±2.70	21.83±5.3	34.17±7.84	291±59.30	70.67±14.15	6.43±1.48
SR59230A (PerT)	11.33±0.98	17.67±2.8	29.00±3.60	305±8.08	81.50±0.92	5.28±0.70
SR59230A (PostT)	13.92±1.05	17.67±3.3	31.58±3.64	326±25.70	82.00±1.09	5.79±0.70
SR59230A (PerT+ PostT)	8.11±2.63*	10.00±3.6*	18.11±6.15*	167±54.57*	46.56±14.73*	3.36±1.14*
L-NAME (PT)	11.00±2.3	19.08±4.9	30.08±7.03	247±51.63	65.45±13.11	5.29±1.24
LNAME+BRL (PT)	9.66±3.07	21.00±6.6	30.67±9.73	224±71.55	50.67±16.70	5.61±1.77
L-NAME (PostT)	11.50±2.9	22.00±6.3	33.50±8.99	271±71.34	66.20±16.73	6.20±1.69
LNAME+BRL (PostT)	10.40±0.97	16.80±2.9	27.20±3.77	351±29.15	81.60±1.28	4.98±0.82

Table 3.2 Haemodynamic parameters (mean ± standard error) of the different treated groups after regional ischaemia, * p-value<0.01 vs NPT.

4. Discussion

The role of β-adrenergic receptor (βAR) activation as a mediator of cardioprotection is undisputed and it has become clear that controlled activation of the β-adrenergic signalling pathway may elicit protective responses in the myocardium. For example, it has been demonstrated that transient activation of either the β1-AR or the β2-AR can act as a trigger in the ischaemic preconditioning (IPC) process (Lochner et al., 1998, 2000). Subsequently, transient pharmacological β1- or β2-AR activation with ligands such as isoproterenol, dobutamine or formoterol was reported to have similar cardioprotective effects as IPC and elicits protection against subsequent long periods of ischaemia (Asimakis et al., 1994; Lochner et al 1999; Salie et al., 2011), the so-called beta-preconditioning. Although transient activation of the β3-AR does not act as trigger of preconditioning (Salie et al., 2011), it should be pointed out that pre-treatment with a β3-AR agonist such as BRL37344, without washout prior to sustained ischaemia, effectively protects the heart against I/R damage.

It is well-established that up-regulation of the β3-AR population and concomitant β1- and β2-ARs down-regulation, in the failing human heart, are induced by the increasing levels of catecholamine (Grazia and Scilimati, 2010). Subsequently, activated β3-AR delivers a more sustained intracellular signal, resulting from its resistance to short term agonist-promoted desensitization (Liggett et al., 1993), making this receptor an ideal target for therapeutic intervention.

Previous studies have investigated the capability of cardioprotective interventions or drugs administered at the onset of reperfusion to reduce infarct size for example postconditioning (IPostC) (Zhao et al., 2003), erythropoietin (Bullard et al., 2005), activators of kinases including insulin, insulin-like growth factor-1 (IGF-1) as well as urocortin, atorvastatin and bradykinin (Hausenloy et al., 2004). All these interventions have been illustrated to confer cardioprotection through activation of the pro-survival kinases, PI3K-Akt and/or ERK p44/p42 cascades, during the first minutes of reperfusion (Bell and Yellon, 2003a; Hausenloy and Yellon, 2004; Hausenloy et al., 2005). Furthermore, inhibitors of the MPTP using sanglifehrin-A (Hausenloy et al., 2003) or

cyclosporin (Hausenloy et al., 2014; Ong et al., 2015), have also been shown to reduce infarct size and improve cardiac functional recovery.

However, from failed clinical trials (Flaherty et al., 1994), it appears that the window of opportunity for successful intervention during reperfusion is very limited. Although protection can be initiated at reperfusion, injury also occurs during ischaemia, and the relative contribution of each event, probably also depends on the duration of ischaemia (Stephanou et al., 2001). Consequently, if cardioprotective strategies can be introduced before or during ischaemia, it is possible that they will enhance protection, particularly with longer durations of ischaemia (Murphy, and Steenbergen, 2007). Therefore, in the present study the effects of β3-AR stimulation with BRL37344 on infarct size were studied at various time intervals, namely, **(1)** *before* 35 minutes of regional ischaemia (Pre-treatment, PT), **(2)** *during* regional ischaemia (only last 10 minutes) (Per-treatment,PerT), **(3)** at the *onset* of reperfusion (Post-treatment,PostT), and **(4)** during both ischaemic and reperfusion phase (PerT +PostT). To investigate the involvement of the RISK pathway as well as the roles of GSK3-β and eNOS in β3-AR modulation at these various time intervals of the perfusion protocol, hearts were freeze-clamped at 10 minutes reperfusion for Western blot analysis, as this point of reperfusion has been shown to be associated with maximal phosphorylation of cardioprotective kinases (Salie et al. unpublished data).

In this study, BRL37344 was used as a selective β3-AR agonist, as it has been shown to be a potent selective β3-AR for rodents, however, it was found to be 60% less effective in humans than in rats (Blin et al., 1994; Dolan et al., 1994). Moreover, recent studies have shown that BRL37344 administration is linked to PKB/Akt activation and NO production (Aragon et al., 2011; Gracia et al., 2014). Furthermore, it has been reported that BRL37344 induced a dose-dependent negative inotropic effect at concentrations ranging from 0.1nM to 1mM (Gauthier et al., 1996). Salie (PhD thesis) also found that BRL37344 at 1μM had a profound negative inotropic effect when administered to the isolated working rat heart. This effect could be washed out which probably accounts for its failure to precondition the heart. It was also illustrated that BRL37344 (1μM) effectively decreased the amplitude of oxytocin-induced myometrial contractions, as well as significantly increased cGMP levels in a BRL37344 group compared to the control group, which

were antagonized with SR59230A (1μM) (Yurtcu et al., 2006). In addition, it was found that β3-receptor antagonists SR59230A (1μM) or L-748,337 (3μM) reduced nebivolol-induced NO release by approximately 50% in human umbilical vein endothelial cells (HUVECs) (Mason RP et al., 2013). The concentration-response curves to isoprenaline in pig bladder showed that SR59230A (30nM to 1μM) effectively inhibits isoprenaline induced contractions (Yamanishi et al., 2002). Consequently, in this study we decided to use BRL37344 at a concentration of **1μM** and SR59230A at **0.1μM.**

4.1 The effect of β3-AR-stimulation/inhibition on the outcome of ischaemia/reperfusion

The data obtained in this study provide significant evidence for the cardioprotective effects of β3-AR stimulation with BRL37344, regardless of its time of administration. This was clearly demonstrated by the significant reduction in infarct size, obtained by stimulation of the receptor at different times during the I/R protocol **(PT, PerT, and/or PostT)**. However, inhibition of β3-AR with the antagonist (SR59230A) had no effect on infarct size (see results section Fig 3.1, 3.2, 3.3 and 3.4). Interestingly, in all interventions, the reduction in infarct size was not associated with an improvement in functional recovery during reperfusion (see results section, tables 3.1 and 3.2). This could be due to concomitant myocardial stunning, namely postischaemic mechanical contractile dysfunction that persists during reperfusion despite the restoration of flow. Myocardial stunning has been proposed to be due to either generation of free radicals or calcium overload, leading to excitation-contraction uncoupling (Bolli et al., 1998).

However, it was suggested that β3-AR activates NOS which would lead to increased production of NO and cGMP (Tavernier et al., 2003). Increased cGMP is associated with decreased intracellular calcium concentration (Hammond and Balligand, 2012) and thus attenuation of myocardial stunning. Therefore, in this study, myocardial stunning could be due to a burst of ROS production during reperfusion and not calcium overload. Thus future investigations need to be done to explore the involvement of ROS production in β3-AR stimulation and its relationship to NO and cGMP activation.

A number of in vivo studies have recently also illustrated a reduction in infarct size with β3-AR agonist administered during reperfusion, confirming our ex vivo data. For example,

administration of BRL37344 during reperfusion after in vivo coronary artery ligation attenuated fibrosis and scar formation (Niu et al 2014) and reduced infarct size in both small and large animal models of MI/R (Garcia-Prieto et al 2014). The latter group also showed improved long-term left ventricular function after β3-AR agonist treatment. Moreover, it was shown that pre-reperfusion treatment was without effect in cyclophilin-D KO mice, implicating the MPTP in the mechanism of protection. It has recently been demonstrated that nebivolol, a third-generation β-blocker, protects against myocardial infarction via stimulation of β3-AR and the eNOS/nNOS pathway (Zhang et al 2014). The study of Aragon and co-workers 2011 also failed to demonstrate any significant improvement in cardiac function after reperfusion with nebivolol as well as with the selective β3-ARs agonists, BRL37344 and CL316243. This is also probably due to stunning since-assessment of left ventricular function was done shortly after the MI/R protocol.

4.2 The effect of β3-AR-stimulation at the end of ischaemic phase and its inhibition during reperfusion on the outcome of ischaemia/reperfusion

With this intervention we intended to investigate whether β3-AR inhibition, using SR59230A at the beginning of reperfusion (PostT), could abrogate the beneficial effects of BRL37344 given at the end of the ischaemic period (PerT) **[BRL37344 (PerT) & SR 59230A (PosT)]**. Although these interventions had no effect on the significant reduction in infarct size elicited by BRL37344 (PerT) alone (see Fig 3.2 and 3.4), it caused the most marked decrease in functional recovery of all manipulations studied (see table Table 3.2). The reason for this marked depression in postischaemic function is probably due to an effect of SR59230A *per se* on contractility, since administration of this inhibitor alone also markedly reduced myocardial function (Table 3.2). These observations clearly illustrate the importance of β3-AR stimulation during the late phase of regional ischaemia to bring about potent cardioprotective effects during reperfusion which, in this particular protocol, may be due to a reduction in ischaemic injury per se.

4.3 The role of NO in the actions of β3-AR stimulation

In the current study, the increased coronary flow observed during administration of BRL37344 during retrograde perfusion of hearts (data not shown), suggests a vasodilatation effect, which

might implicate NOS and NO as important role players in β3-AR mediated cardioprotection. It was previously demonstrated that β3-ARs expressed in the endothelium of human coronary arteries mediate vasodilatation via NO production (Dessy et al., 2004). Subsequently, nebivolol, although it is a selective β1-AR blocker, has been shown to produce vasodilatation in both human and rodent coronary microarteries through stimulation of endothelial β3-ARs and subsequent release of NO (Dessy et al., 2005). In the present study the role of NOS and NO production in β3-AR–mediated cardioprotection was evaluated by using the non-specific NOS inhibitor, L-NAME at concentration of 50 μM, as it has been shown in our laboratory that at this concentration the cGMP levels were significantly reduced (Lochner et al., 2000). L-NAME, administered in combination with the β3-AR agonist (BRL37344) prior to regional ischaemia, **BRL37344+L-NAME (PT)** or at the onset of reperfusion, **BRL37344+L-NAME (PostT)**, abolished cardioprotection as was shown by a significant increase in infarct size in both protocols (Figs 3.5a and 3.5b).

Although several studies suggested that selective β3-AR stimulation elicits its protection by activating NOS pathways and release of NO (Zhang et al., 2014; Niu et al., 2014; Aragon et al., 2011), the exact NOS isoform involved is still being debated. Nebivolol has been shown to increase activation of eNOS and expression of nNOS (Aragon et al 2011; Zhang et al 2014,) while Niu and coworkers showed that BRL37344 has similar effects. In contrast to the proposed dual activation of eNOS and nNOS, other studies suggested that NO release secondary to β3-AR activation was exclusively from eNOS (Brixius et al., 2004; 2006), while nNOS and /or iNOS have also been implicated in the β3-AR mediated cardioprotective signalling (Maffei et al., 2007; Niu et al., 2012; Calvert et al., 2011).

In the current study, Western blot results consistently provide supportive evidence for the involvement of eNOS in the cardioprotection elicited by selective β_3-AR stimulation. Firstly, eNOS was significantly activated when BRL37344 was applied at the end of regional ischaemia and continued at the start of reperfusion, **BRL37344 (PerT+PostT)**. Secondly, eNOS activation also occurred, when the BRL37344 was administered at the end of ischaemia and SR59230A at the beginning of reperfusion with **[BRL37344 (PerT) & SR 59230A (PostT)]** (see Fig.9a and 9b). At the

moment we do not have an explanation why the cardioprotection induced by PT, PerT, and PostT with BRL37344 was not associated with activation of eNOS. It is possible that activation of eNOS occurs very rapidly (within 5-15min after nebivolol administration (Aragon et al 2011) and that is no longer detectable after 10min of reperfusion in our protocol. However, this remains to be further elucidated. Activation of nNOS and iNOS was not evaluated in the present study, but indications are that iNOS is not activated by β3-AR stimulation (Aragon et al 2011; Niu et al 2014).

4.4 Selective β3-AR modulation and the RISK Pathways: PKB/Akt and ERKp44/42

It is well-established that cardioprotective strategies such as IPC, IpostC and pharmacological preconditioning are usually associated with activation of the RISK pathway (ERKp44/p42 and PKB/Akt) as well as phosphorylation of its downstream target GSK-3β (Tong et al., 2002; Hausenloy and Yellon., 2004; Gomez et al., 2008). Although, the exact mechanism by which the RISK pathway inhibits the MPTP is unclear, it has been suggested that phosphorylation of PKB/Akt and ERKp44/p42 may activate downstream signalling, such as PKG, GSK-3β, or Hexokinase II, or through modulation of MPTP inducing mediators such as oxidative stress, calcium overload and pH restoration (for review see Ong et al., 2015). Experimental data has suggested that translocation of kinases such as PKB/Akt, ERKp44/p42 and PKG to the mitochondria are linked to MPTP inhibition (Ong et al., 2015). Interestingly, we found the recruitment of these powerful cardioprotective kinases in our study using pharmacological β3-AR stimulation. However, the fact that we used the whole left ventricle (which included tissue subjected to ischaemia as well as normal tissue) in preparing our samples for blots; this might have masked the protein activation, particularly ERKp44/p42 activation.

4.4.1 PKB/Akt

The present study shows that BRL37344 activates PKB/Akt, regardless of its time of administration (see Figs.3.6a and 3.6b). However, the most significant activation of PKB/Akt was seen when β3-AR stimulation was applied during both the end of ischaemia and at the beginning of reperfusion, **BRL37344 (PerT+PostT)** (Fig.3.6b). Conversely, stimulation of β3-AR with

BRL37344 during late regional ischaemia and its inhibition with SR59230A at the onset of reperfusion **[BRL37344 (PerT) + SR59230A (PostT)]** resulted in a slight, but not significant reduction in phospho-PKB/Akt (Fig.3.6b). This intervention was also associated with a significant infarct size reduction (see Fig 3.4). It should be noted that a slight decrease in PKB/Akt and eNOS activation, even though not significant, can be an important determining factors in cell survival.

The activation pattern obtained with PKB/Akt correlates better with the reduction in infarct size than activation of GSK-3β and eNOS per se. It should be taken into account that PKB/Akt has several downstream substrates, not only GSK-3β and eNOS, and many of which are cardioprotective in their own respect such as Bad, Caspase 9, and FOXOs. Therefore, elucidation the cardioprotective signalling mechanisms, by which the **BRL37344 (PT, PerT and PostT)** groups reduced infarct size, needs intensive investigations to these substrates.

It is possible that activation of PKB/Akt is dependent on the period of administration of the β3-agonist, since the most marked activation of PKB, eNOS and GSK-3β was seen in the **BRL 37344 (PerT+PostT)** group. In a recent study Garcia et al (2014) has reported that BRL37344 exerts Akt-eNOS-NO dependent signals in the isolated adult cardiomyocytes exposed to hypoxia/reoxygenation, which essentially inhibits the MPTP opening and therefore increasing cardiomyocyte survival. These observations are in agreement with previous findings by Garlid and coworkers (2004) that activation of PKB/Akt leads to stimulation of eNOS-NO-cGMP-PKG signalling, which subsequently inhibits MPTP opening. At low concentrations, NO inhibits opening of the mitochondrial permeability transition pore (West et el., 2008), whereas high levels facilitates pore opening.

4.4.2 ERKp44/p42

ERKp44/p42 activation after stimulation of β3-AR with BRL37344 was not significant in **BRL37344 (PT) and BRL37344 (PerT)** groups when compared with **NPT** (Fig.3.7.a). However, **BRL37344 (PostT)** group showed a significant activation (Fig.3.7 b). Furthermore, **BRL37344 (PerT+PostT)** group as well as **[BRL37344 (PerT) & SR59230A (PostT)]** group showed not significant but marginal increases of ERKp44/p42 activation (Fig.3.7 b). Consequently, the role of ERKp44/p42 as an important component of RISK pathways in β3-AR stimulation can not be completely excluded

or ignored. Therefore, significant activation of ERKp44/p42 and differences between the treated groups would be clearly obtained if more samples was used for this analysis.

4.4.3 GSK-3β

The role of phosphorylation and inactivation of GSK-3β leading to inhibition of the mitochondrial permeability transition pore and ultimately cardioprotection is well-established (Hausenloy and Yellon et al., 2004; Juhaszova et al., 2004; Vigneron et al., 2011; Lochner et al., 2011). However, in this study it was found that the reduction in infarct size was not *always* associated with inactivation of GSK-3β: stimulation of β3-AR with BRL37344 before regional ischaemia **BRL37344 (PT)**; at the end of regional ischaemia **BRL37344 (PerT)** or at the onset of reperfusion **BRL37344 (PostT),** did not phosphorylate GSK-3β protein (Fig.3.8a).

Significant phosphorylation of GSK-3β was observed in two protocols only namely when the β3-AR was stimulated at end of ischaemia as well as at the beginning of reperfusion, **BRL37344 (PerT+PostT)** (Fig 3.8b) and in hearts exposed to **[BRL37344 (PerT) & SR59230A (PostT)]** (Fig.3.8b). Finally, it is very interesting that PKB was activated by all cardioprotective protocols while concomitant phosphorylation of eNOS and GSK-3β occurred in the latter two protocols only. Furthermore, the % reduction in infarct size was similar in all BRL37344 groups.

A clear cut association between the extent of reduction in infarct size and activation of these signalling pathways was, however, not forthcoming. Additional studies focussing on the role of eNOS and GSK-3β as well as other downstream targets of PKB/Akt in β3-AR-induced cardioprotection are required.

Summary

The data obtained in this study clearly demonstrates that stimulation of the β3-AR elicits cardioprotection: treatment of hearts with the selective β3-AR agonist **(BRL37344) (1 μM)** at specific time periods (before, at the end of regional ischaemia and / or the beginning of reperfusion), elicited protection against ischaemia/reperfusion injury. This protection was clearly illustrated by the significant reduction in infarct size, activation of the PKB/Akt component of RISK pathways, and phosphorylation of the downstream targets GSK-3β and eNOS. Hence, β3-AR activation provides a convergence of protective pathways which ultimately leads to inhibition of MPTP opening as suggested by Garcia-Prieto and coworkers (2014).

However, in this study the marked reduction in infarct size was not accompanied by an improvement in functional recovery during reperfusion. This is a well- established fact in most studies using infarct size and functional recovery as endpoints and may be due to concomitant stunning which is described as reversible post-ischaemic contractile dysfunction and which may obscure the effect of cardioprotection. However, infarct size is regarded as the "gold standard" for evaluation of cardioprotection (Kloner et al., 1998; Cohen et al., 1999; Lochner et al., 2003).

Our study suggests that the last 10 minutes of regional ischaemia as well as the first 10 minutes of reperfusion are important time intervals to intervene with a selective β3-AR agonist, and this was clearly illustrated with application of BRL37344 during late ischaemia and at the beginning of reperfusion, **BRL37344 (PerT+PostT)**, which consistently reduced infarct size and caused the most significant phosphorylation of the PKB/Akt, GSK-3β and eNOS pathways. Consequently, the negative inotropic effects associated with the β3-adrenergic receptor can be effectively harnessed to bring about cardioprotection through considerable reduction in infarct size.

Novelty of this study

Most recent studies investigating the contribution of this receptor subtype to cardioprotection were done **in vivo**. In this study we made use of an **ex vivo** approach using the isolated perfused rat heart to investigate the cardioprotective effects of stimulation of the β3-AR. This model allows for a number of pharmalogical manipulations which are not possible in an in vivo model: the MI/R protocol was manipulated by using pharmacological activation/inhibition of β3-AR not only during the reperfusion phase but also before and /or during the sustained ischaemic period. Our approach also allowed evaluation of the role of the RISK pathway and its links with NO production and GSK-3β as downstream target.

Limitations of this study

In the current study the effects of only one selective β3-AR agonist and antagonist were studied. Additional agonists should be evaluated, including nebivolol, a third generation beta blocker which is selective for the β1-AR but has been shown to induce the β3-AR-NOS-NO dependent pathway. Furthermore, involvement of other NOS isoforms, nNOS and iNOS, as well as other downstream targets of PKB/Akt, BAD and p70s6K, in the β3-AR cardioprotection needs to be investigated. In addition, the significance of the different role players in β3-AR signalling should be further unravelled, with particular focus on the role of the MPTP in this regard.

Clinical translation and future studies

The discovery of functional β3-AR in the human heart is expected to shed new light on the understanding of the protective effect of therapeutic β-AR inhibition or stimulation in heart failure (Bristow, M.R. 2000). Pharmacological manipulation of β3-AR with a selective agonist could be easily applied in the clinical setting at the effective time periods – for example after an ischaemic episode (or as soon as possible during an ischaemic event) and continued when reflow is established as it was well illustrated in this study. Importantly, BRL37344 has been illustrated to be a more potent and selective agonist for rodents than humans, which critically limit its usage clinically. More studies using large animals are required (as yet there is only one study on pigs by

Garcia-Prieto and his group, 2014). Newly characterized antagonists / agonists with better selectivity for the human β3-AR are required which will allow more intense testing and may lead to the development of new treatment strategies for heart failure.

To achieve the above objectives, the following experimental approaches could be used:

human ventricular myocytes, treated in similar settings but with appropriate and more selective agonist / antagonists; in vivo studies using regional ischaemia / reperfusion, echocardiography and measurement of protein and mRNA levels of inflammatory cytokines; an appropriate cell line stably transfected with the human β3-adrenoceptor mRNA.

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