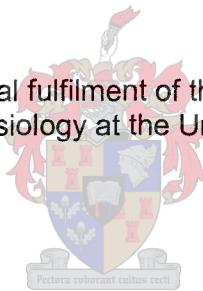


The phenomenon of "second window of protection": effect of beta-adrenergic stimulation and melatonin

Ashraf Davids

Thesis presented in partial fulfilment of the requirements for the degree of
Master of Physiology at the University of Stellenbosch



Project leader : Prof J A Moolman

March 2004

Declaration

I, the undersigned, hereby declare that this study project is my own original work and that all sources have been accurately reported and acknowledged, 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.

Signature

Date

Summary

Background: Myocardial ischaemia causes necrosis and apoptosis of myocytes, which cannot be replaced by division of surviving myocytes. The search for effective therapeutic strategies for the prevention of myocardial ischaemia is therefore an important goal. Clinical circumstances during which myocardial ischaemia arise are represented by the acute phase of coronary artery occlusion and also during cardiac bypass surgery. The main problem with known modalities of cardiac protection is that they have to be employed immediately before or during the acute ischaemic event, which is logistically not possible in most cases.

The phenomenon of ischaemic preconditioning is a newly discovered endogenous mechanism of myocardial protection. Ischaemic preconditioning refers to the phenomenon whereby one or multiple short episodes of ischaemia activates one or several endogenous mechanisms of protection against myocardial ischaemia. Ischaemic preconditioning is associated with two forms of cardioprotection : a classic form which provides immediate protection and lasts up to 2 hours after the preconditioning ischaemia and a delayed form (also called "second window of protection") which becomes apparent 24 hours later and lasts for up to 3 days. The second window of protection is not as powerful as the classic preconditioning phase, but confers much longer protection. This form of myocardial protection is therefore particularly interesting, as the ability to activate it could potentially provide a

clinically useful method to protect against ischaemia without knowing when the ischaemic event will occur.

It is not practically possible to use ischaemia to trigger preconditioning, and therefore there has been interest in elucidating the signal transduction pathways involved, in the hope of developing a pharmacological trigger of the phenomenon. There are numerous non-ischaemic triggers of classic preconditioning, that include beta-adrenergic stimulation, nitric oxide, opioids, bradykinin and adenosine. Most non-ischaemic triggers of classic preconditioning also seem to activate the second window of protection. However, it is currently not known whether beta-adrenergic stimulation can elicit a delayed cardioprotective response.

The mechanism of delayed cardioprotection involves both nitric oxide and reactive oxygen species generation, as the protective effect is abolished by inhibitors of nitric oxide generation as well as by agents that scavenge reactive oxygen species. Beta-adrenergic stimulation with isoproterenol can elicit classic preconditioning, and induces NO synthesis and generates reactive oxygen species.

Aims:

- To investigate whether beta-adrenergic preconditioning with isoproterenol elicits delayed myocardial preconditioning
- To determine whether the mechanism of beta-adrenergic cardioprotection acts via nitric oxide and/or ROS generation.

Methods: Male Wistar rats (ca. 250 g) were used as experimental animals. Isoproterenol was administered on Day 1, and 24 hours later the animals were anaesthetized with a lethal dose of pentobarbital. Hearts were excised and mounted on an aorta cannula of a Neely-Morgan perfusion system. Regional ischaemia was induced by ligation of the left anterior descending coronary artery for 35 minutes. The end-points used to assess myocardial protection were both infarct size and functional recovery after 30 minutes of reperfusion. The latter was assessed by perfusing the hearts in antegrade fashion and determining haemodynamic parameters such as coronary flow, aorta output, peak systolic pressure and calculated total external work. Infarct size was determined using triphenyl tetrazolium staining and expressed as a percentage of the region at risk, as determined by planimetry.

An isoproterenol dose response study was done by treating six groups of rats intraperitoneally with different concentrations of isoproterenol and using different administration protocols: Group 1 (Control) received no treatment, Group 2: 2 X 0.04 mg/kg; Group 3: 1 X 0.04mg/kg; Group 4: 2 X 0.02mg/kg; Group 5: 4 X 0.004mg/kg; Group 6: 4 X 0.0004 mg/kg. Where isoproterenol was administered repeatedly, the interval between administrations was one hour.

To investigate the role of NO in delayed preconditioning, the non-selective NO synthase antagonist N^o-nitro-L-arginine was administered intraperitoneally an hour before preconditioning with isoproterenol 4 X 0.0004mg/kg. The role of the generation of radical oxygen species in isoproterenol induced preconditioning was assessed by administration of the free radical scavengers

Melatonin(5mg/kg), Mercaptopropionylglycine (1 mg/kg) and N-Acetylcysteine (10 mg/kg). These agents were administered 5 minutes before preconditioning with isoproterenol 4X0.0004mg/kg.

Due to the observation that melatonin was a very effective cardioprotective agent, a sub-study was done to investigate its ability to protect the myocardium if administered orally in drinking water for 7 days. The duration of protection against ischaemia of orally administered melatonin was studied by assessing the effect of withdrawal of oral (40 µg/ml) melatonin for 2, 4 and 6 days. Serum levels of melatonin were measured to assess the relationship between protection against infarction and levels of the drug in the circulation. The conditions selected to measure serum levels were a control group (no pretreatment), 2.5 and 5 mg/kg melatonin intraperitoneal groups and 4, 6 day melatonin withdrawal groups.

Results: Isoproterenol induced delayed preconditioning against infarction was successfully demonstrated. Delayed preconditioning was elicited with increasing effectiveness by decreasing the dose and repeating intraperitoneal administrations. The optimal dose and protocol of isoproterenol administration was 4 X 0.0004mg/kg – hearts treated in this fashion had an infarct size of $11.3\pm 1.7\%$, which was significantly smaller than that of control rats ($38.9\pm 2.0\%$).

Following this, the mechanism of isoproterenol induced delayed preconditioning was studied.

Administration of N^o-nitro-L-arginine (17.5 mg/kg) intraperitoneally an hour before preconditioning with isoproterenol (4X0.0004mg/kg), abolished the protective effect of isoproterenol - infarct size was 36.4±3.9%, which was similar to that of the control group (38.9±2.0%). Next, the role of the generation of radical oxygen species in isoproterenol induced delayed preconditioning was studied. When N-Acetylcysteine (10 mg/kg) was administered 5 minutes before preconditioning with optimal isoproterenol dose, cardioprotection was abrogated. Melatonin (5 mg/kg) and Mercaptopropionylglycine (1 mg/kg) both had marked drug effects – control animals treated with the radical oxygen species scavengers alone had similar degrees of protection against infarction as animals preconditioned with isoproterenol. Interestingly, melatonin pre-treatment also resulted in superior functional recovery, apart from its protection against infarct size. Due to this drug effect, both these drugs were not of use to assess the role of ROS in isoproterenol induced delayed preconditioning.

The observation that melatonin was extremely effective to protect against ischaemia 24 hours after its intraperitoneal administration resulted in a sub-study which investigated the ability of orally administered melatonin to protect against myocardial ischaemia. Two different doses of melatonin in the drinking water were studied (20 and 40 µg/ml). The latter concentration proved to be equally as effective against infarct size as intraperitoneal administration of melatonin. Furthermore, withdrawal of melatonin for 2,4 and 6 days resulted in loss of the protective effect of melatonin after 2 days.

Conclusions:

1. Beta-adrenergic stimulation with isoproterenol elicits delayed myocardial preconditioning, and the optimal condition and dose of administration is $4 \times 0.0004 \text{ mg/kg}$ administered at one hourly intervals.
2. The mechanism of beta-adrenergic delayed preconditioning involves the synthesis of both nitric oxide and reactive oxygen species.
3. Melatonin administered intraperitoneally as well as orally provides potent protection against myocardial ischaemia, and results in both a decrease in infarct size and improved functional recovery.

Opsomming

Agtergrond: Miokardiale isgemie veroorsaak nekrose en apoptose van miosiete wat nie deur verdeling van die oorblywende miosiete vervang kan word nie. Die soeke na effektiewe terapeutiese strategieë wat lei tot die voorkoming van miokardiale isgemie is dus 'n belangrike doelwit. Kliniese omstandighede waartydens miokardiale isgemie ontstaan, word deur die akute fase van koronêre okklusie asook tydens hartomleiding chirurgie verteenwoordig. Die hoof probleem met bekende modaliteite van kardiaale beskerming is dat dit toegedien moet word gedurende 'n akute isgemiese voorval, wat in meeste gevalle nie logisties moontlik is nie.

Die verskynsel van isgemiese prekondisionering is 'n nuut ondekte endogene meganisme van miokardiale beskerming. Isgemiese prekondisionering verwys na die fenomeen waarby een of meer kort episodes van isgemie 'n endogene beskermingmeganisme teen miokardiale isgemie aktiveer. Isgemiese prekondisionering word gekenmerk deur twee vorme van kardiaale beskerming: 'n klassieke vorm wat onmiddellike beskerming bied en minstens 2 uur duur en 'n vertraagde vorm ("tweede venster van beskerming") wat 24 uur later ontstaan en tot 3 dae kan duur. Die tweede venster van beskerming is nie so kragtig soos die klassieke fase nie, maar bied langer beskerming. Hierdie vorm van miokardiale beskerming is juis interessant want die vermoë om dit te ontlok, bied juis 'n kliniese relevante metode vir beskerming teen isgemie, sonder om kennis te dra van wanneer die isgemiese gebeurtenis gaan geskied.

Dit is nie prakties moontlik om pre kondisionering met behulp van isgemie te ontlok nie en dus het daar belangstelling ontstaan om die seintransduksie paaie wat betrokke is, te ontrafel met die hoop om 'n farmakologiese sneller van die verskynsel te ontwikkel. Daar is verskeie nie-isgemiese snellers van pre kondisionering, soos byvoorbeeld beta-adrenergiese stimulasie, stikstof oksied, opioïede, bradikinien en adenosien. Die meeste nie-isgemiese snellers van klassieke pre kondisionering blyk om die tweede venster van beskerming te aktiveer. Dit is huidiglik nie bekend of beta adrenergiese stimulasie vertraagde pre kondisionering kan ontlok nie.

Die meganisme van vertraagde miokardiale beskerming sluit in vorming van beide stikstof oksied en vrye radikaal spesies, aangesien die beskermende effek deur inhibitore van stikstof oksied sowel as deur vry radikaal opruimers, opgehef word. Beta adrenergiese stimulasie met isoproterenol kan klassieke pre kondisionering ontlok en induseer NO sintese en genereer suurstof radikaal spesies.

Doel :

- Om te bepaal of beta-adrenergiese pre kondisionering met isoproterenol vertraagde miokardiale pre kondisionering kan ontlok
- Om te bepaal of die meganisme van beta-adrenergiese miokardiale beskerming deur vorming van stikstof oksied en vry radikaal spesies plaasvind.

Metodes: Manlike Wistar rotte (250g) is as eksperimentele diere gebruik. Isoproterenol is toegedien op Dag 1, en 24 uur later is diere verdoof met 'n noodlottige dosis van pentobarbital. Harte is verwyder en gemonteer op die aorta kannule van die Neely-Morgan perfusie sisteem. Streeksisgemie is deur afbinding van die linker anterior dalende koronêre arterie vir 35 minute teweeggebring. Die eindpunte gebruik vir die bepaling van miokardiale beskerming was infarkt grootte en funksionele herstel na 30 minute van herperfusie. Die laasgenoemde is bepaal deur harte volgens die werkhart te perfuseer met bepaling van hemodinamiese parameters soos koronêre vloei, aorta uitset, piek sistoliese druk en berekende totale eksterne werk. Infarkt grootte is bepaal deur gebruik van trifeniel tetrazolium kleuring en uitgedruk as 'n persentasie van risiko area, bepaal deur planimetrie.

'n Isoproterenol dosis respons studie is gedoen deur gebruik van verskillende konsentrasies van isoproterenol (intraperitoneaal toegedien) en gebruik van verskillende protokolle : Groep 1 (kontrole) geen behandeling, Groep 2: 2 X 0.04mg/kg; Groep 3: 1 X 0.04mg/kg; Groep 4: 2 X 0.02mg/kg; Groep 5: 4 X 0.004mg/kg; Groep 6: 4 X 0.0004mg/kg. Waar isoproterenol herhaaldelik toegedien was, is die interval tussen toedienings een uur. Die rol van stikstof oksied in vertraagde prekondisionering is ondersoek deur die nie-selektiewe stikstof oksied antagonist N^ω-nitro-L-arginien intraperitoneaal, 'n uur voor prekondisionering met isoproterenol (4x0.0004mg/kg) toe te dien. Die rol van generasie van vry radikale in isoproterenol geïnduseerde prekondisionering is ondersoek deur toediening van die vrye radikaal opruimers Melatonin (5mg/kg), Merkaptopropionielglisien (1 mg/kg) and N-Asetielsisteïen

(10mg/kg) Hierdie middels is 5 minute voor prekondisionering met isoproterenol (4x0.0004mg/kg) toegedien.

As gevolg van die waarneming dat melatonien die hart baie effektief teen isgemie beskerm het, is 'n substudie gedoen om die beskermingsvermoë van orale melatonin te ondersoek. Die duur van beskerming teen isgemie van oraal toegediende melatonin is na 2, 4 en 6 dae van onttrekking ondersoek. Serum melatonien vlakke is gemeet om die verhouding tussen beskerming teen infarksie en vlakke van middel sirkulasie te bepaal. Die toestande wat geselekteer is om serum vlakke te meet, sluit in 'n kontrole groep, melatonien 2.5 en 5 mg/kg intraperitoneaal groep en 4,6 dae melatonin ontrekkingsgroep

Resultate: Vertraagde isoproterenol geïnduseerde prekondisionering teen infarktgrootte is suksesvol gedemonstreer. Vertraagde prekondisionering is meer effektief deur kleiner dosisse en herhaalde intraperitoneale toedienings ontlok. Die optimale dosis en protokol van isoproterenol administrasie was 4x 0.0004mg/kg-harte wat op hierdie wyse behandel is, se infarktgrootte was $11.3 \pm 1.7\%$, wat beduidend kleiner was as die van kontrole rotte ($38.9 \pm 2.0\%$)($p < 0.05$).

Vervolgens is die meganisme van isoproterenol geïnduseerde vertraagde prekondisionering ondersoek. Intraperitoneale toediening van N^o-nitro-L-arginien (17.5mg/kg) een uur voor prekondisionering met isoproterenol (4x0.0004mg/kg), hef die beskermende effek van isoproterenol totaal op- infarktgrootte was $36.4 \pm 3.9\%$ wat gelyk was aan die van die kontrole groep

($38.9 \pm 2.0\%$). Die rol van generasie van vry radikaal spesies in isoproterenol geïnduseerde vertraagde prekondisionering is ondersoek deur toediening van (10mg/kg) 5 minute voor prekondisionering. Voorafbehandeling met N-Asetielsisteïen het isoproterenol-geïnduseerde beskerming opgehef. Melatonin(5mg/kg) en Merkaptopropionielglisien (1 mg/kg) het egter beide beduidende middel effekte gehad - kontrole diere behandel met suurstof radikaal spesie opruimers alleen het soortgelyke beskerming teen infarksie as geprekondisioneerde diere met isoproterenol getoon. As gevolg van hierdie middeleffekte kon melatonien sowel as merkaptopropionielglisien nie gebruik word om die rol van vry radikaalvorming te evalueer nie Melatonien behandeling het tot beide 'n verhoogde funksionele herstel, sowel as 'n afname in infarkt grootte gelei. In die substudie waar die vermoë van orale melatonin toediening om die harte teen isgemie te beskerm is twee verskillende dosisse van melatonin in die drinkwater bestudeer (20 en $40\mu\text{g/ml}$). Laasgenoemde konsentrasie is bewys om hart net so effektief te beskerm teen infarksie grootte soos intraperitoneale toediening van melatonin. Verder het onttrekking van melatonin vir meer as twee dae die verlies van beskerming tot gevolg gehad.

Opsommend:

1. Beta-adrenergiese stimulasie met isoproterenol ontlok vertraagde miokardiale prekondisionering – die optimale protokol was $4 \times 0.0004\text{mg/kg}$ toegedien elke uur.
2. Die meganisme van beta-adrenergiese vertraagde prekondisionering sluit in generasie van beide stikstof oksied en vry radikaal spesies.

3. Melatonien toediening, beide intraperitoneaal en oraal, bied kragtige beskerming teen miokardiale isgemie gekenmerk deur 'n vermindering in infarkt grootte en verbeterde meganiese herstel.

Acknowledgements

All praise is due to Allah, Most Beneficent, Most Merciful

Sincerest thanks to following persons:

My dearest grandmother (Sietie) for all the love and support

Mom (Ghaironessa) and Dad (Yaseen), all my brothers and my beloved sister
for your unconditional love and support

Extended family and other friends

Professor Johan Moolman for being a great supervisor and excellent mentor

Prof. Johan Koeslag and everyone at the Department Medical Physiology

Sonia Genade for teaching me the perfusion as well as regional ischaemia
technique

Suzel Hattingh for being a great friend and source of encouragement

Index

	Page No.
Declaration	ii
Abstract	iii
Opsomming	ix
Acknowledgements	xv
List of Tables	xxiii
List of Figures	xxv
Alphabetical list of abbreviations	xxvii
Chapter 1: Introduction	1
1.1 Classic preconditioning	4
1.1.1 Triggers of preconditioning with ischaemia	4
1.1.2 Adrenergic receptors and classic preconditioning	6
1.1.3 Beta-adrenergic PC as a trigger of classic PC	7
1.1.4 Mediators of preconditioning with ischaemia	9
1.1.4.1 p38 MAP kinase and classic preconditioning	10
1.1.4.2 PKC as a mediator of classic preconditioning	12
1.1.4.3 K_{ATP} channels and classic PC	13
1.1.4.3.1 The primary functional role of mitochondrial K_{ATP} channels	13
1.1.4.3.2 Mitochondrial K_{ATP} channels as trigger and end effector of cardioprotection	14
1.2 Second Window Of Protection (SWOP)	15
1.2.1 Characteristics of the second window of protection	15
1.2.2 Non-ischaemic delayed PC	17

1.2.3	Pharmacological stimuli of delayed PC	18
1.2.4	Triggers of delayed preconditioning	20
1.2.4.1	Adenosine	20
1.2.4.2	Nitric Oxide	22
1.2.4.3	Reactive Oxygen Species and delayed PC	24
1.2.4.4	Opioids	25
1.2.4.5	Bradykinin	25
1.2.4.6	Delayed cardioprotection by administration of catecholamines	26
1.2.5	Mediators (or Effectors) of Late PC	27
1.2.5.1	Nitric Oxide Synthase	28
1.2.5.2	Cyclooxygenase	32
1.2.5.3	Aldose Reductase and delayed myocardial protection	33
1.2.5.4	Antioxidant Enzymes and delayed preconditioning	34
1.2.5.5	Heat stress Protein and delayed preconditioning	38
1.2.5.6	K_{ATP} Channels	40
1.2.6	The Signaling Pathway of Late PC	41
1.2.6.1	Protein Kinase C	41
1.2.6.2	Protein tyrosine kinases	46
1.2.6.3	Mitogen-Activated Protein Kinases	47
1.2.6.4	Role of Kinases in Mediating Protection	50
1.2.7	Transcription factors	51
1.2.8	Summary of delayed preconditioning	53
1.3	Melatonin effects on the heart	54
1.3.1	Physiology and pharmacology	54

1.3.2	Mechanism of action of melatonin	55
1.3.3	Direct Melatonin Actions on the Heart : Antioxidative Actions	56
1.3.4	Direct Melatonin Actions on the Heart : Cardiac melatonin receptor mediated Actions	58
1.3.4	Effects of melatonin on ischaemia and reperfusion injury	61
1.4	Motivation and aims of study	65
1.4.1	The Specific aims of this study was as follows	66
Chapter 2: Material and Methods		
2.1	Animals	67
2.2	Perfusion technique	67
2.3	Perfusion buffer	68
2.4	Regional ischaemia	68
2.5	End-points of ischaemic damage	69
2.5.1	Myocardial function	69
2.5.2	Determination of infarct size as end point	69
2.6	Chemicals, drugs and reagents	70
2.7	Treatment protocols	70
2.7.1	Eliciting Delayed Beta-Adrenergic preconditioning with isoproterenol dose finding study	71
2.7.2	Investigating the role of NO in β -adrenergic delayed preconditioning	71
2.7.3	Determining the role of Reactive Oxygen Species in mediating β - adrenergic delayed preconditioning (Fig 2.4)	72

2.7.4	Investigating the protective effect of intraperitoneally administrated melatonin (Fig 2.5)	73
	(i) Effect of melatonin injected intraperitoneally on ischaemia/ reperfusion injury 24 hours later (Fig 2.5)	73
	(ii) Investigating the protective effect of orally administrated melatonin (Fig 2.6)	73
	(iii) Evaluation of the duration of the protective effect of oral melatonin of following its withdrawal (Fig 2.7)	74
2.8	Biochemical analysis	75
2.8.1	Blood collection and plasma preparation	75
2.8.2	Melatonin assay principle	75
2.8.3	Melatonin assay procedure	75
2.9	Statistical procedure	76
 Chapter 3: Results		
3.1	Determination of optimal dose of isoproterenol that elicits β adrenergic Delayed Myocardial Preconditioning	77
3.1.1	The effect of isoproterenol pre-treatment on haemodynamic parameters	77
3.1.1.1	Before sustained regional ischaemia (Table 3.1)	77
3.1.1.2	Haemodynamics parameters at reperfusion following sustained regional ischaemia (Table 3.2)	77
3.1.2	The effect of Isoproterenol pre-treatment on myocardial infarct size	78
3.1.2.1	Risk zone volume (Table 3.3)	78

3.1.2.2	Infarct size (Fig 3.1)	78
3.2	The role of eNOS in mediating β -adrenergic delayed myocardial protection	78
3.2.1	Haemodynamic function before sustained regional ischaemia (Table 3.4)	79
3.2.2	Haemodynamic parameters at reperfusion following regional ischaemia (See Table 3.5)	79
3.2.3	The effect of L-NA pre-treatment on infarct size	79
3.2.3.1	Risk zone (Table 3.6)	79
3.2.3.2	Infarct size (Fig 3.2)	80
3.3	Effects of free radical scavenger pre-treatment on delayed β -adrenergic preconditioning	80
3.3.1	The effect of ROS pre-treatment on delayed isoproterenol preconditioning	80
3.3.1.1	Haemodynamic function before ischaemia (Table 3.7)	81
3.3.1.2	Haemodynamic parameters at reperfusion following sustained ischaemia (Table 3.8)	81
3.3.2	The effect of ROS pretreatment on infarct size	81
3.3.2.1	Risk zone (Table 3.9)	81
3.3.2.2	Effect of Melatonin on infarct size (Fig 3.3)	82
3.3.2.3	Effect of MPG on Infarct size (Fig 3.3)	82
3.3.2.4	Effect of NAC on Infarct size (Fig 3.3)	83
3.4	Evaluation of melatonin as a protective agent	83
3.4.1	Intraperitoneally administration of melatonin	83

3.4.1.1	Haemodynamic parameters before sustained regional ischaemia (Table 3.10)	83
3.4.1.2	Haemodynamics after sustained regional ischaemia (Table 3.11)	84
3.4.1.3	The effect on intraperitoneal pretreatment on infarct size	84
3.4.1.3.1	Risk zone (Table 3.12)	84
3.4.1.3.2	Infarct size (Fig 3.4)	84
3.5	Effect of orally administrated doses of melatonin in drinking water	85
3.5.1	Before sustained regional ischaemia	85
3.5.1.1	Haemodynamics (Table 3.13)	85
3.5.2	Haemodynamics after sustained regional ischaemia (Table 3.14)	85
3.5.3	The effect of orally administrated melatonin on infarct size	86
3.5.3.1	Risk zone (Table 3.15)	86
3.5.3.2	Infarct size (Fig 3.5)	86
3.6	The effect of melatonin withdrawal on infarct size (Fig 3.6)	86
3.7	Biochemical results	87
3.7.1	Serum melatonin levels (Fig 3.7)	87
CHAPTER 4: Discussion		
4.1	Introduction	88
4.2	Appropriateness of the end points - infarct size and functional recovery	90

4.3	β-stimulation with isoproterenol elicits delayed cardioprotection	92
4.4	The role of nitric oxide in β-adrenergic delayed preconditioning	96
4.5	The role of oxygen radicals in mediating delayed β-preconditioning	101
4.6	The protective effect of melatonin against ischaemia	102
4.7	Mechanism of action of melatonin	106
4.8	Conclusion	107
	References	109

LIST OF TABLES

Chapter 1: Introduction

- 1.1 Melatonin summary

Chapter 3: Results

- 3.1 Haemodynamic function of isoproterenol treated hearts before regional ischaemia
- 3.2 Haemodynamic function of isoproterenol treated hearts after regional ischaemia
- 3.3 Isoproterenol pre-treated groups: Volume at risk expressed as a percentage of left ventricle volume
- 3.4 Haemodynamic function of L-NA pre-treated hearts before regional ischaemia
- 3.5 Haemodynamic function of L-NA pre-treated hearts at reperfusion following regional ischaemia
- 3.6 LNA pre-treated group: Volume at risk expressed as a percentage of left ventricle volume
- 3.7 Haemodynamic function of ROS scavenger treated hearts before regional ischaemia
- 3.8 Haemodynamic function of ROS scavenger treated hearts after regional ischaemia
- 3.9 ROS pre-treated groups: Volume at risk expressed as a percentage of left ventricle volume

- 3.10 Haemodynamic function of intraperitoneal melatonin treated hearts before regional ischaemia
- 3.11 Haemodynamic function of intraperitoneal melatonin treated hearts after regional ischaemia
- 3.12 Melatonin pre-treated intraperitoneally: Volume at risk expressed as a percentage of left ventricle volume
- 3.13 Haemodynamic function of orally melatonin treated hearts before regional ischaemia
- 3.14 Haemodynamic function of orally melatonin treated hearts after regional ischaemia
- 3.15 Melatonin pre-treated orally: Volume at risk expressed as a percentage of left ventricle volume

LIST OF FIGURES

Chapter 1: Introduction

- 1.1 A schematic illustration of experimental protocols for early and late preconditioning
- 1.2 Schematic presentation of the role of nitric oxide in delayed preconditioning
- 1.3 (a) Schematic illustration showing activation of intercellular signaling pathways classic and delayed preconditioning
- 1.3 (b) β -adrenergic signalling in cardiomyocytes.

Chapter 2: Methods

- 2.1 Perfusion protocol after completion of treatment protocols
- 2.2 Experimental protocols for optimal delayed beta-adrenergic preconditioning with isoproterenol
- 2.3 Experimental protocols for the role of NO in delayed beta - adrenergic preconditioning
- 2.4 Free radical scavenger (Mel, MPG & NAC) pre-treatment and beta-adrenergic delayed myocardial protection
- 2.5 Experimental protocols for delayed preconditioning with intraperitoneal administration of melatonin
- 2.6 Experimental protocols for effects of melatonin supplementation in drinking water and effects on infarct size and functional recovery

- 2.7 Experimental protocols for effects of melatonin withdrawal on delayed myocardial preconditioning

Chapter 3: Results

- 3.1 The effect of isoproterenol pre-treatment on infarct size
- 3.2 The effect of L-NA pre-treatment on infarct size
- 3.3 The effect of ROS scavenger pre-treatment of infarct size
- 3.4 The effect of intraperitoneally administration of Melatonin on infarct size
- 3.5 The effect of orally supplemented melatonin on infarct size
- 3.6 The effect of melatonin withdrawal on infarct size
- 3.7 The effect of melatonin treatment on serum values

ABBREVIATIONS LIST**Units of measurement:**

%	percentage
μ l	microliter
μ g	microgram
ml	millilitre
g	gram
g w w	gram wet weight
M	Molar
mg	milligram
min	minutes
h	hours
pg	picogram
pmol	picomol
mmol	millimol

Chemical compounds

Ca^{2+}	calcium
$\text{CaCl}_2 \cdot \text{H}_2\text{O}$	calciumchloride 2-hydrate
CO_2	Carbondioxide
H_2O	water
K^+	potassium
KCl	potassium chloride
KH_2PO_4	potassium dihydrogenphosphate
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	magnesiumsulphate 7-hydrate
NaCl	sodium chloride
NaHCO_3	sodiumbicarbonate
O_2	Oxygen
TRIS	tris(hydroxymethyl) aminomethane hydrochloride

Other abbreviations:

A/R	Anoxia and reperfusion
ERK	Extracellular regulated kinases
PKC	Protein kinase C
MAPK	Mitogen-activated protein kinase
ROS	Reactive oxygen species
NO	Nitric oxide
NOS	Nitric oxide synthase
I/R	Ischaemia and reperfusion

CHAPTER 1

Introduction

Acute coronary occlusion is the leading cause of morbidity and mortality in the Western world and according to the World Health Organization will be the major cause of death in the world by the year 2020 (Murray & Lopes 1997). Thus, in the United States and other economically developed countries of the world, cardiovascular disease is reported to be the major cause of disability and mortality (Bazzano *et al.*, 2003). The four leading clinical syndromes of ischaemic heart disease are angina pectoris, acute myocardial infarction, chronic post ischaemic cardiac failure and sudden ischaemic death. Ischaemia due to atherosclerotic plaque rupture or vasospasm of sufficient duration results in severe damage to the myocardium which causes cellular injury and eventually cell death due to apoptosis and/or necrosis. Acute myocardial ischaemia also has two other important consequences: failure of contraction and arrhythmias.

Current strategies to combat the deleterious consequences of ischaemic injury include anti-arrhythmic drugs, anti-thrombotic agents, organic nitrates (nitroglycine), beta-receptor antagonists and calcium antagonists. These treatments generally alleviate, but do not prevent ischaemic damage. An endogenous protective mechanism against ischaemia does exist in the myocardium. This mechanism enables the myocardium to adapt to transient ischaemic stress by changing its phenotype in a manner that makes it resistant to ischaemic injury. This powerful endogenous intracellular defence mechanism is known as ischaemic preconditioning.

Ischaemic preconditioning is a phenomenon whereby short periods of ischaemia and reperfusion induce protection of the heart from the deleterious consequences of subsequent prolonged ischaemia (Murray *et al.*, 1986). Two types of preconditioning have been recognized - classic preconditioning and delayed preconditioning (also called "second window of protection"). Classic preconditioning provides immediate cellular protection for one hour of duration (Downey *et al.*, 1997a) but protection disappears altogether 2h after the preconditioning ischaemia (Van Winkle *et al.*, 1991). Ischaemic preconditioning protects myocytes against ischaemic cell death (Murry *et al.*, 1986), and arrhythmias (Shiki & Hearse, 1987). Following this early phase of protection a late (or delayed) phase that becomes apparent 12 to 24 hours later, which lasts 3 to 4 days (Kuzuya *et al.*, 1993, Marber *et al.*, 1993). This late phase of cardioprotection is called delayed preconditioning or "second window of protection" (abbreviated "SWOP").

Classic preconditioning represents an adaptive response to potentially noxious stimuli and involves activation of endogenous defence mechanisms. Although much is known about the cellular basis of this adaptation, there are still large gaps in our knowledge. Different plausible hypotheses have been proposed and several mechanisms seem to be involved. Cell surface receptors, mitochondrial K_{ATP} channels, free radicals, and protein kinase C all play a pivotal role in the signalling of protection.

Due to its potential clinical importance, the second window of protection has generated considerable interest. If the mechanism of this adaptive response can be elucidated, it could be possible to exploit it pharmacologically to develop pre-emptive

strategies to protect the myocardium against situations where ischaemia arise. Clinical examples of this are patients at risk of having a myocardial infarction and patients undergoing to bypass surgery.

Both classic and delayed preconditioning can be elicited by interventions other than brief ischaemia, such as pharmacological manipulations with nitric oxide donors (Takano *et al.*, 1998), opioids receptor agonists (Fryer *et al.*, 1999), adenosine receptor agonists (Baxter *et al.*, 1994), endotoxin (Brown *et al.*, 1990), rapid cardiac pacing (Kaszala *et al.*, 1996), exercise (Yamashita *et al.*, 1999) and heat stress (Currie *et al.*, 1988). Beta-adrenergic stimulation can also elicit classic preconditioning (Lochner *et al.*, 1999, Nasa *et al.*, 1997), but it is currently not known whether it elicits delayed preconditioning.

Over the past 15 years, it has been attempted to elucidate mechanisms of both early and late ischaemic preconditioning (Cohen *et al.*, 2000, Bolli R, 2000). The aim was to develop pharmacological agents that stimulate second messenger pathways thought to be involved in preconditioning (but without causing ischaemia), in order to develop novel approaches to prevent ischaemic damage of the myocardium.

In view of this, it was our aim to:

- (i) investigate whether beta-adrenergic stimulation elicits delayed preconditioning
- (ii) elucidate the mechanism thereof.

1.1 Classic preconditioning

1.1.1 Triggers of preconditioning with ischaemia

A trigger of preconditioning is considered to be a substance released during ischaemia and/or during reperfusion that stimulates signalling pathways in the myocyte and cause cellular changes that allow myocytes to survive a prolonged episode of subsequent ischaemia. Downey *et al.* (1997b) first demonstrated that the protection of ischaemic PC was receptor mediated. Adenosine is a breakdown product of ATP and occurs in high concentrations in ischaemic tissue (Van Wylen *et al.*, 1990). Evidence supporting the role of adenosine as a trigger of classic preconditioning came from Liu *et al.*, 1991 who showed that adenosine receptor blockade with 8-(p-sulphophenyl)-theophylline (8-SPT) abolished the infarct - limiting effect of preconditioning in rabbit heart (this was confirmed by Cohen *et al.*, 1994). It was subsequently shown that transient adenosine A₁ receptor (but not A₂ receptor) activation with selective agonists reproduced the infarct - limiting effect of ischaemic preconditioning in the rabbit, rat and dog (Liu *et al.*, 1991, Thornton *et al.*, 1992, Tsuchida *et al.*, 1992, Auchampach *et al.*, 1993). A 5 min infusion of adenosine or A₁ receptor agonist N-6 (phenyl-2R-isopropyl) adenosine was as effective as 5 min of ischaemia in protecting against infarct size (Cohen *et al.*, 1994). Pharmacological evidence also supports a role for adenosine A₃ receptor activation in classic ischaemic preconditioning (Liu *et al.*, 1994, Armstrong & Ganote, 1994, Armstrong & Ganote, 1995).

The adenosine A₁ receptor also seems to be involved in delayed PC (Baxter *et al.*, 1994). In a study in which non-preconditioned rabbits received a single intravenous bolus of the highly selective adenosine₁ receptor agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA) or saline vehicle 24 hours prior to index coronary artery occlusion, increasing doses of CCPA in the range 25-100 µg/kg resulted in progressive reduction in infarct size compared to the saline treated animals, with maximum protection observed at a dose of 100 µg/kg. CCPA has a 10000-fold selectivity for the A₁ versus A₂ receptor and a subnanomolar affinity. A subsequent study confirmed that this delayed action of A₁ receptor activation was mediated in the myocardium and not via peripheral actions of adenosine, as late protection against ischaemia - reperfusion injury was also evident in the isolated Langendorff-perfused rabbit hearts (Baxter *et al.*, 1997).

Results from experiments in which various receptor agonists and antagonists were used suggest that several others triggers are involved, including: bradykinin (Wall *et al.*, 1996), catecholamines (Bankwala *et al.*, 1998), free radicals (Baines *et al.*, 1997), angiotensin II (Noda *et al.*, 1993) nitric oxide (Vegh *et al.*, 1993) and opiates (Schultz *et al.*, 1997).

All these agonists are produced during ischaemia in the myocardium of experimental animals and are therefore likely to play a role in initiating the protective effects. It seems possible that these breakdown products are released from the ischaemic myocardium, and act in a paracrine fashion to

activate protective cascades. In the rat adrenergic and opioid signalling seem dominant, whilst adenosine and bradykinin signalling are more important in rabbit myocardium (Edwards *et al.*, 2000).

1.1.2 Adrenergic receptors and classic preconditioning

Activation of the adrenergic receptors has been proposed to play a role in ischaemic preconditioning. The possibility that catecholamine release was an important trigger for preconditioning was entertained early. Adrenergic receptors can be divided into three major groups, α_1 , α_2 and β -adrenoceptors. The α_1 group can be sub-divided into four subtypes - α_{1a} , α_{1b} , α_{1c} and α_{1d} respectively. Banerjee *et al* (1993) investigated the role of the α_1 -adrenergic receptor in preconditioning. They reported that norepinephrine induced preconditioning was blocked by an α_1 adrenergic receptor antagonist. Nonselective α_1 agonism with phenylephrine protected rabbit myocardium from subsequent ischaemic injury (Tsuchida *et al.*, 1994). This protection could be blocked by chloroethylclonidine, a selective α_{1b} antagonist, whereas methoxamine, an α_{1a} -selective agonist, failed to protect in this model. These results suggested that activation of α_{1b} receptors alone caused cardioprotection, or that full α_1 agonism was required to exert protection. In a canine model of myocardial infarction, however, methoxamine limited infarct size to a similar degree to that obtained with ischaemic preconditioning (Kitikaze *et al.*, 1994). The dose employed in this particular study, however, may have been sufficiently high to allow methoxamine to act as a less selective agonist. Both preconditioning with ischaemia and pre-treatment with methoxamine

caused an increase in 5' nucleotidase activity. 5' -Nucleotidase can be inhibited by α , β -methyleneadenosine 5-diphosphate, but α , β -methyleneadenosine 5-diphosphate, which inhibits the protection afforded by methoxamine, does not attenuate the cardioprotective effects afforded by ischaemic preconditioning. Thus, the mechanism through which cardioprotection is achieved appears to be different for methoxamine and ischaemia.

1.1.3 **Beta-adrenergic PC as a trigger of classic PC**

Asimakis *et al* (1994) showed that beta-adrenergic receptor activation could elicit cardioprotection, using functional recovery as endpoint. However, it should be noted that in these experiments, the beta-blocker propranolol did not abolish the protective effect of ischaemic preconditioning.

A study by Nasa *et al.* (1997) investigated whether adrenergic stimulation mimicked preconditioning cardioprotection. In this study rat hearts were perfused for 2 minutes with either norepinephrine, phenylephrine or isoproterenol followed by a 10 minute drug free perfusion. Pre-perfusion with norepinephrine (0.25 μ M) or isoproterenol (0.25 μ M), but not phenylephrine (10 μ M) resulted in a better recovery of left ventricular developed pressure in the post-ischaemic reperfused heart. Both the norepinephrine and isoproterenol pretreated groups had a reduction in creatine kinase release (measure of myocardial damage) and a similar improvement of post-ischaemic cardiac contractile dysfunction as the

hearts subjected to 5 minutes of ischaemia followed by 5 minutes of reperfusion (i.e. ischaemic preconditioning) before prolonged ischaemia. Pretreatment of hearts with timolol, a beta-blocker, abolished the protective effects of norepinephrine, whereas pretreatment with bunazosun, an α -1 adrenoceptor blocker, did not affect the protective effects of isoproterenol. These results suggested that brief stimulation of cardiac beta-adrenoceptors were responsible for the preconditioning - mimetic protective effect against post-ischaemic contractile dysfunction in perfused rat hearts.

Lochner *et al* (1999) showed that beta-adrenergic blockade with alprenolol bracketing one ischaemic episode of 5 minutes to trigger ischaemic preconditioning, partially abrogated the protective effect - indicating a role for the beta-adrenergic receptor in ischaemic preconditioning. Furthermore, 5 minutes of transient beta-adrenergic stimulation with isoproterenol at a dose of 10^{-7} M elicits classic preconditioning. The mechanism through which beta- receptor stimulation protects seems to be via the activation of p38 MAP kinase. Evidence for this is provided by observation in our laboratory that bracketing of either a single episode (1 x 5 min) ischaemic preconditioning or isoproterenol-induced pharmacological preconditioning by the p38 MAP kinase inhibitor SB 203580, abolished cardioprotection (Marais *et al.*, 2001). In these experiments, functional recovery was significantly reduced in both SB 203580 pretreated groups - cardiac output was reduced to 15.7 ± 1.6 and 18.9 ± 0.3 ml/min for single episode (1 x 5min) ischaemic preconditioning

and isoproterenol-induced preconditioning respectively by SB203580 pretreatment compared to values of 23.9 ± 1.3 and 29.7 ± 1.5 ml/min respectively if no SB203580 was administered before any of the preconditioning protocols.

The administration of noradrenaline 24h, but not 4h, prior to ischaemia has been shown to result in enhanced contractile function in isolated perfused rat hearts subjected to ischaemia and reperfusion (Meng *et al.*, 1993). It also resulted in reduced arrhythmia severity following coronary artery occlusion (Ravingerova *et al.*, 1995); marked increases in c-fos and c-jun mRNA levels and increased in hsp70 gene expression (Meng *et al.*, 1995) in delayed preconditioning. The noradrenaline activated, and α_1 -adrenoreceptor-mediated, cardiac oncogene and stress protein gene expression is believed to be responsible for the delayed protection. In summary, catecholamine administration results in delayed protection of the heart against myocardial ischaemia. To date there is no evidence that β -stimulation with isoproterenol can trigger delayed myocardial preconditioning in the isolated rat heart.

1.1.4 Mediators of classic preconditioning with ischaemia

Whereas a trigger of preconditioning operates during the short ischaemic period as well as the reperfusion following it, mediators are regarded as those intracellular events that are vital to mediate protection during the sustained ischaemic phase. There are 3 main candidate mediators in classic preconditioning: p38 MAP kinase, the mitochondrial K_{ATP} channel

(Downey *et al.*, 2001, Fryer *et al.*, 2001) and specific activated forms of PKC (Downey, 1997a)

None of these candidate mediators can be explained by the synthesis of new proteins. In fact, there is strong evidence against a role for gene activation and protein synthesis:

- (1) Preconditioning can be induced quickly, e.g. by 3 to 5 minutes of ischaemia and 5 minutes of reperfusion.
- (2) Inhibiting protein synthesis with cyclohexamide and RNA synthesis with actinomycin D (Thornton *et al.*, 1990) have no effect on preconditioning with ischaemia. This indicates that effective gene activation has not occurred in the brief interval required to precondition in classic preconditioning.

1.1.4.1 p38 MAP kinase and classic preconditioning

The role of p38 MAP kinase in classic PC has been investigated extensively (Maulik *et al.*, 1998, Nakano *et al.*, 2000). Published observations regarding the role of p38 MAP kinase in classic PC are controversial and inconsistent. The two lines of studies performed thus far have sought to determine whether (1) preconditioning induces the activation of p38 MAP kinase during sustained ischaemia and (2) whether inhibition of p38 MAP kinase abrogates the cardioprotective effects.

It has been reported (Weinbrenner *et al.*, 1997, Maulik *et al.*, 1998, Nakano *et al.*, 2000) that phosphorylation of p38 MAP kinase during

preconditioning is increased during sustained ischaemia. Weinbrenner and colleagues (1997) preconditioned rabbit hearts with 5 min ischaemia and 10 min reperfusion. They observed enhanced phosphorylation of p38 MAP kinase after 10 and 20 minutes of index ischaemia in protected hearts. In contrast, Ping et al (1999a) observed that p38 MAP kinase activation did not correlate with the preconditioning effect, thereby questioning the significance of p38 MAP kinase in preconditioning. Reports by Saurin et al (2000) also argued against a beneficial role for p38 α MAP kinase activation during sustained ischaemia: inhibition of p38 α MAP kinase during sustained ischaemia reduced reperfusion injury and contributed to preconditioning induced cardioprotection. In our own laboratory it was shown that the protective effect of preconditioning in rats was accompanied by a reduced activation of p38 MAP kinase during 25 min index ischaemia, and that the p38 MAP kinase inhibitor, SB203580, protected effectively against ischaemic damage if infused prior to index ischaemia in non-preconditioned hearts (Marais et al., 2001). Further work in our laboratory showed that p38 MAP kinase activated the small heat shock protein, HSP27, and that this activation actually occurred during the triggering phase of preconditioning (unpublished observations). The precise role of p38 MAP kinase is thus unclear at the moment. Targeted activation of individual p38 MAP kinase isoforms will be essential to provide conclusive evidence to either support or refute the role of p38 MAP kinase in both classic and delayed PC.

1.1.4.2 PKC as a mediator of classic preconditioning

Most of the agonists which generate the signal of (i.e. the triggers) preconditioning bind to heptahelical transmembrane receptors (Sugden *et al.*, 1995). The intracellular messenger systems linked to these receptors are relatively well characterized and have now been investigated with regard to myocardial preconditioning (Cohen & Downey, 1996). When an agonist for example, adenosine or bradykinin binds, a receptor-coupled G protein is activated (Cohen & Downey, 1996). This dissociates and in turn activates a membrane bound phospholipase, which cleaves phosphatidylinositol bisphosphate into inositol trisphosphate and diacylglycerol (DAG). DAG then activates protein kinase C – this step is believed to play a central role in ischaemic preconditioning. Protein kinase C (PKC) is well placed to play a key role in cellular protection. It is known to regulate numerous biological processes such as metabolism, myocyte contraction, ion transport, gene expression and is coupled to the receptors of many agonists of preconditioning (Cohen & Downey, 1996). Ping *et al* (1997) reported PKC ϵ translocation and activation during ischaemic preconditioning, thus implicating an important role for it in the genesis of classic preconditioning. The importance of PKC ϵ translocation was elegantly confirmed in experiments in transgenic mice (Saurin *et al.*, 2002). These authors investigated whether preconditioning still occurred in a mouse line lacking cardiac PKC-epsilon protein due to a targeted disruption within the PKC-epsilon allele. Mice were preconditioned by 4 x 4 min ischaemia/6 min reperfusion, and then underwent 45 min of global ischaemia followed by 1.5 h of reperfusion. In PKC-epsilon (-/-) hearts

preconditioning failed to diminish infarction compared to controls (36.4 ± 2.9 vs. $38.8 \pm 4.5\%$), whereas PKC epsilon (+/-) mice displayed partial protection.

1.1.4.3 K_{ATP} channels and classic PC

1.1.4.3.1 The primary functional role of mitochondrial K_{ATP} channels

The K_{ATP} channels have been described in many tissues including pancreatic β -cells, neurons, vascular smooth muscle, skeletal muscle and cardiomyocytes. K_{ATP} channels are closed by ATP in the low μ -molar-concentration range and open as ATP levels fall (Trapp & Ashcroft, 1997). The physiological function of K_{ATP} channels remains conjectural, but two such functions have been proposed. First, a concerted action of the electrophoretic K⁺ uniport and the electro neutral K⁺/ H⁺ exchange is believed to maintain K⁺ homeostasis within the mitochondrion and therefore control mitochondrial volume. The regulatory control of volume changes is important for metabolic control at mitochondrial level (Halestrap, 1989). Observations that ATP inhibits swelling, whereas K_{ATP} channels opens potentiate swelling, make it likely that this channel, perhaps together with other K⁺ pathways, is involved in mitochondrial regulatory volume changes. The respiring mitochondria transports H⁺, thus generating both the transmembrane potential and the pH gradient. The second putative role of mitochondrial K_{ATP} channels is based on the observation that energization of mitochondria is accompanied by uptake of K⁺, which can be partially inhibited by glibenclamide and activated by

potassium channel openers. This fact is consistent with the hypothesis that K^+ uptake upon energization is responsible for partial compensation of the electric charge transfer produced by the proton pump, thus enabling the formation of ΔpH along with $\Delta \Psi$. Despite the fact that the mitochondrial K_{ATP} channel has been characterized pharmacologically in cells, to date it has not been cloned and its molecular structure remains unknown and its very existence is questioned (Das *et al.*, 2003, Lim *et al.*, 2002).

1.1.4.3.2 Mitochondrial K_{ATP} channels as trigger and end effectors of cardioprotection

Gross and Fryer (1999) reported that sulphonylurea receptor antagonist could abolish ischaemic preconditioning induced protection, suggesting that these channels might be effectors of cardioprotection. This idea was reinforced by the observation that K_{ATP} channel openers like cromakalim mimicked protection (Grover & Garlid, 2000). This indicated an important trigger action of these channels. The exact mechanism through which mitochondrial channel opening results in cardioprotection needs to be investigated further.

The end effector(s) of ischaemic preconditioning have been very elusive. Considerable evidence suggests that opening of K_{ATP} channels represent the final step in this signal transduction process. Evidence supporting the K_{ATP} channels hypothesis is based on blockade of protective effects of both ischaemic and pharmacological preconditioning by inhibitors of the

K_{ATP} channels (Grover, 1997). Structurally diverse mitochondrial K_{ATP} channel openers exert cardioprotective effects in various animal models of ischaemia (Grover, 1994). These protective effects on ischaemic myocytes are direct and independent of vasodilatory activity. These cardioprotective effects are universally abolished by the K_{ATP} blockers such as glibenclamide (Auchampach *et al.*, 1992a).

There are, however, divergent views on the role of the mitochondrial K_{ATP} channel as end effector. Thornton *et al* (1993b) were unable to prevent the anti-infarct effect of preconditioning with glibenclamide in pentobarbital-anaesthetized rabbits. However, with ketamine-xylazine anaesthesia of rabbits, glibenclamide completely abolished protection (Walsh *et al.*, 1994). The exact cardioprotective role of mitochondrial K_{ATP} channels is not clear and needs to be investigated further.

1.2 Second Window Of Protection (SWOP)

1.2.1 Characteristics of the second window of protection as elicited by ischaemia

The protective effect of ischaemic preconditioning has a bimodal distribution. As stated above, the initial or classic window of preconditioning begins within minutes of the initial preconditioning insult, but is lost after 1-2h. (Li *et al.*, 1992, Murry *et al.*, 1986). However, 12-24h following the preconditioning ischaemia or stimulus, a delayed phase of protection can be observed (Kuzuya *et al.*, 1993). The protective effect of

classic preconditioning is strong, but of limited duration (See Figure 1). In contrast, this second window of protection (SWOP) (Yellon, 1995), is not as powerful as the classic phase, but confers protection lasting for up to 72h (Marber *et al.*, 1993, Kuzuya *et al.*, 1993, Baxter *et al.*, 1997). Following initial reports in dogs (Kuzuya *et al.*, 1993) and rabbit (Marber *et al.*, 1993), delayed preconditioning against infarction *in vivo* has been observed in most species examined so far (pig, rat and mouse). In almost all studies, several cycles of preconditioning have been adopted, with an index ischaemic insult of 30-60 minutes applied 24 hours later. Reduction in infarct size with such protocols was around 45%. Miki *et al* (1999) reported that in conscious rabbits, preconditioning induced a delayed modest reduction in infarct size. However, others failed to demonstrate delayed preconditioning against infarction studies in rabbits - Tanaka *et al* (1994) demonstrated the protective effects of classic preconditioning in this model (a 72% reduction in infarct size), but no protection was observed 24 or 48 hours later. Delayed preconditioning has also been demonstrated in large animals - Qui *et al* (1997b) examined the effects of repeated brief coronary occlusions (2 x 10 minutes) in conscious pigs 24 hours before 40 minute occlusion. In the preconditioned group a modest, 26% reduction in infarct size was reported, which was not statistically significant from controls in the study.

Delayed preconditioning has also been described in the rat, the experimental animal used in our study. Yamashita *et al.* (1998b) showed that preconditioning with two 3 minutes coronary artery occlusions, each

separated by 5 minute reperfusion, protected against a 30 minute occlusion 24 hours later. Infarct size was reduced by 34% in the preconditioned group relative to the controls. In a subsequent study, the same group reported that preconditioning with four 3 minute coronary artery occlusions induced delayed protection with a 46% relative reduction in infarct size (Yamashita *et al.*, 2000a).

The second window of protection confers protection against other endpoints of injury apart from infarction such as reperfusion arrhythmias and myocardial stunning. Global preconditioning of canine heart with four 5 minutes periods of rapid ventricular pacing resulted in marked protection against coronary occlusion and reperfusion arrhythmias 20 hours later (Vegh *et al.*, 1994). Reduction of stunning, another end point of delayed preconditioning, has been described by the Bolli group (Sun *et al.*, 1995). These workers used conscious pigs to investigate post-ischaemic myocardial stunning lasting 3-4 hours. Repetition of the protocol in the same animals 24 hours later revealed that post-ischaemic recovery of contractile function was significantly accelerated compared with recovery following the initial protocol. It therefore appeared that the initial stunning protocol preconditioned against a subsequent stunning protocol 24h later.

1.2.2 Non-ischaemic delayed PC

Delayed protection against myocardial ischaemia/reperfusion injury can be induced by a wide variety of non-ischaemic stimuli, which can broadly be classified as nonpharmacological and pharmacological. The former

includes heat stress (Currie *et al.*, 1988), rapid ventricular pacing (Kaszala *et al.*, 1996), and exercise (Yamashita *et al.*, 1999). Reports on infarction demonstrate that non-ischaemic delayed preconditioning protects similarly to ischaemic delayed PC.

1.2.3 Pharmacological stimuli of delayed PC

Pharmacological agents that can elicit delayed preconditioning consist of naturally occurring and often noxious agents such as endotoxin (Brown *et al.*, 1989), interleukin-1 (Brown *et al.*, 1990), TNF α (Brown *et al.*, 1992), TNF- β (Nelson *et al.*, 1995), leukemia inhibitory factor (Nelson *et al.*, 1995), ROS (Sun *et al.*, 1995), and of clinically applicable drugs such as NO – releasing agents (Takano *et al.*, 1998b), adenosine receptor agonists (Baxter *et al.*, 1994), endotoxin derivatives such as monophosphoryl lipid A [MLA] (Yoa *et al.*, 1993) and opioid receptor agonists (Fryer *et al.*, 1999).

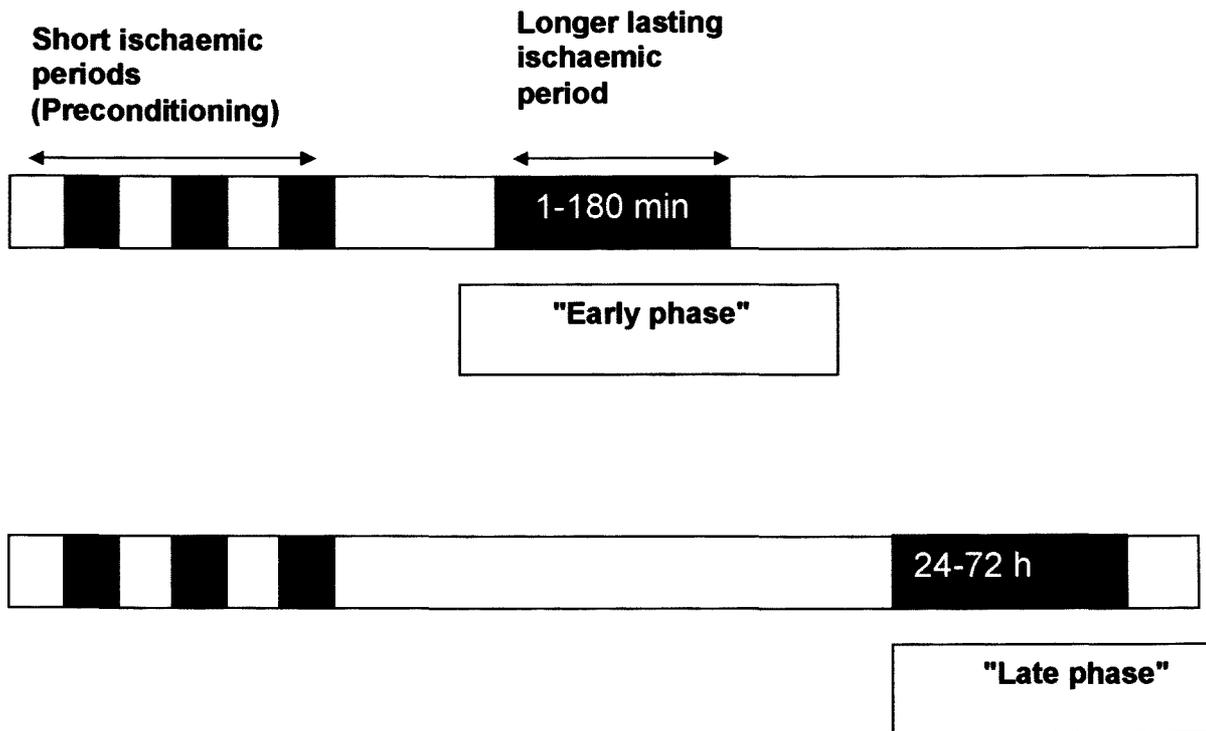


Figure 1.1: A schematic illustration of experimental protocols for early and late preconditioning (white = ischaemia, blue = ischaemia)

1.2.4 Triggers of delayed preconditioning

Brief myocardial ischaemia and ensuing reperfusion are associated with major metabolic perturbations that result in generation of a wide variety of metabolites and ligands. These substances warn the myocardium that a danger is imminent, essentially acting as a cellular alarm system to which the heart responds by switching to a defensive phenotype.

1.2.4.1 Adenosine

Adenosine has long been proposed as a "regulatory metabolite" in ischaemia (Bern *et al.*, 1963), in recognition of its ability to limit myocardial oxygen demand by causing negative inotropy and chronotropy and by increasing oxygen delivery through vasodilation.

The concept that adenosine released during the PC stimulus triggers the development of delayed protection was first proposed by Baxter *et al.* (1994). Activation of the adenosine receptors protects against infarct size, but is neither necessary nor sufficient to trigger late PC against stunning (Sun *et al.*, 1995, Auchampach *et al.*, 1999, Maldonado *et al.*, 1997). Whether only one or both of these adenosine receptor subtypes (A₁ and A₃) contributes to triggering ischaemia-induced late preconditioning is still unknown, because 8-p-(sulfophenyl) theophylline (the only adenosine receptor antagonist shown to block the development of late preconditioning after ischaemic stress) (Baxter *et al.*, 1994) is not selective for either of the two receptors. The adenosine agonist 2-chloro-N⁶ cyclopentyladenosine (CCPA) is highly selective for A₁ receptors. Baxter and co-workers (1994) were the first to demonstrate adenosine receptor

involvement in the delayed phase of myocardial protection 24h after ischaemic preconditioning. They also demonstrated the temporal nature of this delayed effect with adenosine A₁ receptor agonist, which lasted up to 72 hours (Baxter *et al.*, 1997). Recent studies indicate that delayed protection against infarction can also be triggered by a selective adenosine A₃ receptors agonist (Takano *et al.*, 2001). Thus, it appears that pharmacological stimulation of either A₁ or A₃ receptors can elicit late preconditioning against infarction.

The majority of pharmacological studies imply an anti-ischaemic effect of acute adenosine A₃ receptor activation (Liu *et al.*, 1994, Armstrong and Ganote, 1994, Armstrong and Ganote, 1995, Carr *et al.*, 1997, Auchampach *et al.*, 1997, Tracey *et al.*, 1997, Dougherty *et al.*, 1998). Although recent evidence suggests that the protective actions of A₃ receptor agonists could be mediated by A₁ receptor activation, this has not been conclusively proven (Guo *et al.*, 2001). Takano *et al* (2001) have undertaken the pharmacological and molecular characterisation of adenosine receptor participation in delayed protection. They reported that the A₃ receptor agonist IB-MECA (100 or 300 µg/kg), given to rabbits 24h before coronary artery occlusion, resulted in limitation of infarction, comparable to that seen with the A₁ agonist, CCPA.

1.2.4.2 Nitric Oxide

The first indication that NO triggered late PC was provided by a study in which administration of *N*^o-nitro-L-arginine (L-NA), a nonselective inhibitor of all 3 NO synthase(NOS) isoforms (neural (nNOS), endothelial (eNOS) and inducible(iNOS), before the PC ischaemic stimulus was found to block the development of delayed protection against myocardial stunning (Bolli *et al.*, 1997). A subsequent study demonstrated that NO was also necessary to trigger ischaemia induced late preconditioning against myocardial infarction (Qui *et al.*, 1997). Pre-treatment with NO donors in the absence of ischaemia induced a delayed protective effect against both myocardial stunning and infarction that was indistinguishable from that observed during the late phase of ischaemic preconditioning (Takano *et al.*, 1998, Banerjee *et al.*, 1999, Guo *et al.*, 1999 and Hill *et al.*, 2000). Administration of nitroglycerine can elicit late PC both by the intravenous and transdermal route (Hill *et al.*, 2000), and this effect is not abrogated by the development of nitric tolerance, indicating that different mechanisms underlie the haemodynamic and preconditioning actions of nitrates (Hill *et al.*, 2000). The ability of NO - releasing agents such as nitrates to faithfully mimic late phase of ischaemic PC despite nitrate tolerance supports the possibility of novel clinical applications of these drugs.

Recent studies (Xuan *et al.*, 2000) have provided direct evidence of enhanced biosynthesis of NO in myocardium subjected to brief episodes of ischaemia/reperfusion. The source of increased NO formation during

the PC ischaemia is likely to be eNOS, since the development of late PC is blocked by pretreatment with the nonselective NOS inhibitor L-NA, but not with the relatively selective iNOS inhibitors aminoguanidine and S-methylisothiurea (Bolli *et al.*, 1997).

Interestingly, the development of ischaemic late PC is not affected by pretreatment with the guanylate cyclase inhibitor ODQ (Kodani *et al.*, 2000) but is completely prevented by pre-treatment with the antioxidant mercaptopropionyl glycine (MPG)(Takano *et al.*, 1998, Tang *et al.*, 1997). NO is known to react rapidly with O_2^- to form peroxynitrite anion (ONOO⁻), which then protonates and decomposes to generate the hydroxyl radical (\cdot OH) or some other potent oxidant with similar reactivity (Bechman *et al.*, 1990, Crow *et al.*, 1995). Because MPG scavenges both ONOO⁻ and \cdot OH(Crow *et al.*, 1995, Sun *et al.*, 1993), the ability of MPG to block late preconditioning (Takano *et al.*, 1998, Tang *et al.*, 1997) coupled with the failure of ODQ to do so, (Kodani *et al.*, 2000) suggest that NO triggers this response via formation of (ONOO⁻) and/ or secondary ROS, rather than via cGMP-dependent pathways.

In the rabbit, Dana *et al* (2002) found that the NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME) administered prior to an adenosine A₁ agonist did not prevent the development of tolerance to ischaemia, suggesting that adenosine A₁ receptor activation and NO are independent co-activators of the delayed ischaemic preconditioning response.

1.2.4.3 Reactive Oxygen Species and delayed PC

The concept that the generation of ROS during the PC ischaemia was essential to trigger delayed protection was first proposed by Sun *et al.* (1996). These investigators demonstrated in conscious pigs that the administration of a combination of the antioxidants superoxide dismutase (SOD) plus catalase plus MPG during the initial ischaemic challenge prevented the development of late preconditioning against stunning. Similar findings were obtained in rabbits with MPG alone. (Tang *et al.*, 1997). MPG has also been found to prevent ischaemia-induced late PC against infarction (Yamashita *et al.*, 1998), arrhythmias (Yamashita *et al.*, 1998), and coronary endothelial injury (Kaeffer *et al.*, 1997), as well as heat stress-induced (Yamashita *et al.*, 1998) and exercise-induced (Yamashita *et al.*, 1999) late preconditioning against infarction. These findings implicate ROS as initiators of these forms of delayed protection as well. Conversely, intracoronary infusion of a ROS-generating solution in rabbits elicits a late PC response (Takano *et al.*, 1997). Taken together, these results suggest that sublethal oxidative stress plays a useful role by triggering delayed cardioprotection. Further studies will be necessary to determine the source(s) and the identity of the ROS responsible for initiating the late PC, and whether NO and ROS are parts of the same mechanism (i.e. whether ROS are derived from the reaction of NO with O₂, as discussed above) or act in parallel as an independent trigger.

1.2.4.4 Opioids

The evidence for a role for opioids in delayed preconditioning is still limited, but a number of studies have recently shown that opioids can act as a trigger for this phenomenon. Recent data in rats (Fryer *et al.*, 1999) and mice (Guo *et al.*, 2000) indicate that pharmacological activation of δ_1 -opioid receptors induces a delayed infarct-sparing effect 24 to 48 hours later. In this regard Gross and colleagues (1999) demonstrated that δ_1 -opioid receptor agonist (TAN-167) induced protection via activation of mitochondrial K_{ATP} channels as cardioprotection was abolished by 5HD but not glibenclamide. It was demonstrated that protection was dependent upon δ_1 receptor stimulation immediately following administration of the opioid agonist and upon receptor reoccupation immediately prior to index ischaemia (Fryer *et al.*, 1999). Opioids are thought to mediate cardioprotection in rats (Fryer *et al.*, 2001), pigs (Schulz *et al.*, 1998) and rabbits (Miki *et al.*, (1998) It was later shown that this cardioprotective effect was mediated by activation of the MAP kinases, ERK and p38 MAP kinase. These ongoing studies are important, as the use of agents such as δ_1 -opioid receptor agonists may be of clinical relevance in the setting of patients with acute coronary symptoms who are at risk of myocardial ischaemia

1.2.4.5 Bradykinin

Bradykinin is well established as a mediator of classic preconditioning and there is limited evidence to suggest that it may also contribute to delayed preconditioning. Ebrahim *et al* (2001) have shown that administration of

bradykinin to rats caused very brief haemodynamic perturbation (<60 seconds) but resulted in protection against infarction 24 hours later. If these rats were pre-treated with L-NAME to inhibit NO synthase activity prior to bradykinin administration, the protective effect 24 hours later was abolished. Further indirect evidence for a role of endogenous bradykinin liberation in delayed preconditioning in pigs comes from work by Jaberansari *et al* (2001), who showed that a 2 min coronary artery occlusion was insufficient to induce delayed myocardial protection 24 hours later. However, if an angiotensin converting enzyme inhibitor was given prior to this sublethal threshold preconditioning stimulus, a fully protective response was observed. This finding is compatible with the notion that bradykinin, whose breakdown is inhibited by ACE-inhibitors, is a mediator of delayed preconditioning. To date, no work has reported the effects of bradykinin receptor antagonists in delayed preconditioning models. Such observations are required to confirm that endogenous bradykinin participates in triggering delayed preconditioning.

1.2.4.6 Delayed cardioprotection by administration of catecholamines

Historically, studies resulting from catecholamine administration almost certainly represent the earliest examples of delayed cardioprotection. Of particular interest was the finding that myocardial resistance developed against toxic doses of isoprenaline not only if rats were pretreated with smaller doses of isoprenaline (Rona *et al.*, 1963) but that coronary artery ligation, either of the left or the right coronary artery, also protected against the toxic effects of isoprenaline (Selye *et al.*, 1960). This phenomenon,

referred to by Rona (Dusek *et al.*, 1971) as myocardial resistance or protection, was not associated with beta-receptor down regulation and lasted several days or even weeks (Joseph *et al.*, 1983). It has not been possible to determine whether at this time these workers attempted to demonstrate whether isoprenaline also protected against the consequences of coronary artery occlusion, but this was demonstrated several years later (Beckman *et al.*, 1981). Beckman and colleagues observed that dogs that developed long-term tolerance to intravenously administered adrenaline were also resistant to coronary embolization with microspheres (Beckman *et al.*, 1981). For example, only 1 of 14 tolerant dogs fibrillated on coronary artery occlusion compared to 11 of 31 in the control group; all deaths occurred early, that is within 15 minutes of occlusion. The time course of this delayed protection was not evaluated.

1.2.5 Mediators (or effectors) of late preconditioning

Ischaemic preconditioning causes an increase in the rate of myocardial protein synthesis. If this increase is blocked by cyclohexamide; the development of delayed preconditioning is also blocked (Rizvi *et al.*, 1999). Thus, unlike early preconditioning, late preconditioning requires increased synthesis of new proteins, not simply activation of pre-existing proteins. The time course of the enhanced tolerance to ischaemia, which requires 12 to 24 hours to develop and lasts for 3 to 4 days, (Tang *et al.*, 1996, Baxter *et al.*, 1997) is also consistent with the synthesis and degradation of cardioprotective proteins. Several proteins have been proposed as possible mediators (effectors) of the protection afforded by

late preconditioning including NOS, cyclooxygenase-2 (COX-2), aldose reductase, antioxidant enzymes (particularly Mn SOD), and heat stress proteins (HSP's).

1.2.5.1 Nitric Oxide Synthase

The first demonstration that the cardioprotective effects of the late phase of ischaemic PC are mediated by NOS was provided by studies in conscious rabbits, in which delayed protection against both myocardial stunning (Bolli *et al.*, 1997) and infarction (Takano *et al.*, 1998) was found to be completely abrogated when preconditioned animals were given L-NA 24 hours after ischaemic PC, just before the second ischaemic challenge. The same effects were observed with the relatively selective iNOS inhibitors aminoguanidine and S-methylisothiourea, implicating iNOS as the specific NOS isoform involved in mediating the protective effects of late preconditioning (Bolli *et al.*, 1997, Takano *et al.*, 1998). These results were subsequently confirmed by others (Imagawa *et al.*, 1999).

Using an *in vivo* murine model of myocardium infarction, Guo *et al.*, (1999) demonstrated that the late phase of ischaemic PC was associated with upregulation of myocardial iNOS (whereas eNOS remains unchanged). Furthermore, that targeted disruption of the iNOS gene completely abrogated the delayed infarct sparing effect, providing unequivocal molecular genetic evidence for an obligatory role of iNOS in the cardioprotection afforded by the late phase of ischaemic PC. Immunohistochemical and *in situ* hybridization studies identified cardiac

myocytes as the specific cell type that expressed iNOS during late preconditioning (Wang *et al.*, 2000). Thus, NO appears to play a dual role in the mechanism of the delayed ischaemic preconditioning, acting initially as the trigger (Takano *et al.*, 1998, Bolli *et al.*, 1997, Hill *et al.*, 2000) and subsequently as the mediator (Bolli *et al.*, 1997, Takano *et al.*, 1998, Imagawa *et al.*, 1999, Guo *et al.*, 1999, Wang *et al.*, 2000) of this adaptive response. This concept has become known as the "NO hypothesis of late preconditioning" (Bolli *et al.*, 1998). The finding that both ischaemic preconditioning and nitroglycerin induce a rapid increase in steady-state levels of iNOS mRNA, which is abolished by administration of L-NA before ischaemia (Jones *et al.*, 1999), is congruent with the concept of NO - dependent iNOS induction. Taken together, the studies reviewed above (Takano *et al.*, 1998, Bolli *et al.*, 1997, Qiu *et al.*, 1997, Banerjee *et al.*, 1999, Guo *et al.*, 1999, Hill *et al.*, 2000, Xuan *et al.*, 2000, Bolli *et al.*, 1997) support a complex paradigm in which 2 different NOS isoforms are sequentially involved in the pathological cascade of late PC, with eNOS generating the NO that initiates the development of the late PC response on day 1 and iNOS then generating the NO that protects against recurrent ischaemia on day 2 (Bolli *et al.*, 1998). The quantitative aspects of the upregulation of iNOS after ischaemic PC are noteworthy. The increase in cardiac iNOS protein expression in the Guo *et al.* (1999) study was mild: 18 fold less than that observed after a lethal dose of lipopolysaccharide.

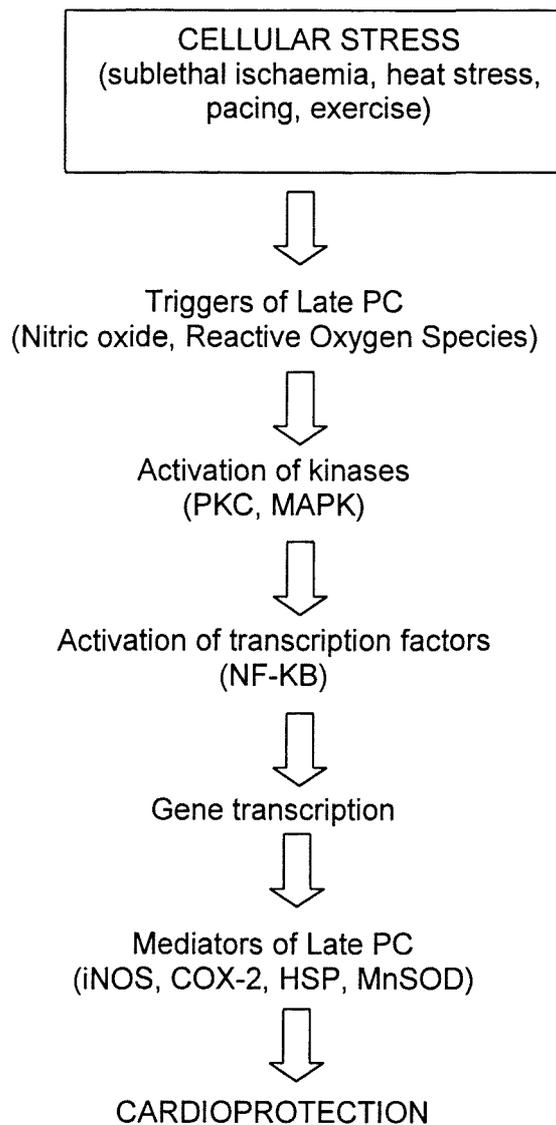


Figure 1.2: Schematic presentation of the role of nitric oxide in delayed preconditioning

This supports the hypothesis (Guo *et al.*, 1999) that induction of iNOS after ischaemic PC is protective because it is relatively modest, in contrast to other situations (such as inflammation or septic shock) in which iNOS induction is massive and promotes tissue injury. The precise mechanism(s) whereby iNOS -derived NO protects against ischaemia

remains to be elucidated, but appears to involve activation of guanylate cyclase, given that both the alleviation of stunning and the reduction in infarct size are abrogated if ODQ was administered on day 2. Administration of ODQ on day 1 did not alleviate the infarct sparing effects (Kodani *et al.*, 2001).

Besides ischaemia-induced PC, there is now evidence that other forms of late PC are also iNOS dependent. Guo *et al* (1999) found that the delayed infarct -sparing effects elicited by S -nitroso - N -acetylpenicillamine and nitroglycerine (Guo *et al.*, 1999), the selective δ_1 - opioid receptor agonist TAN- 670, (Guo *et al.*, 2000) and by exercise (Y, Guo and R.Bolli, unpublished observations, 2000) were completely abrogated in iNOS -/- mice. This implies that iNOS serves as an obligatory mediator of NO donor-induced, δ_1 -opioid receptor induced, and exercise induced late PC against infarction. Similarly, iNOS activity has been found to be necessary for the delayed infarct size limitation observed after pre-treatment with the endotoxin derivatives MLA and RC-552 (Xi *et al.*, 1999, Zhao *et al.*, 1997) and diazoxide (Ockaili *et al.*, 1999).

Recent reports on the role of iNOS in CCPA - induced late PC have arrived at conflicting conclusions (Guo *et al.*, 2000, Zhao *et al.*, 2000, Bell *et al.*, 1999). Administration of CCPA 24 hours prior to a lethal ischaemic insult resulted in a marked reduction of infarction (Kukreja *et al.*, 2000). However, Bell and co-workers (2002) demonstrated that iNOS knockout mice (with no demonstrable iNOS protein) display a similar degree of

infarct limitation following CCPA administration 24 hours earlier. This study is in contrast to findings of others (Bolli *et al.*, 1997) and implies that delayed cardiac protection can be achieved via an iNOS independent mechanism. Clearly, the relationship between adenosine-induced late PC and the role of iNOS in this scenario needs to be further investigated.

1.2.5.2 Cyclooxygenase

An obligatory role for COX-2 in late PC was first shown by Shinmura *et al* (2000). Utilising conscious rabbits, this study found that COX-2 protein expression was upregulated 24 hours after ischaemic preconditioning, concomitant with an increase in the myocardial levels of prostaglandin (PG) E₂, 6-keto-PGF_{1α}. Administration of 2 unrelated COX - 2 - selective inhibitors (NS - 398 and celecoxib) 24 hours after ischaemic preconditioning abolished the increase in prostanoids and completely blocked the cardioprotective effects of late PC, demonstrating that COX-2 activity is necessary for this phenomenon to occur (Shinmura *et al.*, 2000). Similar results were subsequently obtained in mice (Guo *et al.*, 2000). These observations identify COX-2 as a cardioprotective protein and strongly point to PGE₂ and/or PGI₂ as the likely effectors of COX-2-dependent protection. Induction of COX-2 is generally thought to be detrimental (Vane *et al.*, 1998). The finding that COX-2 mediates the anti-stunning and anti-infarct effects of late PC impels a reassessment of current views regarding this enzyme and supports a more complex paradigm in which COX-2 can play either a beneficial or a deleterious role depending on various factors (e.g., the intensity of its induction, the

pathophysiological setting, and the ability of specific cell types to metabolize PGH₂ produced by COX-2 into cytoprotective prostanoids) (Shinmura *et al.*, 2000).

The recognition that iNOS and COX-2 are co - induced and serve as co - mediators of late PC logically leads to the question of whether there is an interaction between these 2 proteins or whether they act as independent effectors of cytoprotection. Previous studies have suggested that NO can directly activate COX-2 (Salvemini *et al.*, 1993). Further work is needed to confirm all the above findings.

1.2.5.3 Aldose Reductase and delayed myocardial protection

Oxidative stress, osmotic stress, cytokines and NO are known to upregulate the expression of aldose reductase, an enzyme that catalyzes not only the metabolism of glucose to sorbitol, but also the detoxification of ROS - derived lipid aldehydes (Srivastava *et al.*, 1998). Shinmura *et al* (2000) have recently found that the protein expression of aldose reductase is upregulated 24 hours after ischaemic PC in conscious rabbits and that inhibition of this enzyme abrogates the infarct-sparing effects observed in untreated animals. Thus, in addition to iNOS and COX-2, aldose reductase is a third candidate for mediator of cardioprotective actions of the late phase of ischaemic PC. The mechanism whereby aldose reductase enhances resistance to ischaemia/reperfusion remains to be determined but could involve the removal of toxic byproducts of lipid peroxidation such as 4-hydroxy-*trans*-nonenal (Srivastava *et al.*, 1998).

1.2.5.4 Antioxidant enzymes and delayed preconditioning

The oxidants, superoxide and/or hydrogen peroxide, are generated as a consequence of normal cellular aerobic metabolism (Sies, 1991). Since these oxidants participate in reactions which result in cytotoxicity, detoxification of these oxidants is a prerequisite for successful aerobic life. Mammalian cells contain superoxide dismutase (SOD) (which decomposes superoxide), and glutathione peroxidase and catalase, which decompose hydrogen peroxide. These antioxidant enzymes prevent excessive accumulation of oxidants during normal aerobic metabolism. I/R is one pathologic event which disturbs this oxidant/antioxidant balance (Reilly *et al.*, 1991, Korthuis and Granger, 1993). In general, it is believed that the oxidant production upon reperfusion of ischaemic tissues overwhelms the detoxifying capacity of the enzymatic antioxidants and initiates a cascade of events that eventually leads to tissue injury.

Exogenous administration of SOD and/or catalase offers protection against I/R-induced tissue injury in a variety of *in vivo* and *in vitro* models (Granger 1988, Kvietys and Granger, 1997). Thus it is not surprising that consideration has been given to the possibility that delayed preconditioning is in part, a result of an enhanced antioxidant status of tissue or cell. Evidence for this idea comes from extra cardiac tissue. In the gut, mucosal epithelial barrier dysfunction was induced by I/R; an event that was completely prevented by a previous (24 h earlier) I/R challenge (Osborne *et al.*, 1994). The antioxidant status of the mucosa

was increased (increased activities of SOD, glutathione peroxidase and catalase) at 24h, but not at 1 and 72 hours after initial I/R challenge.

These findings indicate that the development of delayed preconditioning is associated with an enhanced antioxidant status of the target tissue (gut mucosa of rat). There is evidence that delayed preconditioning induced by I/R is specific for oxidant stress. In the gut, an initial insult imposed 24h earlier protected against hydrogen peroxide-induced, but not acid- or ethanol-induced mucosal epithelial barrier dysfunction (Osborne *et al.*, 1994). A similar phenomenon was noted in the kidneys, where an I/R insult resulted in an enhanced antioxidant status of the glomeruli and prevented the typically observed proteinuria induced by a subsequent exogenous administration of hydrogen peroxide (Yoshioka *et al.*, 1990). Similar correlation between the development of delayed preconditioning and an enhanced antioxidant status have been demonstrated in the kidney and heart (Yellon and Baxter, 1995, Steare and Yellon, 1995). In the heart, I/R-induced myocardial stunning (Sun *et al.*, 1996) and decreased responsiveness of coronary arteries to acetylcholine (Kaeffer *et al.*, 1997) were largely prevented by a previous I/R insult. The induction of this delayed preconditioning was largely attributed to the generation of oxidant stress during initial I/R challenge. Exposing isolated cardiomyocytes to anoxia/reoxygenation (A/R; the in vitro equivalent to I/R) results in superoxide generation by the myocytes (Zhou *et al.*, 1996). Twenty-four hours later myocyte Mn-SOD was increased and a subsequent A/R challenge no longer resulted in superoxide production by the myocytes. In

addition, delayed preconditioning was evident, in that the second A/R challenge no longer resulted in myocyte cytotoxicity (LDH release). The delayed preconditioning was prevented in this system by exposing the myocytes to Mn-SOD during the initial A/R challenge. Delayed preconditioning could also be induced if the myocytes were initially exposed to superoxide/hydrogenperoxide generating system (xanthine plus xanthine oxidase) rather than A/R. Finally, exposure of endothelial cells in culture to hydrogen peroxide results in (1) an increase in SOD, catalase, and glutathione peroxidase; and (2) protection against the cytotoxic effects of the second challenge with hydrogen peroxide 18-24h later (Lu *et al.*, 1993). Taken together, these studies indicate that an initial I/R insult results in an oxidative stress which enhances the target tissues antioxidant status and provides a specific protection against a second oxidant stress, i.e. I/R.

The induction of antioxidant enzymes may be an important mechanism involved in delayed preconditioning, though the relative contribution of specific antioxidant enzymes is unclear. The importance of catalase in protecting the myocardium against I/R - induced injury (as measured with infarct size and creatinine kinase release) is provided by studies using transgenic mice (Li *et al.*, 1997). The hearts from isolated transgenic mice in which myocardial catalase is increased 60-fold (while SOD glutathione peroxidase was unchanged) were resistant to I/R injury.

Studies in dogs have shown that ischaemic preconditioning induces an increase in the protein expression and activity of Mn-SOD 24 hours later

while other antioxidant enzymes are unchanged (Hoshida *et al.*, 1993) and that the time course of Mn-SOD induction parallels that of protection against lethal ischaemia/reperfusion injury (Kuzuya *et al.*, 1993). A similar association between the time course of Mn-SOD induction and that of delayed cardioprotection has been observed after heat stress (Yamashita *et al.*, 1998), exercise (Yamashita *et al.*, 1999) and administration of CCPA (Dana *et al.*, 1998). In one study however, (Xi *et al.*, 1998) heat stress did not augment Mn-SOD activity. The increase in Mn-SOD activity after heat stress and exercise appears to be caused by the production of ROS, TNF- α and interleukin -1 β (Yamashita *et al.*, 1999, Yamashita *et al.*, 1998, Yamashita *et al.*, 2000). Importantly, in vivo administration of antisense oligonucleotides to Mn-SOD has been reported to block heat stress induced (Yamashita *et al.*, 2000), exercise induced (Yamashita *et al.*, 1999), and CCPA induced (Dana *et al.*, 2000) delayed PC, indicating that Mn-SOD upregulation is essential for these 3 forms of delayed cardioprotection. No such data are available to determine whether Mn-SOD is necessary for ischaemia induced late PC in vivo (although evidence supporting this concept has been obtained in isolated neonatal myocytes) (Yamashita *et al.*, 1994). In contrast to this finding, in isolated rat cardiac myocytes, delayed preconditioning appears to be strongly dependent on induction of Mn-SOD. When hypoxia/reperfusion was used as a challenge, delayed preconditioning was associated with an increase in cellular Mn-SOD (Yamashita *et al.*, 1994)

An increase in activity of various antioxidant enzymes (Mn-SOD, Cu-ZN SOD, catalase and/or glutathione peroxidase) has also been reported 24 to 72 hours after pharmacological PC with interleukin-1 (Maulik *et al.*, 1993), and endotoxin (Maulik *et al.*, 1995), concomitant with increased myocardial resistance to ischaemia/surperfusion injury, but a cause-and-effect relationship remains to be established.

Not all studies have found upregulation of antioxidant defenses during late preconditioning. In conscious pigs (Tang *et al.*, 1997) and rabbits (Tang *et al.*, 2000) subjected to ischaemic PC, no increase in anti-oxidant enzymes (Mn-SOD, Cu-ZN SOD, catalase, glutathione peroxidase or, glutathione reductase) activity could be detected 24 hours after PC stimulus, when the delayed cardioprotection was fully manifest. Thus, the role of antioxidant proteins in ischaemia induced late PC remains unclear.

1.2.5.5 Heat stress Protein and delayed preconditioning

Studies in transgenic mice overexpressing HSP70 have shown that this protein confers protection against ischaemia/reperfusion injury, (Plumier *et al.*, 1995, Marber *et al.*, 1995, Radford *et al.*, 1996). However, it remains controversial whether ischaemic or pharmacological PC upregulates HSPs *in vivo*. Earlier investigators reported an increase in myocardial HSP70 content 24 hours after ischaemic preconditioning (Marber *et al.*, 1993, Knowlton *et al.*, 1991). Subsequent studies found no HSP70 induction in rabbits preconditioned with ischaemia, (Baxter *et al.*, 1998), CCPA (Baxter *et al.*, 1997, Heads *et al.*, 1998, Bernado *et al.*, 1999) or monophosphoryl

lipid A (MLA) (Yoshida *et al.*, 1996, Baxter *et al.*, 1996). Furthermore, several studies in rats subjected to whole body hyperthermia (Yamashita *et al.*, 1997, Qian *et al.*, 1998, Kukreja *et al.*, 1999) or to ischaemic preconditioning (Qian *et al.*, 1999) have shown that the changes in myocardial HSP70 and HSP27 content do not correlate with protection against infarction. In addition, induction of HSP70 by heat stress fails to confer cardioprotection in mice (Xi *et al.*, 1998).

Recently heat shock protein 27 (HSP27), has been suggested to participate in late preconditioning. Studies in rabbits have shown that CCPA-induced late PC is associated with a redistribution of HSP27 from the membranous to the cytosolic fraction of the homogenate (Heads *et al.*, 1998) as well as with increased phosphorylation of this protein. The latter is abolished by pre-treatment with protein kinase C γ -(PKC) or tyrosine kinase inhibitors (Dana *et al.*, 2000). Since HSP 27 is a substrate for the p38 MAP kinase pathway, which is activated 24 hours after CCPA (Dana *et al.*, 2000) it has been proposed that posttranslational modification of HSP27 may play an important role in mediating the delayed cardioprotection afforded by CCPA (Dana *et al.*, 2000).

The evidence reviewed above suggests the induction of the heat shock proteins is responsible for the cardioprotection of delayed preconditioning. Whether only the small (HSP27) or large (HSP 70) or both of these proteins participate in delayed myocardial preconditioning needs to be investigated further.

1.2.5.6 K_{ATP} Channels

There is persuasive evidence that the opening of K_{ATP} channels is an important mechanism of classic preconditioning as discussed earlier, but what is the evidence for its participation in delayed PC? Bernado *et al* (1999) reported that delayed preconditioning was abolished when either glibenclamide (which antagonize opening of sarcolemmal K_{ATP} channels) or 5-HD (which antagonize opening of mitochondrial K_{ATP} channels) was administered prior to ischaemia. This suggests a critical role for K_{ATP} channels opening in delayed myocardial protection. However, it has been concluded that the sarcolemmal, and not the mitochondrial K_{ATP} channels were more important, since they elicited differences in monophasic action potential duration. In this regard Cole *et al* (1991) demonstrated that glibenclamide, a nonselective K_{ATP} channel antagonist, attenuated the action potential duration shortening, which occurs during ischaemia. Work done by Tanako and colleagues (2000) have confirmed that 5-HD given prior to index ischaemia abolished delayed preconditioning against infarction but did not alter the anti-stunning effect of preconditioning. A further interesting development is that K_{ATP} channel openers such as diazoxide are able to induce pharmacological delayed preconditioning (Ockaili *et al.*, 1999, Takano *et al.*, 2000).

Takashi *et al* (1999) have reported that diazoxide triggered delayed preconditioning via a PKC dependant mechanism. This group found that pretreatment with diazoxide (7mg/kg i.v.) 12, 24 and 72 hours before

ischaemia/reperfusion resulted in a significant reduction in infarct size. This cardioprotection against infarction was blocked if hearts were pretreated with 5-HD (5mg/kg i.v.) and the PKC blocking agent chelerythrine (5mg/kg i.v.) on the first day before diazoxide pretreatment or 10 minutes before I/R on the second day. This group therefore concluded that activation of mitochondrial K_{ATP} channel with diazoxide produced late preconditioning against reperfusion injury. The mitochondrial K_{ATP} channels may play an important role in delayed myocardial adaptation but its precise role remains to be elucidated.

1.2.6 The signaling pathway of delayed preconditioning

The stimuli (mentioned above) trigger late PC by activating a complex cascade of signalling events that ultimately results in increased transcription of cardioprotective genes. Among various families of cellular kinases, there is now convincing evidence that PKC and Src protein tyrosine kinases (PTKs) play an essential role in the development of late PC (See Fig 1.3 a and b).

1.2.6.1 Protein Kinase C

The notion that PKC is essential for the genesis of late PC was first proposed by Baxter *et al* (1995), who found that the delayed infarct-sparing effects of ischaemic preconditioning in rabbits was abrogated by pre-treatment with the PKC inhibitor chelerythrine. Conversely, administration of the PKC activator dioctanoyl-sn-glycerol induced cardioprotection 24 hours later (Baxter *et al.*, 1997). Subsequent studies

have also implicated PKC in the development of CCPA-induced (Dana *et al.*, 1997) and heat stress-induced (Yamashita *et al.*, 1997, Kukreja *et al.*, 1999, Joyeux *et al.*, 1997) late PC against infarction. Direct evidence that PC stimuli activate PKC in vivo, however, was lacking. Furthermore, no information was available as to which PKC isoform was involved.

These issues were addressed in a series of studies in conscious rabbits (Ping *et al.*, 1997, Qui *et al.*, 1998, Ping *et al.*, 1999, Ping *et al.*, 1999) in which it was found that ischaemic PC caused selective translocation (and activation) (Ping *et al.*, 2000) of PKC ϵ and PKC η (2 members of the subfamily of novel PKC isoforms) but does not affect the other 8 isoforms expressed in the rabbit heart (PKC isoforms α , β , γ , δ , ι , ζ , λ and μ) and does not significantly change total PKC activity (Ping *et al.*, 1997). Inhibition of PKC ϵ translocation (and activation) by chelerythrine blocked the development of late PC against myocardial stunning (Ping *et al.*, 2000), whereas inhibition of PKC η translocation did not have an effect, indicating that the translocation of PKC ϵ (but not PKC η) was necessary for late PC to occur (Qui *et al.*, 1998). Thus, activation of PKC after ischaemic preconditioning is isoform selective, and PKC ϵ appears to be the specific PKC isotype responsible for the development of delayed cardioprotection.

The ischaemic preconditioning induced activation of PKC ϵ is caused by the generation of NO during the initial ischaemic stress, as it is blocked by pre-treatment with L-NA (Ping *et al.*, 1999). Interestingly, administration of

NO donors in the absence of ischaemia induces a selective activation of PKC ϵ quantitatively similar to that induced by ischaemic preconditioning (Ping *et al.*, 1999). This event is essential for NO donor - induced late preconditioning, since co-administration of chelerythrine blocks both the activation of PKC ϵ and delayed protection elicited by the NO donors (Ping *et al.*, 1999). Thus, the recruitment of the ϵ isoform of PKC is a critical signalling event in the development of both ischaemia - induced and NO donor - induced late preconditioning in rabbits. The recent finding that transgenic expression of constitutively active PKC ϵ mimics both the signaling events and the cardioprotective effects of late PC supports a role of PKC ϵ in mice as well (Ping *et al.*, 2000).

The precise mechanism whereby NO activates PKC ϵ remains to be elucidated. Because MPG blocks NO donor-induced late PC (Takano *et al.*, 1998), it seems reasonable to postulate that NO-derived reactive species (ONOO $^-$ and/ or ROS) may activate PKC ϵ either by direct oxidative modification or via activation of phospholipases (Ping *et al.*, 1999).

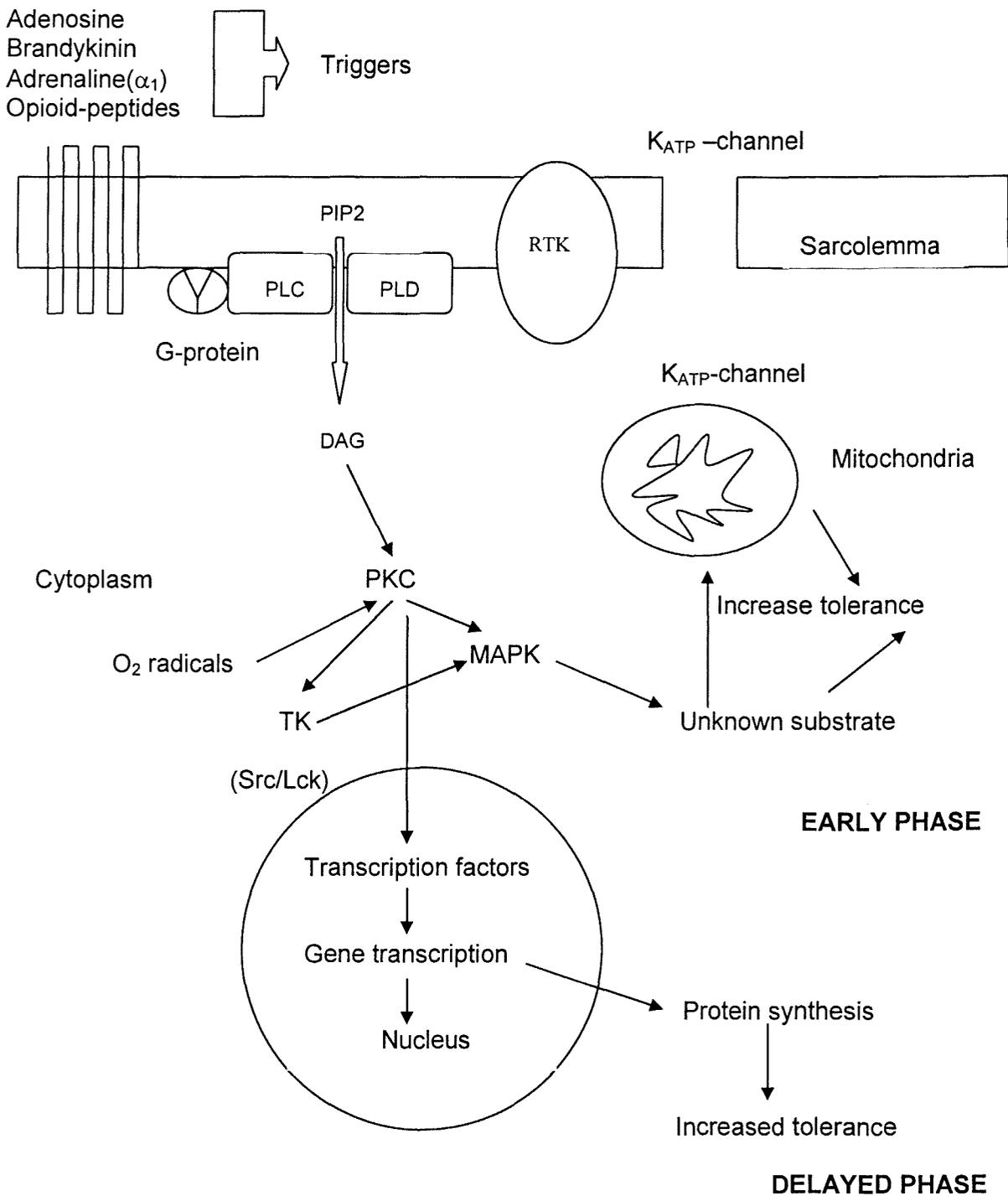


Figure 1.3a: Schematic illustration showing activation of intracellular signalling pathways during classic and delayed preconditioning. DAG, Diacyl glycerol; MAPK, Mitogen-activated protein kinases; PIP2, Phosphatidyl inositol diphosphate; PKC, Protein kinase C; PLC, Phospholipase C; PLD, Phospholipase D; TK, Tyrosine kinase

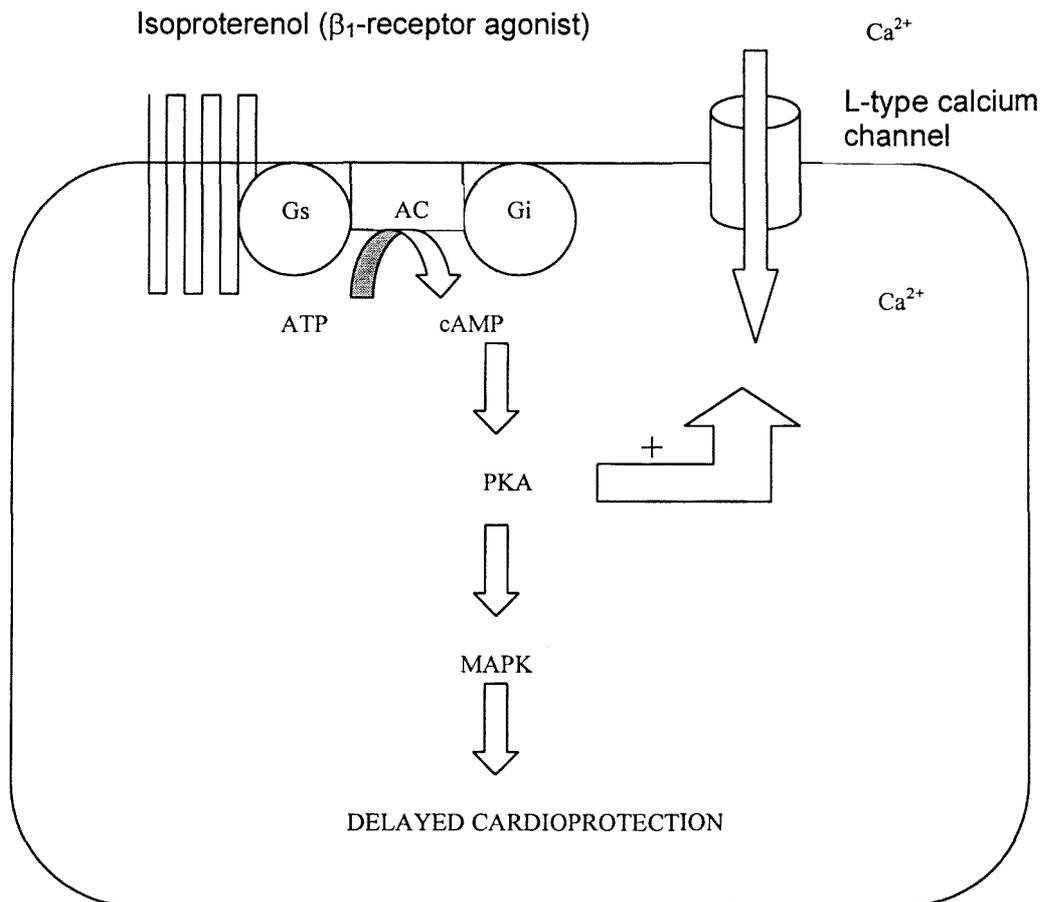


Fig 1.3 b β -Adrenergic signalling in cardiomyocytes. Gs, stimulatory G-protein; Gi inhibitory G-protein; AC, Adenyl cyclase; PKA; Protein kinase A; MAPK, Mitogen activated protein kinase; ATP, Adenosine triphosphate; , cAMP, cyclic adenosine monophosphate.

1.2.6.2 Protein tyrosine kinases

Since more than a 1000 different PTK's have been identified so far, assessing the contribution of this class of enzymes to late preconditioning represents a gargantuan task. A role of PTK's in the genesis of ischaemia - induced late PC was proposed on the basis of studies using genistein (a tyrosine kinase inhibitor) (Imagawa *et al.*, 1997), which, however, is a broad inhibitor of most known PTKs and other kinases. Recent studies in conscious rabbits (Ping *et al.*, 1999 , Dawn *et al.*, 1999) have focused on 2 major families of PTKs - the Src PTKs and the epidermal growth factor (EGF) receptor PTKs. It was found that ischaemic PC selectively activates 2 members of the Src PTKs (Src and Lck) (among the 7 members expressed in rabbit heart) and that this activation is blocked by chelerythrine, suggesting that these kinases are distal to the activation of PKC (Ping *et al.*, 1999). Inhibition of Src and Lck activation with lavendustin (LD-A) completely abrogates the development of late PC against myocardial stunning (Dawn *et al.*, 1999), indicating that Src and /or Lck signaling plays a causative role in this phenomenon. In contrast, EGF receptor PTK's are not activated by ischaemic preconditioning (Ping *et al.*, 1999). Thus, Src and/ or Lck (but not EGF receptor PTK's) appear to be essential components of the signalling pathway responsible for the development of ischaemia induced late PC and appear to be downstream targets of PKC phosphorylation in rabbits. Subsequent studies in mice have corroborated these concepts by demonstrating that Lck is a direct substrate of PKC ϵ (Song *et al.*, 2000) and that the targeted disruption of the Lck gene abolishes ischaemia induced late PC (Ping *et al.*, 2000). LD-

A has also been found to block CCPA - induced late PC (Dana *et al.*, 1997) implicating tyrosine kinases in the genesis of this form of adaptation as well. In contrast, genistein failed to block heat stress - induced late PC (Joyeux *et al.*, 1997).

1.2.6.3 Mitogen-Activated Protein Kinases

Another potential downstream target of PKC - dependent signaling during the development of late PC is the MAPK superfamily, which includes 3 major subfamilies, the p44/p42 MAPKs (or extracellular signal - regulated kinases [ERKs]), p38 MAP kinases, and the p46/p54 MAPK (or c-jun N-terminal kinases [JNKs]). Several studies in hearts have documented that brief myocardial ischaemia/ reperfusion is associated with activation of p44/42 MAPKs, p38 MAP kinases and JNK (Sugden *et al.*, 1998 , Ping *et al.*, 2000) but it is unknown whether the ischaemic protocols used in those investigations elicit late preconditioning. Recent studies in conscious rabbits have demonstrated that an ischaemic protocol known to induce delayed cardioprotection activates all of the 3 MAPK subfamilies, although the activation of p38 MAP kinase is of short duration (Ping *et al.*, 1999, Ping *et al.*, 1999). The activation of p44/p42 MAPKs and JNKs is abolished by chelerythrine, indicating that it is downstream of, and dependent on, activation of PKC (Ping *et al.*, 1999, Ping *et al.*, 1999). Interestingly, selective overexpression of PKC ϵ in adult rabbit myocytes induces activation of p44/p42 MAPKs and protects against simulated ischaemia, an effect that can be abolished by p44/p42 MAPK inhibitors (Ping *et al.*, 1999).

Among the mitogen -activated protein (MAP) kinase families, the role of stress activated kinase, p38 MAP kinase, has received special attention in relation to adenosine A₁ receptor induced delayed protection. Adenosine treatment of the isolated rat heart was observed to cause rapid phosphorylation of MAP kinase - activated protein kinase - 2 (MAPKAPK-2, a downstream substrate of p38 MAP kinase) (Nakano *et al.*, 2000). Dana *et al* (2000) observed that myocardial p38 MAP kinase catalytic activity was markedly elevated 24h after treatment with CCPA. This increase in p38 activity correlated with phosphorylation of the small heat shock protein, HSP 27. The time course of this p38 MAP kinase induction is unknown as is the identity of the active isoenzyme and the mechanisms of its regulation. It is also unknown if this apparent increase in p38 MAP kinase activity was sustained throughout the period following A₁ agonist stimulation until the period of protection 24-72 hours later. If this was not the case, it remains to be determined how this late increase in kinase activity occurred in the absence of any reinforcing stimulus such as ischaemia (Ping *et al.*, 2000).

In support for a role of p38 MAP kinase are the findings by Kukreja *et al* (2001), that SB203580 given prior to CCPA abolished the late protection afforded by CCPA in the isolated mouse heart. It is of particular interest that in this study A₁ receptor associated delayed protection required the phosphorylation of p38 MAP kinase at two time points: as an early event following CCPA administration and also as a late event during the

ischaemic episode 24h later. Enhanced phosphorylation of p38 MAP kinase during index ischaemia was observed in the hearts from mice pretreated with CCPA 24h earlier. This differs from the observation of Dana *et al* (2000) who reported an increase in the basal activity of p38 MAP kinase before regional ischaemia. This enhanced phosphorylation of p38 MAP kinase correlated with protection against infarction because SB203580 and genistein given before CPPA abolished and also blocked the phosphorylation of p38 MAP kinase during the subsequent ischaemia. Another intriguing observation was that late phosphorylation of p38 MAP kinase in CCPA-pretreated hearts was abolished by 5-hydroxydecanoate (5-HD) given at the time of index ischaemia, suggesting that opening of the mitochondrial K_{ATP} channel participated in the distal signalling mechanism. This implies coupling between mitochondrial K_{ATP} and p38 MAP kinase in delayed preconditioning.

The critical unresolved issue now is whether the recruitment of the MAPKs contributes to the development of late PC or whether it is merely an epiphenomenon. Elucidation of this issue will require studies in an *in vivo* model of late preconditioning in which the activity of MAPKs is inhibited either with transgenic approaches (e.g., gene targeting or transgenesis of dominant - negative mutants) or with pharmacological agents more specific than currently available compounds such as SB202190.

1.2.6.4 Role of Kinases in Mediating Protection

In addition to their role in initiating the development of late PC shortly after the stimulus (day 1), there is mounting evidence that cellular kinases are also necessary for the protection to become manifest 24 hours later (day 2). This concept is supported by the recent finding that administration of the PTK inhibitor LD-A before the second ischaemic challenge (on day 2) completely abrogates the protective effects of late preconditioning against myocardial stunning and concomitant increase in iNOS activity (Dawn *et al.*, 1999). It appears; therefore, that PTKs play a bifunctional role in ischaemia induced late PC, contributing not only to its development shortly after the initial ischaemic stress but also to the occurrence of the protection 24 hours later. The mechanism whereby PTKs participate in the protection on day 2 remains to be established. Because inhibition of PTKs with L- DA on day 2 abolishes the increase in iNOS activity, it has been suggested that posttranslational modulation of iNOS proteins via tyrosine phosphorylation is necessary to activate this enzyme and to confer tolerance to ischaemia/reperfusion injury (Dawn *et al.*, 1998). In addition the recent finding that p38 MAPK activity is markedly increased 24 hours after administration of CCPA (concomitant with increased phosphorylation of HSP27) raises the possibility that this subfamily of MAPKs may be involved in mediating the protective effects of late preconditioning (Dana *et al.*, 2000).

1.2.7 Transcription factors

The recruitment by the preconditioning stimulus of PKC, Src PTKs, and other as-yet-unidentified kinases leads to the activation of transcription factors that govern the expression of the cardioprotective genes responsible for delayed preconditioning. The first transcription regulatory element to be identified as an integral component of the late preconditioning response was nuclear factor- κ -B (NF- κ -B) (Xuan *et al.*, 1999), which is known to be a major modulator of iNOS, COX-2, and aldose reductase gene expression. Using conscious rabbits, Xuan *et al.* (1999) demonstrated that ischaemic preconditioning induces rapid activation of NF- κ -B and that this event can be mimicked by infusing NO donors in the absence of ischaemia. Inhibition of NF- κ -B with diethylthiocarbamate completely abrogated the cardioprotective effects observed 24 hours later, indicating that NF- κ -B plays a critical role in the genesis of late PC (Xuan *et al.*, 1999). The ischaemic PC- induced activation of NF- κ -B was blocked by pre-treatment with L-NA, MPG, chelerythrine and LD-A (all given at doses previously shown to block late PC), indicating that the cellular mechanism whereby ischaemic preconditioning activates NF- κ -B involves the formation of NO and ROS and the subsequent activation of PKC- and PTK- dependent signalling events (Xuan *et al.*, 1999). Thus, NF- κ -B appears to be a common downstream pathway through which multiple signals elicited by ischaemic stress (NO, ROS, PKC, and PTKs) act to induce gene expression in the heart. Subsequent studies have shown that ischaemic PC induces both serine and tyrosine phosphorylation of I- κ -B α (the inhibitor of NF- κ -B)

concomitant with PKC - dependent activation of IKK α and IKK β (the serine -threonine kinases that phosphorylate I- κ -B α (Zhang *et al.*, 1999), suggesting that a dual mechanism accounts for the activation of NF- κ -B induced by ischaemia, as this event is absent in Lck -/- mice (Ping *et al.*, 2000).

A molecular link between PKC ϵ (the isoform implicated in the genesis of late preconditioning) (Ping *et al.*, 1997, Qiu *et al.*, 1998, Ping *et al.*, 1999) and NF- κ -B is further supported by studies in adult cardiac myocytes, in which selective overexpression of PKC ϵ has been found to induce activation of NF- κ -B (Li *et al.*, 2000), activation of IKK α and IKK β (Li *et al.*, 1997) and serine tyrosine phosphorylation of I- κ -B α (Li *et al.*, 1997). Interestingly, PKC ϵ - induced recruitment of NF- κ -B in cardiomyocytes is abolished by inhibition of either p44/p42 MAPKs or JNKs (Li *et al.*, 2000).

Recruitment of NF- κ -B has also been reported in rat hearts subjected to short-term protocols of ischaemia/ reperfusion *in vivo* (Chandrasekar *et al.*, 1997) and *in vitro* (Maulik *et al.*, 1998) but it is not clear whether these protocols elicit a late preconditioning response in the models used. In addition, activating protein 1 (AP-1) has been found to be activated by brief myocardial ischaemia in rats (Chandrasekar *et al.*, 1997) and by overexpression of PKC ϵ in cardiomyocytes (Li *et al.*, 2000). Whether this transcriptional factor plays a functional role in the development of the late PC response remains unknown. Given the fact that specific combinations of 2 or more trans-regulatory proteins are obligatorily required for iNOS

gene expression and given the multiplicity of enzymes that co-mediate late PC (e.g. COX-2, aldose reductase and MN-SOD), it seems likely that the upregulation of iNOS, COX-2 and other co-mediators after a preconditioning stimulus involves simultaneous activation of multiple stress-responsive transcription factors acting in an additive or synergistic manner.

1.2.8 Summary of delayed preconditioning

A complex sequence of cellular events is mobilized by the heart in reaction to stress such as ischaemia. The heart elicits both early and delayed protective response. The exact cellular and molecular mechanisms underlying the phenomenon of delayed preconditioning remains to be deciphered, but remarkable progress has been made in understanding this powerful protective adaptation as discussed above. It is now clear that this delayed adaptation is a polygenic process that requires the synthesis of multiple proteins. Chemical signals released by ischaemic stress (such as NO, ROS, and adenosine) are transduced by a cascade of signalling elements that includes protein kinase C and NF- κ -B to the nucleus where they direct the transcription of iNOS, COX-2, aldose reductase, and other cardioprotective genes. An analogous sequence of events (probably involving MnSOD) can be triggered by a wide variety of stimuli, including heat stress, exercise, and cytokines. Delayed preconditioning appears to be a universal response of the heart in response to stress. A sustained cardioprotection similar to that afforded by the late phase of ischaemic PC can also be induced pharmacologically with clinically relevant agents, such

as NO donors, adenosine A₁ and A₃ receptor agonists, endotoxin derivatives and δ 1-opioid receptor agonists. This suggests that this endogenous adaptive response might be exploited for therapeutic purposes in treating patients exposed to myocardial ischaemia.

1.3 Melatonin effects on the heart

1.3.1 Physiology and pharmacology

In humans the pineal gland lies in the center of the brain, behind the third ventricle. The gland consist of two cell types: pinealocytes, which predominates and produce both indolamines (mostly melatonin) and peptides (such as arginine and vasotocin), and neuroglial cells. The gland is highly vascular.

Melatonin, or acetyl-N-formyl-5-methoxytryptamine was first identified in bovine pineal extracts on the basis of its ability to aggregate melanin granules and thereby lighten the color of frog skin (Lener *et al.*, 1958). In the biosynthesis of melatonin, tryptophan is first converted by tryptophan hydroxylase to 5-hydroxytryptophan, which is decarboxylated to serotonin. Melatonin synthesis is catalized by two enzymes (arylalkylamine N-acetyltransferase and hydroxyindole-O-methyltransferase) that are largely confined to the pineal gland (Axelrod and Weissbach , 1960).

The mammalian pineal gland is a neuroendocrine transducer. The photic information from the retina is transmitted to the pineal gland through the suprachiasmatic nucleus of the hypothalamus and the sympathetic

nervous system. The neural input to the gland is norepinephrine, and the output is melatonin. Melatonin synthesis is stimulated by darkness and inhibited by light. With onset of darkness, the photoreceptors release norepinephrine, thereby activating the system, and the number of α_1 - and β_1 -adrenergic receptors in the gland increases (Pangerl *et al.*, 1990). The activity of arylalkylamine N-acetyltransferase, the enzyme that regulates the rate of melatonin synthesis, is increased, initiating the synthesis and release of melatonin. Melatonin is rapidly metabolized by the liver via hydroxylation (6-hydroxymelatonin). It is conjugated with sulphuric or glucuronic acid and excreted in the urine.

1.3.2 Mechanism of action of melatonin

Melatonin partially synchronises circadian rhythmicity in the heart rate during constant light conditions (Witte *et al.*, 1998). In the rat heart, tyrosine hydroxylase activity is highest at early night and acetylcholine synthesis peaks in the afternoon (Brusco *et al.*, 1998). The above rhythms deteriorate with ageing and melatonin may partially restore the deteriorated rhythms (Brusco *et al.*, 1998). In *in vitro* systems, melatonin has an anti-adrenergic effect causing a decrease in contractility of the isolated rat papillary muscle (Abete *et al.*, 1997) and decreases ischaemia/reperfusion - induced arrhythmias in the isolated rat heart (Tan *et al.*, 1998). This indole also suppresses the iron-induced lipid peroxidation in many rat tissues including the heart (Tang *et al.*, 1997). The cardiomyopathic hamster develops heart disease leading to congestive heart failure early in life. Inhibition of pineal function prolonged the life

span of this animal with congenital congestive heart failure (Natelson *et al.*, 1997).

1.3.3 Direct melatonin actions on the heart : antioxidative actions

The modern concept of the free radical theory of ageing suggests that free radicals influence the primary and several secondary age- associated ageing processes such as carcinogenesis, decline of immune function, atherosclerosis, and heart diseases. With ageing, there is a shift in the antioxidative/prooxidative balance that leads to increased oxidative stress, dysregulation of cellular function, and ageing (Meydani *et al.*, 1998). Melatonin has a significant antioxidative action. Accumulated evidence suggests that it may be beneficial for the ageing heart, arteriosclerosis and heart disease.

Melatonin is an electron-rich molecule. It may interact with free radicals to form metabolites (e.g. N-acetyl-N-formyl-5methoxykynuramine and n-acetyl-5-methoxykynuramine) which are also effective free radicals scavengers. These continuous processes of trapping of unpaired electron free radicals by melatonin and its metabolites have been defined as a scavenging cascade reaction (Reiter *et al.*, 2000). The stable end-products such as cyclic-3-hydroxymelatonin radical (Equation 1) are excreted. (Tan *et al.*, 1999, Tan *et al.*, 1998) Alternatively, melatonin may stimulate several antioxidative enzymes such as glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and superoxide

dismutase and inhibit the prooxidative enzyme, nitric oxide synthase (Reiter *et al.*, 1999 , Reiter *et al.*, 2000, Tan *et al.*, 1999 , Tan *et al.*, 2000)



The protective antioxidant capacity of melatonin has been implicated to be responsible for:

- (1) The reversal of Ca^{2+} - stimulated ATP-ase activity reduced by alloxan in the rat myocyte in vitro (Chen *et al.*, 1994 a , Chen *et al.*, 1994 b).
- (2) An increase in glutathione peroxidase, a detoxifying enzyme, in the chick heart (Pablos *et al.*, 1995).
- (3) The protective effect against adriamycin-induced cardiomyopathy in rats (Morishima *et al.*, 1998).
- (4) A suppressive action on the iron-induced lipid peroxidation by reducing the concentration of cellular membrane malondialdehyde of the rat heart cell membrane (Tang *et al.*, 1997).
- (5) The prevention of ischaemia/reperfusion-induced arrhythmias in the isolated rat heart (Tan *et al.*, 1998).

The protective action of melatonin on the isolated model indicates clinical application of melatonin in patients with coronary heart diseases. In addition to melatonin, other methoxyindoles such as 5-methoxytryptamine also have radioprotective actions (Lebkova, 1966 , Feher *et al.*, 1968, Hara *et al.*, 1997). 5-Methoxytryptamine and other methoxyindoles are

present in the serum, pineal gland and retina of animals (Tsang *et al.*, 1996 , Li *et al.*, 1997a, Li *et al.*, 1997b , Zawilska *et al.*, 1998 , Vivien *et al.*, 1999). 5-Methoxyindoles are potent antioxidants and melatonin is a relatively safe molecule with potential for many clinical applications (Bubenik *et al.*, 1998) and little side effects in humans. Therefore, the protective effect of melatonin and/or its analogues on coronary heart diseases should be further examined in animal models and human studies.

1.3.4 Direct melatonin actions on the heart : cardiac melatonin receptor mediated actions

In addition to its antioxidative actions (Tan *et al.*, 1998), there is evidence for receptor mediated melatonin actions. Melatonin receptors were characterized in the duck, quail and chick heart using the melatonin radioligand, 2[¹²⁵I]iodomelatonin for radioreceptor studies on membrane preparations, as well as autoradiography and immunohistochemistry on tissues sections. The 2[¹²⁵I]iodomelatonin binding to bird heart membranes was rapid, saturable, stable, reversible, of high affinity and low density and modulated by the nonhydrolyzed GTP analog, guanosine 5'-O-(3-thiotriphosphate) (Pang *et al.*, 1993 , Pang *et al.*, 1996 , Pang *et al.*, 1998). Similar results were obtained with fish hearts (Pang *et al.*, 1994). In addition, Paul *et al* (1999) reported the binding of 2[¹²⁵I]iodomelatonin to the hamster heart.

The biochemical and pharmacological characteristics of hamster $2[^{125}\text{I}]$ iodomelatonin binding sites remains to be investigated. The picomolar affinity receptors in birds and fish correlates well with the circulating levels of melatonin indicating that these binding sites in chick myocytes are physiological melatonin receptors (Pang *et al.*, 1993, Pang *et al.*, 1996, Pang *et al.*, 1998, Pang *et al.*, 1994). Moreover these receptors are highly specific for melatonin, melatonin analogues (2-iodomelatonin, 6-chloromelatonin and melatonin) and naphthalenic ligands (N-[2-(7-methoxy-1-naphthyl)ethyl] cyclobutane carboxamide; S20642; N-propyl-N-[2-(7-methoxy-1-naphthyl) ethyl] urea; S20753; N-[2-(7-methoxy-1-naphthyl)ethyl] :S20750] (Pang *et al.*, 1993, Pang *et al.*, 1996, Pang *et al.*, 1998, Pang *et al.*, 1994).

Using a specific antibody (anti-mt₁) against the third intramembrane loop (TIL3) of the human melatonin receptor mt₁ (Song *et al.*, 1997, Chan *et al.*, 1997), it has been estimated that the molecular weight of the immunoreactive protein (melatonin receptor) was 35 kDa by Western blotting (Pang *et al.*, 2000). To date no melatonin receptors have been identified in human myocardium.

Alternately, the target tissues of melatonin was detected as early as 8 day old chick embryonic brain (Chong *et al.*, 1992), earlier than the development of the melatonin synthesizing enzyme, NAT. Whether the early appearing receptor is functional is not known. The melatonin receptor is expressed early (embryonic day 11) in the chick heart (Song *et al.*, 1995)

and brain (Song *et al.*, 1997), before the appearance of NAT (Zeman *et al.*, 1990). Its peak density, expressed either as per mg protein or μg DNA, is around embryonic day 15 (Pang *et al.*, 2000) which coincides with the appearance of melatonin rhythm and also when adrenergic and cholinergic innervation are fully functional in the embryonic heart (Pappano *et al.*, 1974). This is true for glucocorticoid receptors in the chick heart. However, among all melatonin target tissues in the chicken, the heart is the only tissue that expresses melatonin receptors most abundantly in its embryonic stage. Those of the kidney, spleen and brain peaked at 2 weeks, 2 days and 3 months posthatched respectively (Chong *et al.*, 1992, Song *et al.*, 1995).

The biological significance of these cardiomyocyte melatonin receptors remains to be elucidated. Melatonin affected $[\text{Ca}^{2+}/\text{Mg}^{2+}]$ -dependent ATPase (Ca^{2+} pump) activities in the sarcolemma of rat heart tissue *in vivo* and *in vitro* (Chen *et al.*, 1993, Chen *et al.*, 1994a, Chen *et al.*, 1994 b). Ca^{2+} plays an important role in normal physiology. In view of the above Mei *et al* (2000) studied the effect of melatonin on calcium current in the chick embryonic myocyte, where 2[125I] iodomelatonin binding sites have been demonstrated (Pang *et al.*, 2000). They demonstrated that melatonin-induced increase in high-voltage calcium current may increase myocyte contractility and enhance cardiac output. Therefore, a regulatory role for melatonin on cardiac function should be considered further.

1.3.5 Effects of melatonin on ischaemia and reperfusion injury

Melatonin has significant protective effects against the cardiac damage and altered physiology that occur during ischaemia/reperfusion injury (reviewed recently by Reiter & Tan, 2003). These actions were apparent even when melatonin was given as a single dose. Tan *et al* (1998) used a Langendorff isolated perfused rat heart model. The left descending coronary artery was ligated for 10 minutes, which reduced the total coronary flow by more than 25%. A 10 minute reperfusion period was allowed during which most hearts (80 to 90%, respectively) exhibited premature ventricular contractions (PVC) and/or ventricular fibrillation (VF). When melatonin was infused throughout the period of occlusion and after reopening the coronary artery it significantly reduced both PVC and VF. The melatonin concentration in the perfusate was either 1,10 or 50 μM . Anticipating that the effects of melatonin in this study were likely related to its antioxidant actions, Tan *et al* (1998) infused another free radical scavenger i.e. vitamin C as a positive control in a separate group of hearts. At a concentration of 500 μM (i.e. 500 times melatonin concentration) ascorbate was significantly less effective than melatonin in preventing the cardiac arrhythmias in this model.

Investigations regarding the antioxidant actions of melatonin are increasing. Work done in our laboratory (Salie *et al.*, 2001) did a thorough *in vitro* analysis of melatonin action in a setting of the hypoxia/reoxygenation in cardiomyocytes. Adult cardiomyocytes were isolated and grown in culture and chemical hypoxia was induced by the

addition of 1.5 mM KCN and 20 mM deoxyglucose to the superfusion fluid. Cells were then preloaded with either tetramethylrhodamine (TMRM) in combination with either dichlorodihydrofluorescein (DCDHF), dihydorhodamine 123 (DHR) or Fluo 3 to determine the effects of melatonin on H_2O_2 , ROS and Ca^{2+} respectively using the confocal microscope. Chemical hypoxia resulted in significantly increased H_2O_2 , ROS and Ca^{2+} fluorescence. Melatonin at doses 50 and 100 μM significantly reduced all these changes. These findings strongly implicate and support the antioxidant and protective properties of melatonin. Kaneko and workers (2000) used the isolated perfused working heart model, and subjected hearts to 30 minutes ischaemia followed by a 30 minutes of reperfusion. In this study melatonin concentration in the perfusion medium was 100 μM . This group also found a significant reduction in the duration of ventricular tachycardia relative to the duration of these arrhythmias in control hearts. Additionally, melatonin significantly restored the functional recovery of the left ventricle (LV) : LV developed pressure ,+dp/dt and - dp/dt were 63.0, 60.3 and 59.4 respectively compared to the values of the controls - 30.2, 29.7, 31.5 respectively. They then further extended their study by measuring OH^\cdot generation (by estimating levels of 2,3 - dihydroxybenzoic acid after salicylate administration) and observed a highly significant reduced amount of metabolite in the perfusate in melatonin treated hearts. At the end of reperfusion, tissue myocardial levels of lipid peroxidation products were measured. Melatonin again reduced the levels of lipid peroxidation in the heart significantly. This

study concluded that melatonin was beneficial during I/R and the results were consistent with the free radical scavenger actions of melatonin.

Lagneux *et al* (2000) recently published their findings in which both melatonin and an analogue, 5-methoxy-carbonylamino-N-acetyl tryptamine (5-MCA-NAT), were compared in terms of their efficiencies in reducing both reperfusion arrhythmias and infarct volume after I/R. Rats were treated with melatonin (1 or 10 mg/kg i.p.) or with 5-MCA-NAT (10 mg/kg i.p.) 30 minutes before the hearts were perfused using the Langendorff method. In terms of both the reperfusion arrhythmias and infarct size, this group reported that melatonin and 5-MCA-NAT both provided 'spectacular protection' against I/R injury. Taking in to consideration the large amount of experimental evidence implicating free radicals in cardiac arrhythmias during hypoxia/reoxygenation (Shiki & Hearse, 1987), Lagneux *et al* (2000) surmised that free radical scavenging activity of melatonin accounted for the ability to restore cardiac function. However since the role of free radicals in determining infarct size is some what less clear (Jeroudi *et al.*, 1994) the authors were cautious in assuming that the reduction in the size of the lesion was exclusively related to this indole's antioxidant properties.

Lee *et al* (2002) performed the first in vivo study in which melatonin was tested for its ability to protect the heart when blood supply was transiently interrupted. A bolus intravenous injection of either 0.5, 1.0, 5.0 mg/kg was administered before left coronary artery occlusion. Melatonin markedly

suppressed ventricular tachycardia and fibrillation and also reduced the total number of PVC's upon reperfusion. Finally, while eight of the nine rats subjected to ischaemia/reperfusion died, none of those treated with melatonin died.

Most studies reported cardiac protection if protocols included melatonin pretreatment. Only a few studies conducted with pretreatment of melatonin reported no myocardial protection. For example, Dave *et al* (1998) conducted a study in which anaesthetized rabbits were treated with melatonin 10 mg/kg intravenously 10 min before coronary artery occlusion. All rabbits underwent 30 minutes of occlusion and 3 hour reperfusion. The control rabbits received vehicle. Melatonin at this dose had no limiting effect on infarct size (0.38 ± 0.06 , compared to the controls, 0.34 ± 0.07). Effects of melatonin are summarized in Table 1.1

Table 1.1: A literature review of melatonin function during ischaemia/reperfusion injury

Model	Duration of I/R (min)	Melatonin actions	Reference
Langendorf model (rat)	10/10	Reduced premature ventricular contraction and fibrillation	Tan et al., 1998
Langendorf model (rat)	30/30	Reduced ventricular tachycardia and fibrillation; Restored ventricular function; Reduced lipid peroxidation; lowered OH [·] generation	Kaneko et al., 2000
Langendorf model (rat)	5/30	Reduced reperfusion arrhythmias; reduced infarct size	Lagneux et al., 2000
Langendorf model (rat)	20/40	Reduced fibrillation	Szarszoi et al., 2001
In situ heart (rat)	45/60	Reduced ventricular tachycardia and fibrillation; Lowered total ventricular contractions, reduced O ₂ and MPO activity, lowered infarct size, increased survival	Lee et al., 2002
In situ heart (rat)	7/7	Reduced ventricular fibrillation, Lowered mortality	Sahna et al., 2002
In situ heart (rat)	3/120	Reduced infarct size	Sahna et al., 2003

In situ heart (rabbit)	30/180	No improvement in cardiac function , no reduction in infarct size	Dave et al., 1998
Cardiomyocytes in culture(rats)	12.5 or 27.5/1.5	Reduced morphological damage, lowered oxygen free radical generation , reduce $[Ca^{2+}]_i$	Salie et al., 2001

1.4 Motivation and aims of study

Evidence exist that adenosine, nitric oxide, reactive oxygen species and opioids elicit both classic and delayed preconditioning. It has been established in our laboratory that transient β -adrenergic stimulation with isoproterenol mimics classic ischaemic preconditioning (Lochner *et al.*, 1999). Beta-adrenergic stimulation increases both nitric oxide (Balligand *et al.*, 1999) and reactive oxygen species generation (Opie *et al.*, 1979). The mechanism of delayed preconditioning includes both NO synthesis and ROS production (Bolli 1999). In view of this it is plausible that isoproterenol could elicit delayed preconditioning. However, to date there is no evidence that β -stimulation with isoproterenol can trigger delayed myocardial preconditioning in the isolated rat heart.

We hypothesise that :

- (1) Beta-adrenergic stimulation with isoproterenol can elicit delayed myocardial preconditioning.
- (2) The mechanism of beta-adrenergic preconditioning mediated delayed preconditioning will include nitric oxide synthesis and generation of radical oxygen species .

1.4.1 The specific aims of this study was as follows

- (1) To set up a model for second window of protection using beta-adrenergic stimulation with isoproterenol
- (2) To establish whether beta-adrenergic preconditioning was mediated by NOS activation and NO release (using the non-selective NOS inhibitor, N ω -nitro L-arginine, L-NA)
- (3) Investigate whether beta-preconditioning with isoproterenol protects via ROS generation or ROS mediated mechanisms by eliciting delayed PC in the presence of ROS scavengers such as melatonin, MPG and N-acetyl-cysteine.
- (4) Due to the findings obtained in (3) a substudy was done in which the protective effects of melatonin and specifically its ability to protect the myocardium after oral administration, was investigated

Chapter 2

Material and Methods

2.1 Animals

Male Wistar rats weighing 250-350 grams were utilized in this study. Institutional ethical guidelines were observed with respect to the handling of experimental animals. Rats were allowed free access to food and water. All animals were weighed in order to determine the correct dose for body weight before the intraperitoneal administration of isoproterenol, melatonin, L-nitro arginine, MPG or NAC. Injection volume did not exceed 3ml for any drug mentioned. Pentobarbital (50mg/kg) was administered intraperitoneally before excision of hearts.

2.2 Perfusion technique (Fig. 2.1)

At the end of the experimental protocol all hearts were rapidly excised and arrested in ice-cold (4 °C) normal saline solution. These hearts were then mounted on the aortic cannula and perfused retrogradely in a noncirculating manner at 100cm H₂O for 15 minutes to allow stabilization of the heart. The left atrium was cannulated to allow atrial perfusion (atrial pre-load 15 cmH₂O) according to the working heart model of Neely as modified by Opie and coworkers (1971). When switched to working heart perfusion, the left ventricle ejected against a hydrostatic pressure of 100cm H₂O. Myocardial temperature was thermostatically controlled by inserting a temperature probe into the pulmonary artery and was checked

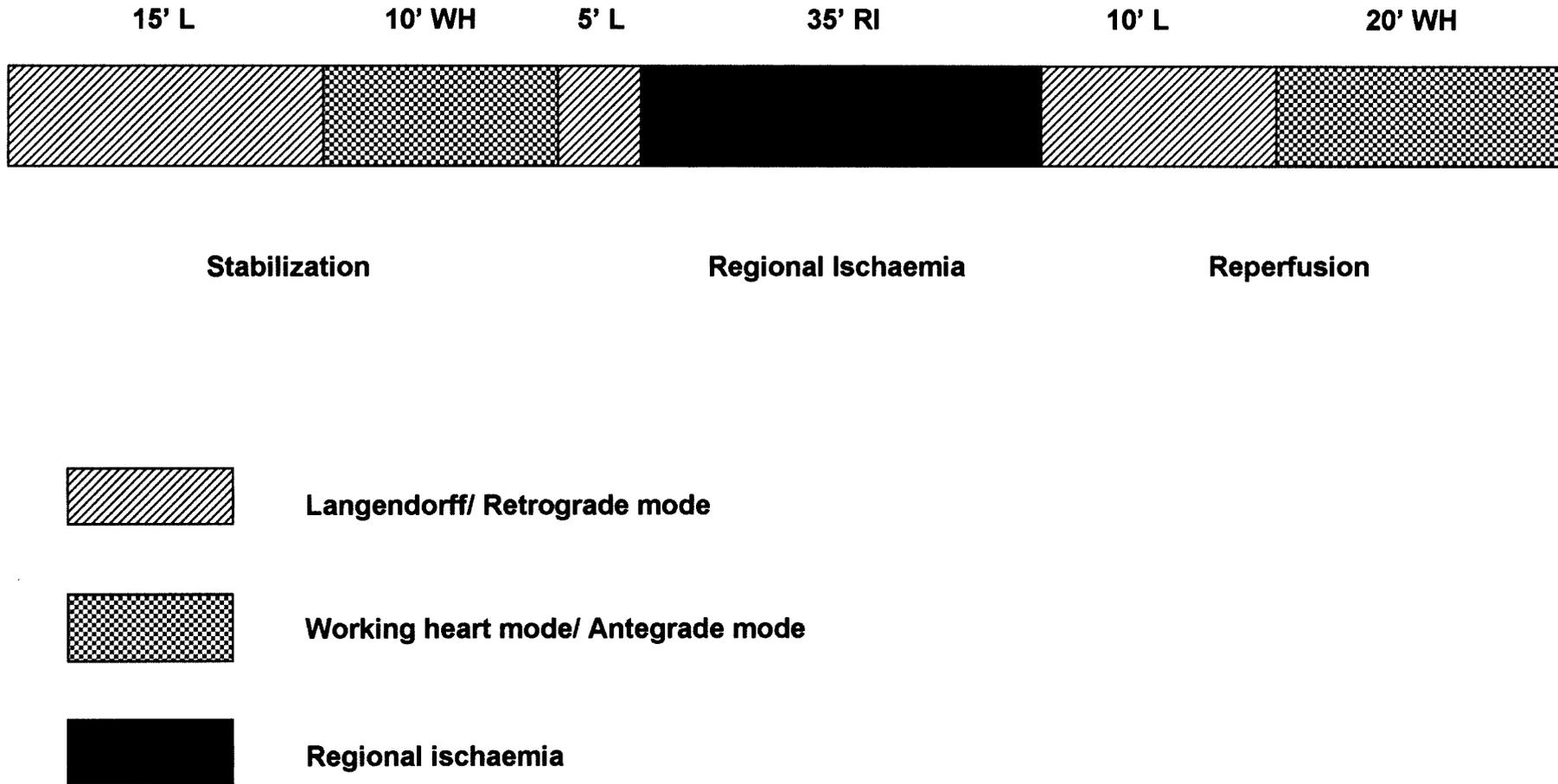


Fig. 2.1 Perfusion protocol after completion of treatment protocols

at regular intervals to ensure that it remained constant during the experiment.

2.3 Perfusion buffer

A modified Krebs-Henseleit bicarbonate buffer was used containing (in mM/l) NaCl 119, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.2, MgSO₄.7H₂O 0.59, Na₂SO₄ 0.59, CaCl₂.H₂O 1.25 and Glucose 10.

2.4 Regional ischaemia

Hearts were perfused for a control period of 30 minutes -15 min retrogradely and 10 minutes in a working heart (antegrade) mode. Hearts were then perfused retrogradely for 5 minutes, after which a silk suture was inserted underneath the proximal LAD. After measuring coronary flow the suture was tightened and the LAD occluded for 35 minutes at 36.5°C. Coronary flow was measured again at the beginning of regional ischaemia and a 33% reduction in coronary flow in comparison to the 15 minute control coronary flow period indicated adequate regional ischaemia. Another confirmation of regional ischaemia was regional cyanosis of myocardial surface. Reperfusion consisted of 10 minutes retrograde and 20 minutes working heart perfusion. Perfusion protocols will be described in the experimental protocol section (2.7) below.

2.5 End - points of ischaemic damage

2.5.1 Myocardial function

The coronary (Q_e) and aortic (Q_a) flow rates in ml/minute were measured manually. The aortic pressure was obtained through a side-arm of the aortic cannula which was connected to a Viggo-Spectramed pressure transducer and was recorded in mm Hg. The peak systolic pressure (PSP) and heart rate (HR) were obtained from the recordings made. Total work was determined according to the formula of Kannengieser *et al.* (1979). To determine functional recovery of all hearts the post - ischaemic aortic output was expressed as a percentage of pre - ischaemic aortic output.

2.5.2 Determination of infarct size as end point

Following regional ischaemia and reperfusion the silk suture under the left anterior descending coronary artery branch was reoccluded. A 0.25% Evans blue solution was infused into the remaining coronary bed using a sterile syringe to delineate the area at risk. Hearts were removed and frozen. After 24 hours the frozen heart was cut into transverse slices of 2mm thickness. After slices had thawed, they were stained by incubation for 20 minutes in a 1% triphenyl tetrazolium choride buffer (TTC) with a pH of 7.4. Tetrazolium reacts with NADH and dehydrogenase enzymes and causes all vital tissue still containing the enzymes and co-factors to stain a deep red color. The infarcted area (dead tissue) of the heart remains unstained. After staining was completed, the slices were fixed by immersion in a 10% formalin solution. The heart slices were then

sandwiched between two glass plates to a uniform 2 mm thickness. The region of infarcted tissue and the risk zone was traced in each ventricular section. The volume of infarcted tissue and risk zone of each heart was calculated by multiplying the planimetered areas with the slice thickness and summing the products (Summa sketch II, Summa graphics). Infarct size was expressed as a percentage of zone at risk for infarction.

2.6 Chemicals, drugs and reagents

Melatonin (5-methoxy -N-tryptamine), isoproterenol, N-acetyl -cysteine and L-NA (N ω L-arginine) were purchased from Sigma (St. Louis, MO, USA). The melatonin radioimmunoassay test kit was obtained from Amersham. MPG (mercaptpropionyl-glycine) was obtained from Aldrich (St.Louis, USA).

2.7 Treatment protocols

Delayed preconditioning was elicited by intraperitoneal administration of isoproterenol 24 hours before induction of sustained regional ischaemia. The effect of pharmacological manipulations was studied 24h later by subjecting all hearts to regional ischaemia as described in 2.4.

2.7.1 Eliciting Delayed Beta-Adrenergic Preconditioning with isoproterenol - dose finding study (Figure 2.2)

Group 1: No previous treatment

Group 2: Two repeated intraperitoneal administrations of isoproterenol (0.04mg/kg) 3 hours apart.

Group 3: One intraperitoneal injection of isoproterenol (0.04mg/kg).

Group 4: Two intraperitoneal administrations of isoproterenol (0.02mg/kg) three hours apart.

Group 5: Four repeated administrations of isoproterenol dose (0.004mg/kg) intraperitoneally, at one hour intervals.

Group 6: Four injections of isoproterenol (0.0004 mg/kg) also at 1 hourly intervals.

2.7.2 Investigating the role of NO in β - adrenergic delayed preconditioning (Fig. 2.3)

To investigate whether β - adrenergic delayed preconditioning was mediated through NO generation, the non – selective NOS inhibitor (L-NA) was administered an hour before preconditioning with isoproterenol

Group 1 : No previous treatment

Group 2 : Pre-treatment with 17.5 mg/kg L-NA 24 hours before regional ischaemia

Group 3 : Four injections of isoproterenol dose (0.0004 mg/kg) also at 1 hourly intervals.

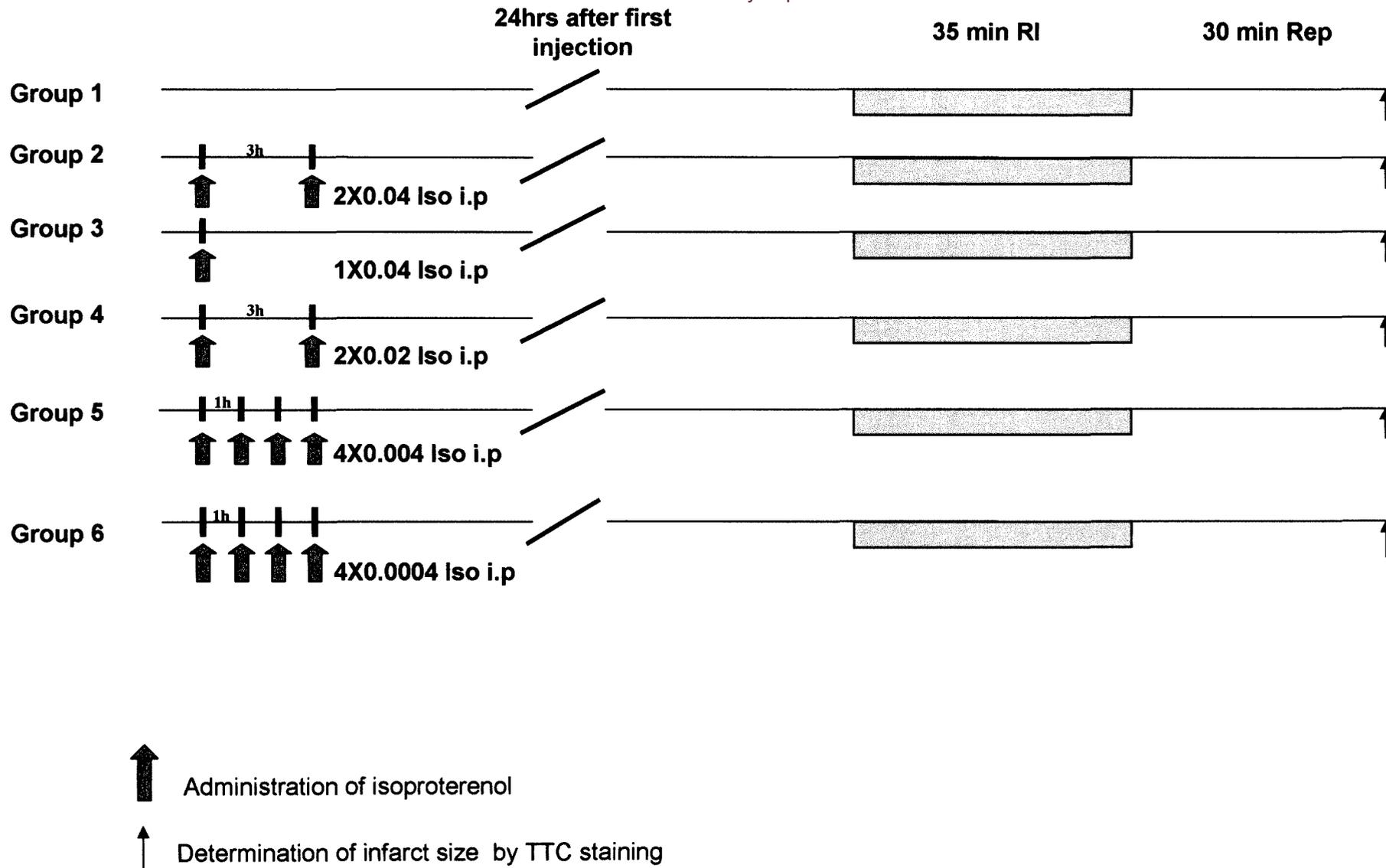


Fig. 2.2 Experimental protocols for optimal delayed beta-adrenergic preconditioning with isoproterenol

Group 4 : Pre-treatment with L-NA (N ω L-arginine) an hour before pharmacological preconditioning with four repeated administrations of isoproterenol (0.0004 mg/kg) an hour apart.

2.7.3 **Determining the role of Reactive Oxygen Species in mediating β -adrenergic delayed preconditioning (Fig 2.4)**

The free radical scavengers Melatonin, MPG and NAC was administered before preconditioning with isoproterenol to investigate the role of ROS in β -adrenergic delayed preconditioning.

Group 1 : No previous treatment.

Group 2: Treatment with 5 mg/kg melatonin alone

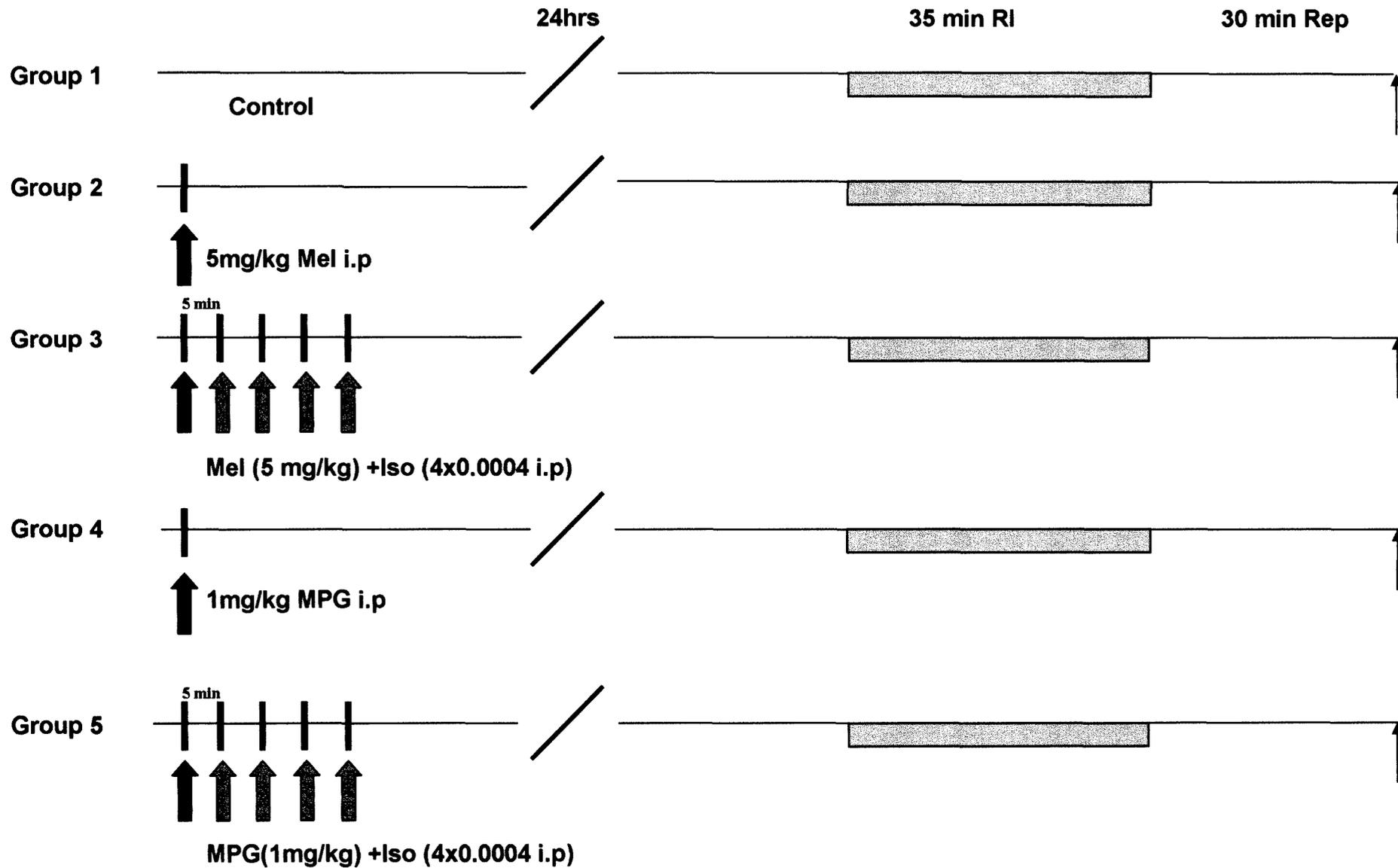
Group 3: Pre-treatment with melatonin (5 mg/kg) 5 minutes before pharmacological preconditioning with four repeated injections of isoproterenol (0.0004 mg/kg) an hour apart.

Group 4: Treatment with MPG (1 mg/kg) alone

Group 5: Administration of MPG (1 mg/kg) 5 minutes before preconditioning with isoproterenol (four repeated injections of isoproterenol 0.0004 mg/kg an hour apart).

Group 6: Administration NAC (10 mg/kg) alone before exposure to ischaemia /reperfusion protocol.

Group 7: One injection of NAC (10 mg/kg) 5 minutes before pharmacological preconditioning with four repeated injections of isoproterenol (0.0004 mg/kg) an hour apart



(Protocols continued on next page)

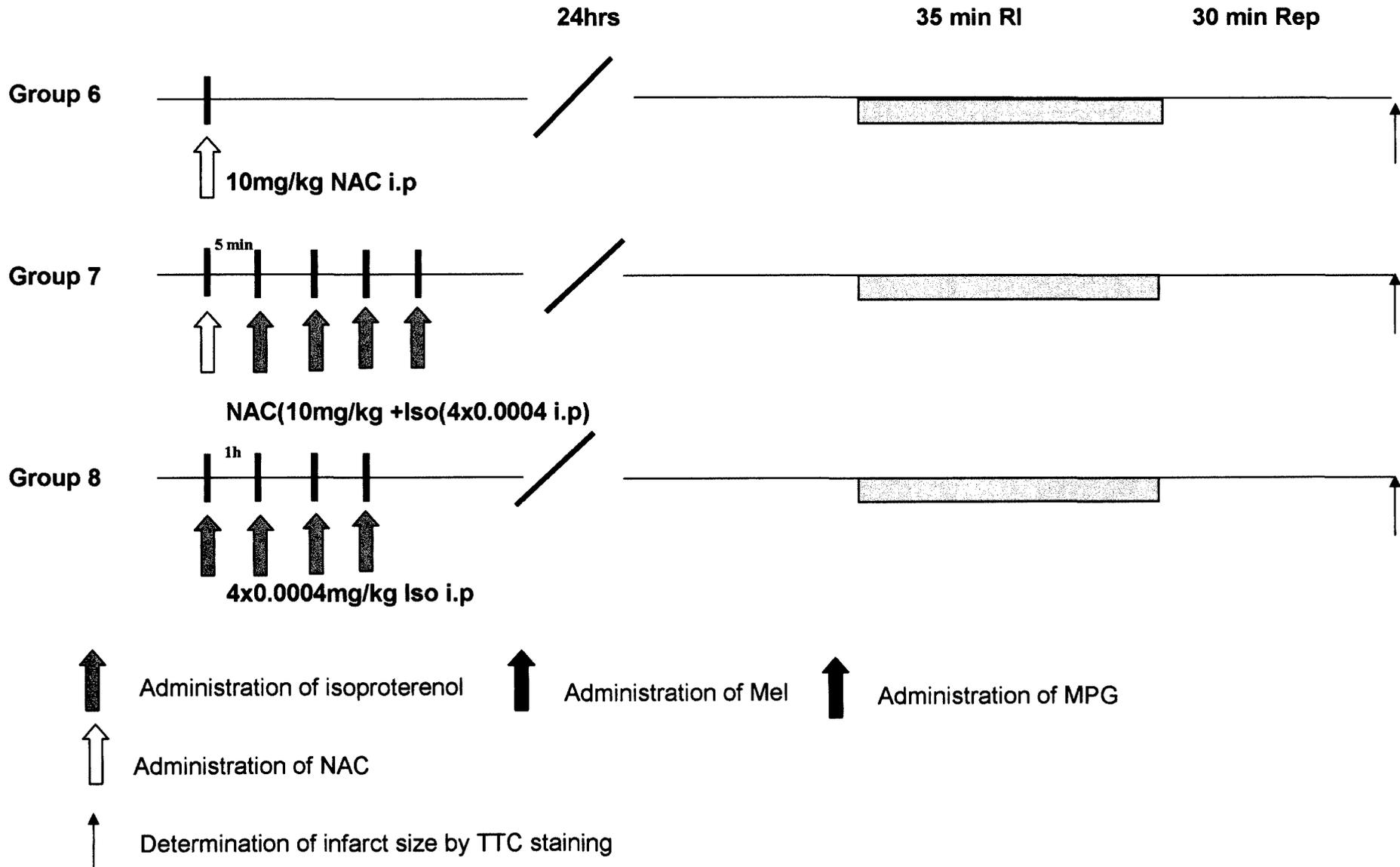


Fig. 2.4 Free radical scavenger (Mel, MPG & NAC) pretreatment and β -adrenergic delayed myocardial protection

Group 8: Administration of 4 repeated injections of isoproterenol at 0.0004 mg/kg (an hour apart)

2.7.4 Investigating the protective effect of intraperitoneally administered melatonin (Fig 2.5)

(i) Effects of melatonin injected intraperitoneally on ischaemia/reperfusion injury 24 hours later (Fig 2.5)

Group 1 : Control group no pretreatment .

Group 2 : Pretreatment with 2.5mg/kg melatonin intraperitoneally .

Group 3 : 5 mg/kg melatonin administered intraperitoneally.

(ii) Investigating the protective effect of orally administered melatonin (Fig 2.6)

Melatonin was dissolved in ethanol and added to drinking water. The final ethanol concentration (10 μ l/100ml) was 0.01% for both vehicle and melatonin treated rats. All rats received this treatment for seven days. The water bottles were covered with aluminium foil because melatonin is light sensitive and fresh solutions were prepared daily for seven days.

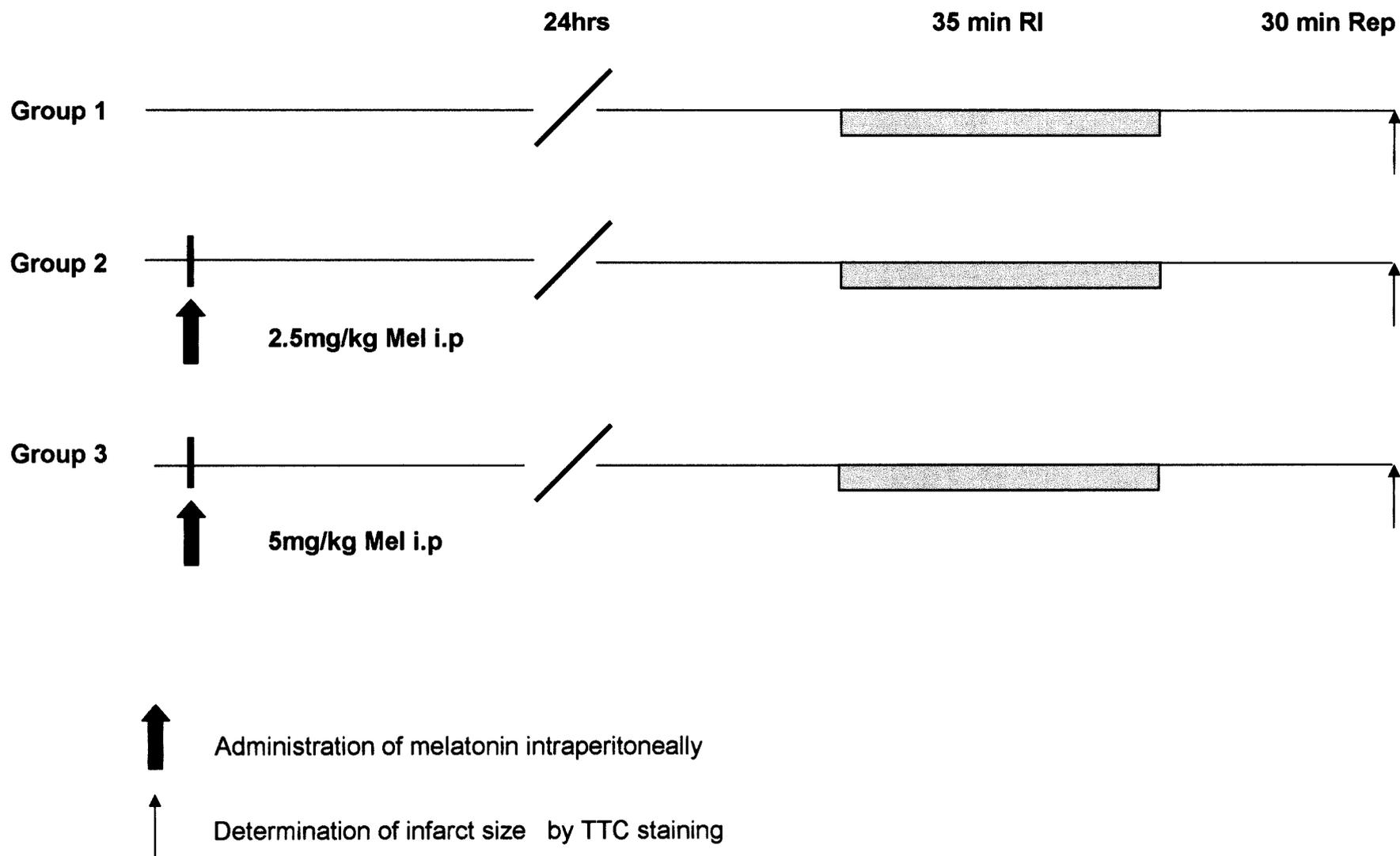


Fig. 2.5 Experimental protocols for delayed preconditioning with intraperitoneal administration of melatonin

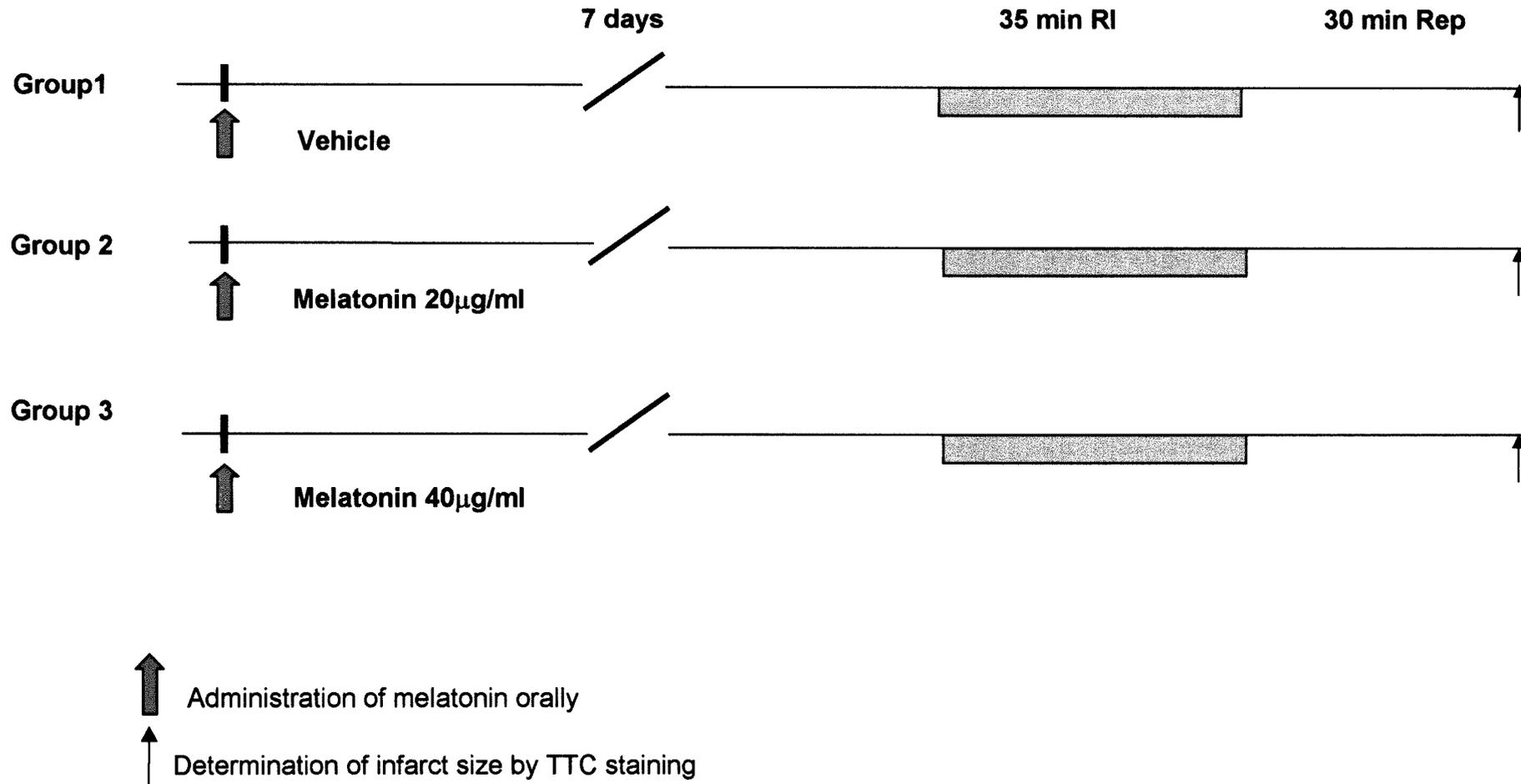


Fig. 2.6 Experimental protocols for melatonin supplementation in drinking water and effects on infarct size and functional recovery

Experimental groups

Rats were randomly assigned to one of three groups described below:

Group 1 : (Control group) Vehicle-ethanol (0.01%) that was added to drinking water for seven days.

Group 2 : Melatonin 20 μ g/ml drinking water for seven days.

Group 3 : Melatonin 40 μ g/ml drinking water for seven days.

(iii) Evaluation of the duration of the protective effect of oral melatonin of following its withdrawal (Fig 2.7)

Group 1: Control (0.01% ethanol) in drinking water.

Groups 2, 3, 4: All animals received melatonin 40 μ g/ml in their drinking water. Melatonin drinking water was replaced with normal water for 2, 4 and 6 days respectively, after which the effect of regional ischaemia was studied.

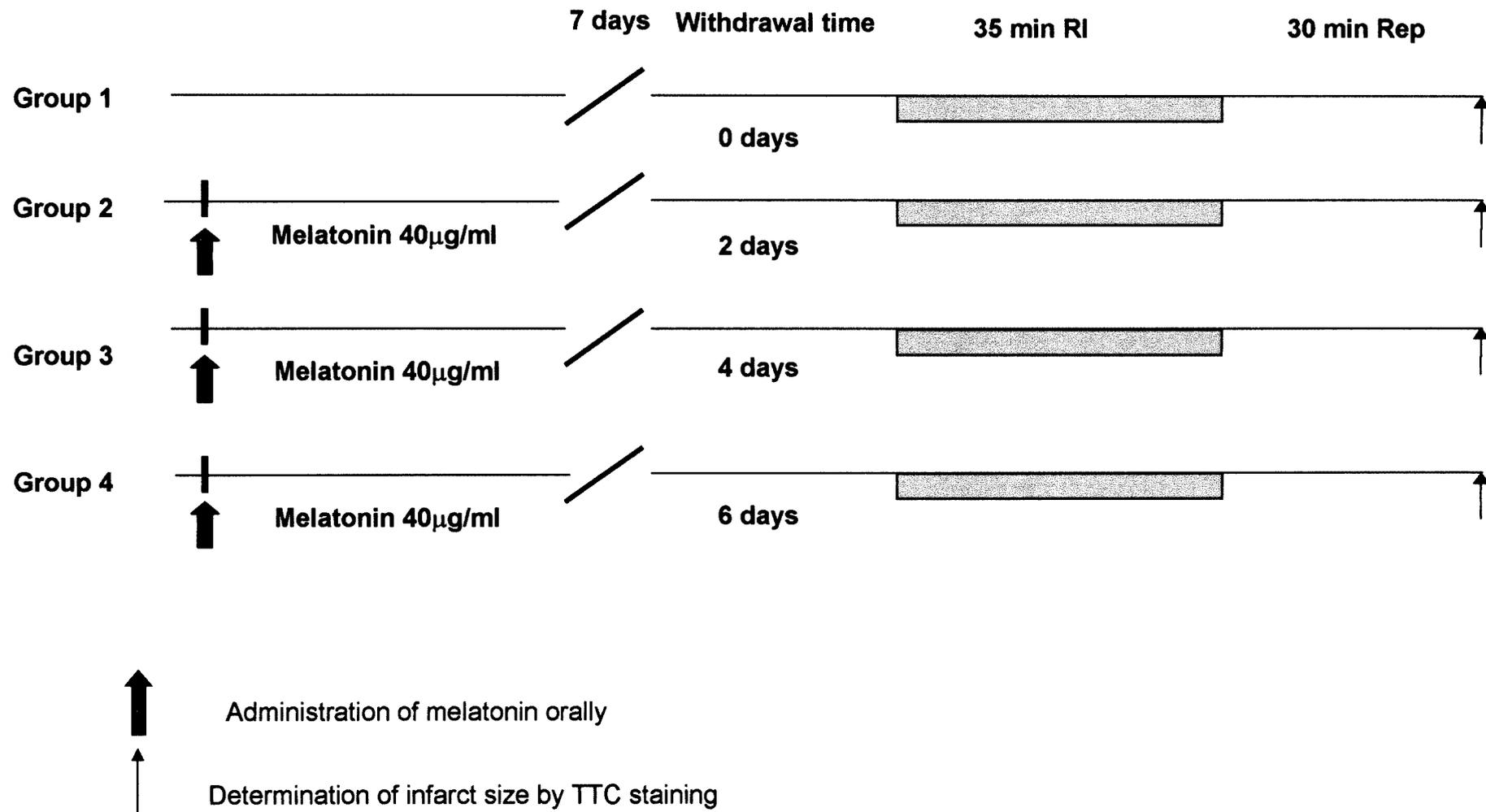


Fig.2.7 Experimental protocols for effects of melatonin withdrawal on delayed myocardial preconditioning

2.8 Biochemical analysis

2.8.1 Blood collection and plasma preparation

Rats were sacrificed after completion of the respective protocols. Peripheral blood was collected into sterile vacutainer tubes containing K₃EDTA. Blood samples were centrifuged at 26X100g for 10 minutes at 4°C. The supernatant was removed and stored in sterile vacutainer tubes at -20°C.

2.8.2 Melatonin assay principle

This assay procedure uses the basic principle of a radioimmunoassay. The radioactive labeled melatonin and unbound melatonin (sample melatonin) compete for a defined number of binding sites on the antibody. The separation between antibody-bound and free tracer is accomplished by the double antibody technique. The antibody-bound tracer is precipitated with a second antibody in the presence of glycol. The precipitate is counted in a gamma counter.

2.8.3 Melatonin assay procedure

Ten polystyrene tubes were labeled in duplicate. The first four polystyrene tubes were labeled for total counts (TC) and Nonspecific binding (NSB). The next fourteen tubes were labeled from A through to F (polystyrene tubes coated with antibody to melatonin). The remaining four tubes were controls (1,2) in duplicate. An additional forty tubes coated with anti-body were labeled for samples, also in duplicate. A 100 µl of standard A was

pipetted into tubes mark for NSB. Dispensed into the rest of the polystyrene tubes were 100 μ l of standards A-F or 100 μ l of plasma/tissue samples into respective tubes. To each tube was added 25 μ l of enzyme solution except for TC. All tubes were vortexed briefly and centrifuged at 500 g for 1 minute. This was followed by incubation of all tubes at room temperature for one hour. Then 50 μ l of assay buffer was added to every tube which was followed by vortexing every tube. 50 μ l of I-125 tracer was added to all tubes and all tubes were vortexed thoroughly. To each sample 50 μ l of diluted antiserum was added (except TC and NSB) and the content of each tube was mixed. All tubes were incubated overnight (18-20 hrs) at room temperature. A 500 μ l of cold (2-8 °C) precipitating reagent was added to all tubes (except TC), this was followed by vortexing all tubes thoroughly. An incubation period of 15 minutes at 2-8°C followed. All samples were centrifuged at 3000 g for 15 minutes. The supernatant was aspirated/decanted, and the radioactivity of the pellet counted for 60 seconds with a gamma counter.

2.9 Statistical procedure

All values were presented as mean \pm standard error of the mean. One way analysis of variance (ANOVA) combined with a Newman -Keuls post hoc test was used to test for difference between groups. Infarct size was expressed as a percentage of area at risk zone infarcted and an analysis of covariance was performed to detect differences among groups. Significant differences were defined as $P < 0.05$. All statistical procedures were performed using PRISM software.

Chapter 3

Results

3.1 Determination of optimal dose of isoproterenol that elicits β - adrenergic Delayed Myocardial Preconditioning

3.1.1 The effect of isoproterenol pre-treatment on haemodynamic parameters

3.1.1.1 Before sustained regional ischaemia (Table 3.1)

There was no significant difference in the basal heart rate, peak systolic pressure, coronary flows, cardiac output, total work as measured amongst all groups at 30 minutes of stabilisation. However, the aortic output of group 5 was significantly lower in comparison with the control group (* $p < 0.03$)(Fig 3.1).

3.1.1.2 Haemodynamic parameters at reperfusion following sustained regional ischaemia (Table 3.2)

Regional ischaemia of 35 minutes caused a significant reduction in all parameters during reperfusion. Heart rate, peak systolic pressure, coronary flow, aortic output, %functional recovery, cardiac output, and the total work were not significantly different amongst all groups after sustained regional ischaemia followed by 30 minutes of reperfusion.

Table 3.1 : Haemodynamic function of isoproterenol treated hearts before regional ischaemia

	Group 1 n=17	Group 2 n=6	Group 3 n=9	Group 4 n=5	Group 5 n=7	Group 6 n=7
HR (beats/min)	248.4±4.2	210.9±12.9	216.8±6.8	229.0±30.3	226.0±25.1	242.0±12.0
PSP(mmHg)	114.9±2.0	114.5±1.7	114.4±3.1	109.8±6.4	118.1±8.0	123.6±5.9
Coronary flow (ml/min)	17.1±2.1	16.1±1.8	17.8±2.5	14.4±2.0	14.6±4.0	16.7±1.6
Aortic output (ml/min)	36.9±0.6	34.3±0.9	34.1±0.9	32.6±2.6	28.3±3.1*	33.1±1.3
Cardiac output (ml/min)	54.7±0.9	50.4±1.8	51.9±1.0	47.0±3.9	42.5±3.8	49.8±1.9
Total work (Watt)	14.2±0.4	12.9±0.4	13.4±0.5	11.6±1.1	11.6±1.3	10.3±0.9

All data presented as Mean ± SEM

P* < 0.05 "vs" Group 1

Abbreviations

Group 1 = Control

Group 2 = 2 x 0.04 mg/kg Isoproterenol

Group 3 = 1 x 0.04 mg/kg Isoproterenol

Group 4 = 2 x 0.02 mg/kg Isoproterenol

Group 5 = 4 x 0.004 mg/kg Isoproterenol

Group 6 = 4 x 0.0004 mg/kg Isoproterenol

Table 3.2 : Haemodynamic function of isoproterenol treated hearts after regional ischaemia

	Group 1 n=17	Group 2 n=6	Group 3 n=9	Group 4 n=5	Group 5 n=7	Group 6 n=7
HR (beats/min)	244.1±18.8	222.9±6.1	219.2±28.3	235.0±19.7	187.5±34.1	251.7±11.2
PSP(mmHg)	87.8±5.5	92.5±2.1	78.7±9.9	88.9±3.4	80.5±13.4	95.9±2.1
Coronary flow (ml/min)	17.4±0.7	15.8±1.1	17.8±2.0	15.0±1.2	15.2±1.2	14.7±0.7
Aortic output (ml/min)	15.6±0.7	18.4±2.7	10.6±2.0	12.3±2.7	10.9±2.1	14.6±1.7
% Functional recovery	45.8±2.4	54.0±3.6	35.1±5.2	38.7±9.1	41.5±4.6	43.3±4.3
Cardiac output (ml/min)	31.7±2.4	34.2±2.8	27.4±4.1	27.3±4.0	26.0±2.9	29.3±2.4
Total work (watt)	6.7±0.5	7.2±0.6	5.4±0.8	5.5±0.9	5.0±0.9	6.4±0.6

All data presented as Mean ± SEM

P > 0.05 "vs" Group 1

Abbreviations

Group 1 = Control

Group 2 = 2 x 0.04 mg/kg Isoproterenol

Group 3 = 1 x 0.04 mg/kg Isoproterenol

Group 4 = 2 x 0.02 mg/kg Isoproterenol

Group 5 = 4 x 0.004 mg/kg Isoproterenol

Group 6 = 4 x 0.0004 mg/kg Isoproterenol

3.1.2 The effect of isoproterenol pre-treatment on myocardial infarct size

3.1.2.1 Risk zone volume (Table 3.3)

Risk zone volume expressed as a percentage of left ventricle volume, was the same in all groups, and were therefore comparable.

3.1.2.2 Infarct size (Fig 3.1)

Infarct size expressed as a percentage of area at risk (I/R) in the 6 experimental groups is presented in Fig (3.1). Infarct size of the control animals was $38.9 \pm 2.0\%$. All protocols of isoproterenol pre-treatment except that of group 2 caused a significant decrease in infarct size. Optimal protection was achieved at a dose of $4 \times 0.0004\text{mg/kg}$ - infarct size was reduced by 70.9% in comparison to the controls. Therefore all further interventions aimed at elucidating the mechanisms involved in β - adrenergic delayed preconditioning utilised this protocol.

3.2 The role of eNOS in mediating β - adrenergic delayed myocardial protection

To investigate whether beta adrenergic late preconditioning was mediated via nitric oxide generation, an exogenous NOS inhibitor (L-NA) was administered an hour before pharmacological preconditioning with four repeated intraperitoneal injections of 0.0004mg/kg isoproterenol an hour apart.

Table 3.3 : Isoproterenol pre-treated groups : Volume at risk expressed as a percentage of left ventricle volume

	Group 1 n=17	Group 2 n=6	Group 3 n=9	Group 4 n=5	Group 5 n=7	Group 6 n=7
R/L	43.4%±2.3	48.4%±3.2	41.3%±4.5	36.6%±3.6	35.3%±4.6	40.4%±4.0

R = Risk volume

L = Left ventricular volume

All data presented as Mean ± SEM

P>0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group 2 = 2 x 0.04 mg/kg Isoproterenol

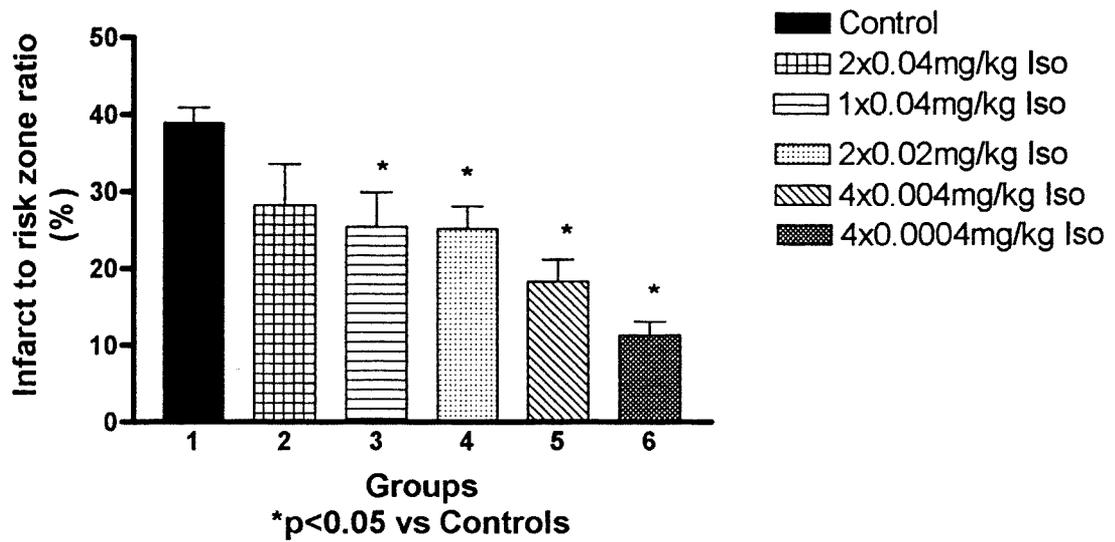
Group 3 = 1 x 0.04 mg/kg Isoproterenol

Group 4 = 2 x 0.02 mg/kg Isoproterenol

Group 5 = 4 x 0.004 mg/kg Isoproterenol

Group 6 = 4 x 0.0004 mg/kg Isoproterenol

Fig. 3.1 The effect of Isoproterenol pre-treatment on infarct size



Abbreviations

Iso = Isoproterenol

3.2.1 Haemodynamic function before sustained regional ischaemia

(Table 3.4)

At the end of the stabilization period the cardiac output and coronary flow of the L-NA + Isoproterenol pre-treated group (group 4) were significantly reduced in comparison to the controls (Group1)($p < 0.05$). In group 2, that was pre-treated with L-NA alone 24 hours before regional ischaemia, the coronary flow and the peak systolic pressure were significantly lower than controls (Group1). Heart rate, peak systolic pressure, aortic output, cardiac output and total work were not significantly different amongst all other groups.

3.2.2 Haemodynamic parameters at reperfusion following regional ischaemia (See table 3 .5)

There was no significant difference in heart rate, peak systolic pressure, aortic output (absolute or as a percentage of control value), total work, and cardiac output after 30 minutes of reperfusion. The coronary flow of Group 4 remained significantly lower than the coronary flow of control group ($*p < 0.02$), but was not significantly different from other treated groups.

3.2.3 The effect of L-NA pre-treatment on infarct size

3.2.3.1 Risk zone (Table 3.6)

The left ventricular risk zone volumes were comparable in all four groups.

Table 3.4 : Haemodynamic function of L-NA pre- treated hearts before regional ischaemia

	Group 1 n=17	Group2 n=5	Group 3 n=7	Group 4 n=5
HR (beats/min)	248.4±4.2	227.0±6.7	242.0±12.0	218.3±16.4
PSP(mmHg)	114.9±2.0	97.0±0.6*	123.6±5.9	106.8±5.4
Coronary flow (ml/min)	17.1±2.1	12.9±0.8*	16.7±1.6	12.3±0.7*
Aortic output (ml/min)	36.9±0.6	34.4±1.3	33.1±1.3	34.0±2.0
Cardiac output (ml/min)	54.7±0.9	47.3±1.2	49.8±1.9	46.9±2.5*
Total work (Watt)	14.2±0.4	10.4±0.2	10.3±0.9	11.1±0.7

All data presented as Mean ± SEM

*P<0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group2 = 17.5 mg/kg L-NA

Group 3= 4 x 0.0004 mg/kg Isoproterenol

Group 4= 17.5mg/kg L-NA + 4 X 0.0004mg/kg Isoproterenol

Table 3.5 : Haemodynamic function of L-NA pre- treated hearts at reperfusion following regional ischaemia

	Group 1 n=17	Group 2 n=5	Group 3 n=7	Group 4 n=5
HR (beats/min)	244.1±18.8	224.0±2.3	251.7±11.2	233.4±9.0
PSP(mmHg)	87.8±5.5	88.9±0.8	95.9±2.1	88.3±2.7
Coronary flow (ml/min)	17.4±0.7	15.1±1.9	14.7±0.7	13.4±1.6*
Aortic output(ml/min)	15.6±1.2	14.4±2.2	14.6±1.7	16.4±3.4
% Functional recovery	46.0±2.6	42.0±8.4	43.3±4.3	46.2±8.2
Cardiac output (ml/min)	31.7±2.4	29.5±1.1	29.3±2.4	29.8±3.8
Total work (Watt)	6.7±0.5	5.9±0.2	6.4±0.6	6.2±0.7

All data presented as Mean ± SEM

P>0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group 2 = 17.mg/kg L-NA

Group 3 = 4 x 0.0004 mg/kg Isoproterenol

Group 4= 17.5mg/kg L-NA + 4 X 0.0004mg/kg Isoproterenol

Table 3.6 : LNA -pretreated group : Volume at risk expressed as a percentage of left ventricle volume

	Group 1 n=17	Group 2 n=5	Group 3 n=7	Group 4 n=5
R/L	43.1%±1.9	41.2%±3.7	40.4%±4.0	38.8%±3.8

R = Risk volume

L = Left ventricular volume

All data presented as Mean ± SEM

P>0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group 2 = 17.5mg/kg L-NA

Group 3 = 4 x 0.0004 mg/kg Isoproterenol

Group 4 = 17.5mg/kg L-NA + 4 X 0.0004 mg/kg Isoproterenol

3.2.3.2. Infarct size (Fig 3.2)

In the isoproterenol pre-treated group (group 3), infarct size was significantly smaller in comparison to controls (Group1) ($11.3\pm 1.6\%$ and $38.9\pm 1.9\%$ respectively). The group pre-treated with L-NA alone had an infarct size that did not differ from the control treated group ($31.4\pm 3.8\%$ and $38.9\pm 1.9\%$ respectively). Treatment of hearts (Group 4) with 17.5mg/kg L-NA an hour before preconditioning with 4 repeated injections of isoproterenol at dose 0.0004mg/kg abrogated the infarct sparing effect of pharmacological preconditioning with isoproterenol at dose ($4\times 0.0004\text{mg/kg}$) 24 hours earlier.

These results suggest that NO generation during repeated β - stimulation is necessary to trigger a delayed cardioprotective response.

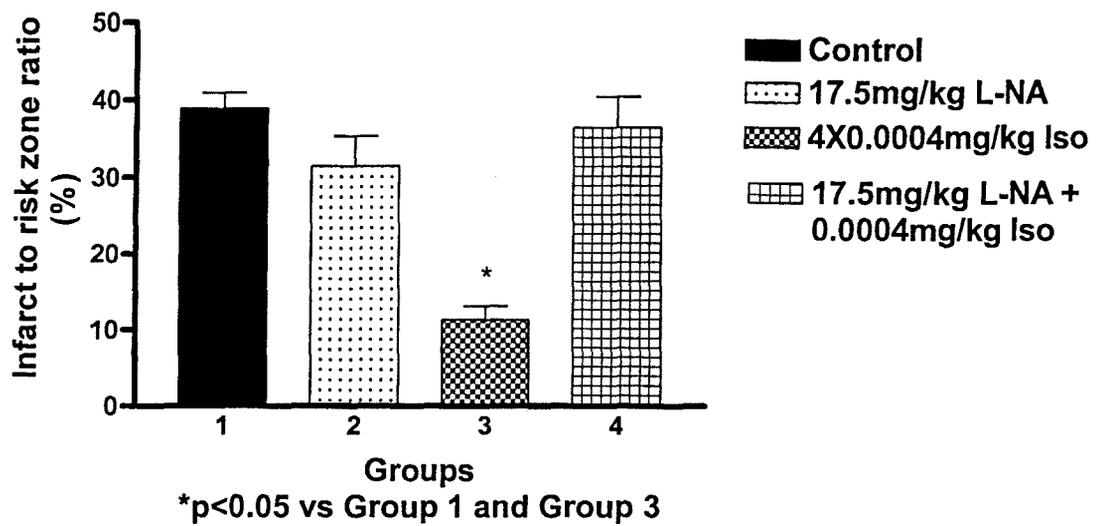
3.3 Effects of free radical scavenger pre- treatment on delayed β - adrenergic preconditioning

In order to investigate the role of ROS generation in β -adrenergic delayed PC the following scavengers of ROS were used - Melatonin, MPG and NAC.

3.3.1 The effect of ROS scavenger pre- treatment on delayed isoproterenol preconditioning

Exogenous melatonin , MPG and NAC were administered in different doses and time intervals before preconditioning with 4 intermittent administrations of isoproterenol intraperitoneally and an hour apart, with the aim to determine whether free radical scavengers pre-treatment before

Fig 3.2 The effect of L-NA pre-treatment on infarct size



Abbreviations

Iso = isoproterenol

L-NA = N^ω-nitro-L-arginine

preconditioning with repeated β -adrenergic stress would eliminate protection afforded by this protocol.

3.3.1.1 Haemodynamic function before regional ischaemia

(Table 3.7)

Pretreatment with melatonin at dose 5mg/kg, MPG (1mg/kg) and NAC (10mg/kg) did not have any effect on baseline parameters. However, in isoproterenol pretreated group hearts (group 8) had a peak systolic pressure that was significantly higher than group 1 and group 2.

Heart rate, coronary flow, cardiac output and total work measured at the end of the stabilization period were not significantly different amongst all groups (1-8) (Table 3.7).

3.3.1.2 Haemodynamic parameters at reperfusion following sustained ischaemia (Table 3.8)

The heart rate, peak systolic pressure, coronary flow, aortic flow, cardiac output, % functional recovery, and total work were similar in all groups.

3.3.2 The effect of ROS scavenger pretreatment on infarct size

3.3.2.1 Risk zone (Table 3.9)

The risk zone volumes expressed as a percentage of the left ventricle were not significantly different from the normal control treated or vehicle controls.

Table 3.7 : Haemodynamic function of ROS scavenger treated hearts before regional ischaemia

	Group 1 n=6	Group 2 n=6	Group 3 n=6	Group 4 n=5	Group 5 n=4	Group 6 n=6	Group 7 n=6	Group 8 n=6
HR (beats/min)	249.0±13.5	240.0±7.4	243.0±6.7	264.0±19.9	250.0±3.6	261.0±4.9	262.0±15.1	242.0±12.0
PSP(mmHg)	102.9±2.5	108.6±2.4	109.4±4.6	106.8±2.4	105.3±1.3	109.9±5.4	101.4±2.1	123.6±5.9*
Coronary flow (ml/min)	15.0±1.2	14.8±0.9	14.8±1.1	15.9±0.8	13.1±0.6	15.3±0.8	17.0±0.8	16.7±1.6
Aortic output (ml/min)	41.6±2.4	39.0±3.2	31.7±1.1	39.2±1.7	40.0±0.7	42.8±1.2	39.8±1.7	33.1±1.3
Cardiac output (ml/min)	56.6±3.3	53.8±3.6	46.5±2.0	55.1±2.1	53.1±1.2	58.1±2.0	56.8±1.9	49.8±1.9
Total work (Watt)	13.3±1.0	13.8±0.9	11.6±0.8	13.5±0.7	14.0±0.6	14.5±0.7	13.1±0.7	10.3±0.9

All values presented as mean ± SEM

*P<0.05 " vs" Group1

Group 1 = Control

Group 2 = 5mg/kg Melatonin

Group 3 = 5mg/kg Melatonin + 0.0004mg/kg Isoproterenol

Group 4 = 1mg/kg MPG

Group 5 = 1mg/kg MPG+4 x 0.0004 mg/kg Isoproterenol

Group 6 =10mg/kg NAC

Group 7 = 10mg/kg NAC+4 x 0.0004 mg/kg Isoproterenol

Group 8 = 4 x 0.0004 mg/kg Isoproterenol

Table 3.8 : Haemodynamic function of ROS scavenger treated hearts after regional ischaemia

	Group 1 n=6	Group 2 n=6	Group 3 n=6	Group 4 n=5	Group 5 n=4	Group 6 n=6	Group 7 n=6	Group 8 n=6
HR (beats/min)	256.0±19.3	244.0±8.9	254.0±15.4	240.0±10.4	227.0±23.7	240.0±3.9	258.0±12.4	251.7±11.2
PSP(mmHg)	88.3±2.4	92.6±1.65	96.0±2.4	95.4±1.3	96.1±2.9	92.4±1.0	94.9±3.5	95.9±2.1
Coronary flow (ml/min)	12.3±1.0	17.30±1.4	13.8±0.7	12.9±0.5	12.4±0.3	15.8±0.6	14.0±0.8	14.7±0.7
Aortic output (ml/min)	16.8±2.2	19.7±2.5	18.2±1.5	22.4±1.3	19.0±2.9	21.5±1.9	15.5±2.4	14.6±71.7
%Functional recovery	46.0±2.6	47.7±4.7	57.4±5.2	57.9±5.4	47.4±8.3	50.0±4.0	39.9±6.6	43.3±4.3
Cardiac output (ml/min)	29.1±2.5	36.9±3.6	31.7±1.2	38.8±2.7	31.4±2.6	37.3±2.2	29.5±2.7	29.3±2.4
Total work (Watt)	5.8±0.7	7.7±0.8	6.8±0.3	8.2±0.3	6.8±0.6	7.6±0.5	8.1±1.4	6.4±0.6

All values presented as mean ± SEM

P>0.05 " vs" Group1

Group 1 = Control

Group 2 = 5mg/kg Melatonin

Group 3 = 5mg/kg Melatonin + 0.0004mg/kg Isoproterenol

Group 4 = 1mg/kg MPG

Group 5 = 1mg/kg MPG+4 x 0.0004 mg/kg Isoproterenol

Group 6 =10mg/kg NAC

Group 7 = 10mg/kg NAC+4 x 0.0004 mg/kg Isoproterenol

Group 8 = 4 x 0.0004 mg/kg Isoproterenol

Table 3.9 : ROS scavenger pre-treated groups : Volume at risk expressed as a percentage of left ventricle volume

	Group 1 n=6	Group 2 n=6	Group 3 n=6	Group 4 n=5	Group 5 n=4	Group 6 n=6	Group 7 n=6	Group 8 n=6
R/L	40.9%±3.2	37.0%±4.1	33.5%±3.8	39.3%±4.8	45.9%±4.9	44.9%±4.7	44.4%±3.9	40.4%±4.0

R = Risk volume

L = Left ventricular volume

All data presented as Mean ± SEM

P>0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group 2 = 5mg/kg Melatonin

Group 3 = 5mg/kg Melatonin + 0.0004mg/kg Isoproterenol

Group 4 = 1mg/kg MPG

Group 5 = 1mg/kg MPG+4 x 0.0004 mg/kg Isoproterenol

Group 6 =10mg/kg NAC

Group 7 = 10mg/kg NAC+4 x 0.0004 mg/kg Isoproterenol

Group 8 = 4 x 0.0004 mg/kg Isoproterenol

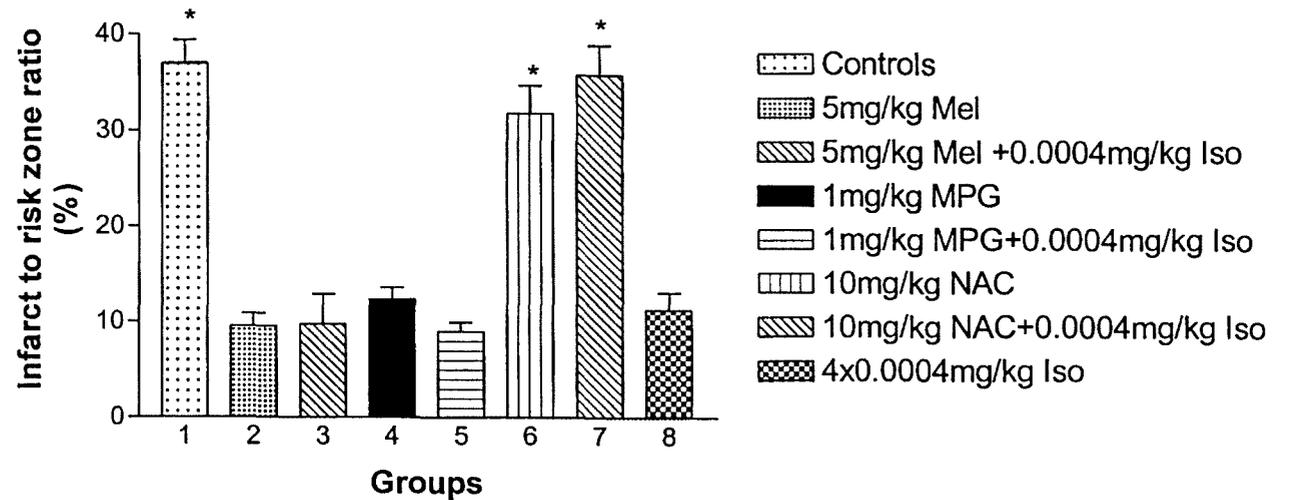
3.3.2.2 Effect of Melatonin on infarct size (Fig 3.3)

Melatonin administered at dose 5 mg/kg intraperitoneally, 24 hours before regional ischaemia resulted in a highly significant decrease in infarct size compared to the controls ($9.5 \pm 1.3\%$ and $36.9 \pm 2.3\%$ respectively). Pretreatment with combination of melatonin (5mg/kg) + isoproterenol (0.0004mg/kg) and isoproterenol (4X0.0004mg/kg) alone both resulted in a highly significant decrease in infarct size ($p < 0.0001$). These results indicate that melatonin could not be used to study the effect of ROS generation in isoproterenol induced delayed PC. Another important deduction made from these results is the important role for melatonin as a free radical scavenger that is so effective that it elicits an infarct sparing effect 24 hours after pre-treatment. This observation led to a sub-study that investigated the protective effect of i.p. and oral melatonin as reported in section 3.4 and 3.5 of this thesis.

3.3.2.3 Effect of MPG on Infarct size (Fig3.3)

Pretreatment with combination of MPG(1mg/kg) + isoproterenol (0.0004mg/kg) and MPG (1mg/kg) alone resulted in a significant reduction in infarct size ($p < 0.0001$). Similar to melatonin, our results of this protocol and model indicated that MPG cannot be used to study the effect of ROS generation in isoproterenol induced delayed PC.

Fig 3.3 The effect of ROS scavenger pretreatment on infarct size



*p<0.05 vs Group 2, 3, 4, 5 and 8

Abbreviations

Mel = Melatonin

Iso=Isoproterenol

MPG = mercaptopropionylglycine

NAC= N-Acetylcysteine

3.3.2.4 Effect of NAC on Infarct size (Fig 3.3)

Infarct sizes of Group1 did not differ from that of animals treated with 10 mg/kg NAC only on day 1 (Group 6). Values were $36.9 \pm 2.3\%$ and $31.8 \pm 2.8\%$ respectively. Pre-treatment with 10 mg/kg NAC 5 minutes before preconditioning with four repeated administrations of 0.0004mg/kg isoproterenol i.p. abolished the delayed infarct sparing effect of repeated isoproterenol stimulation - infarct size $36.9 \pm 2.3\%$ for controls (group 1) and 35.9 ± 2.8 for NAC + Iso combination group (group 7). These results indicate that the mechanism of delayed β -adrenergic myocardial protection involves generation of reactive oxygen species.

3.4 Evaluation of melatonin as a protective agent

3.4.1 Intraperitoneal administration of melatonin

3.4.1.1 Haemodynamic parameters before sustained regional ischaemia (Table 3.10)

Intraperitoneal melatonin treatment at doses 2.5 mg/kg and 5 mg/kg significantly reduced pre-ischaemic coronary flows in comparison to the control treated group. The coronary flows were 14.7 ± 0.9 and 15.1 ± 0.5 in group 2 and 3 respectively and 18.0 ± 0.70 in the controls (Group1). Peak systolic pressure measured at the end of the stabilization period was significantly higher in controls compared to both melatonin treated groups ($p < 0.009$). Heart rate, aortic output, cardiac output, and total work were

Table 3.10 : Haemodynamic function of intraperitoneal melatonin treated hearts before regional ischaemia

	Group 1 n=10	Group 2 n=7	Group 3 n=6
HR (beats/min)	245.0±7.1	250.7±5.4	240.1±8.1
PSP(mmHg)	118.6±2.7	103.1±1.7*	108.6±2.6*
Coronary flow (ml/min)	18.0±0.7	14.7±0.9*	15.1±0.5*
Aortic output (ml/min)	36.9±0.6	45.1±01.1	39.0±03.2
Cardiac output (ml/min)	57.5±0.6	60.2±1.4	53.7±3.9
Total work (Watt)	14.3±0.4	12.7±0.5	13.8±0.9

All data presented as Mean ± SEM

*P<0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group 2 = 2.5mg/kg Melatonin

Group 3 = 5mg/kg Melatonin

not affected by treatment with melatonin at both doses 24 hours earlier as shown in table 3.10.

3.4.1.2 Haemodynamics after sustained regional ischaemia (Table 3.11)

There was no difference measured in heart rate, peak systolic pressure, coronary flows, aortic flow, % functional recovery, cardiac output and total work after 35 minutes of sustained regional ischaemia followed by 30 minutes of reperfusion.

3.4.1.3 The effect of intraperitoneal pretreatment on infarct size

3.4.1.3.1 Risk zone (Table 3.12)

The left ventricular risk zone was similar in all three groups.

3.4.1.3.2 Infarct size (Fig 3.4)

Pre - treatment with melatonin 5mg/kg or 2.5 mg/kg significantly reduced infarct size expressed as a percentage of the risk zone when compared to the infarct size of the control group ($p < 0.0001$). In group 2 the infarct size was reduced by 53.4% and in Group3 by 76.3% in comparison to the control treated group ($P < 0.001$). It is concluded that both melatonin doses used afforded delayed cardioprotection.

Table 3.11 : Haemodynamic function of intraperitoneal melatonin treated hearts after regional ischaemia

	Group 1 n=10	Group 2 n=7	Group 3 n=6
HR (beats/min)	232.0±18.1	243.3±6.7	244.2±9.7
PSP(mmHg)	93.4±1.3	92.3±1.4	92.6±1.8
Coronary flow (ml/min)	17.1±1.1	13.5±1.0	17.2±1.5
Aortic output (ml/min)	15.6±0.7	20.3±01.9	19.7±2.5
%Functional recovery	46.0±2.6	45.5±5.5	47.7±4.7
Cardiac output (ml/min)	33.1±2.1	33.7±2.3	36.9±4.0
Total work (Watt)	6.7±0.5	7.0±0.5	7.7±0.8

All data presented as Mean ± SEM

P>0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group 2 = 2.5mg/kg Melatonin

Group 3 = 5mg/kg Melatonin

Table 3.12 : Melatonin pre-treated intraperitoneally : Volume at risk expressed as a percentage of left ventricle volume

	Group 1 n=10	Group 2 n=7	Group 3 n=6
R/L	42.8%±1.8	46.9%±2.2	37.0%±4.1

R = Risk volume

L = Left ventricular volume

All data presented as Mean ± SEM

P>0.05 "vs" Group1

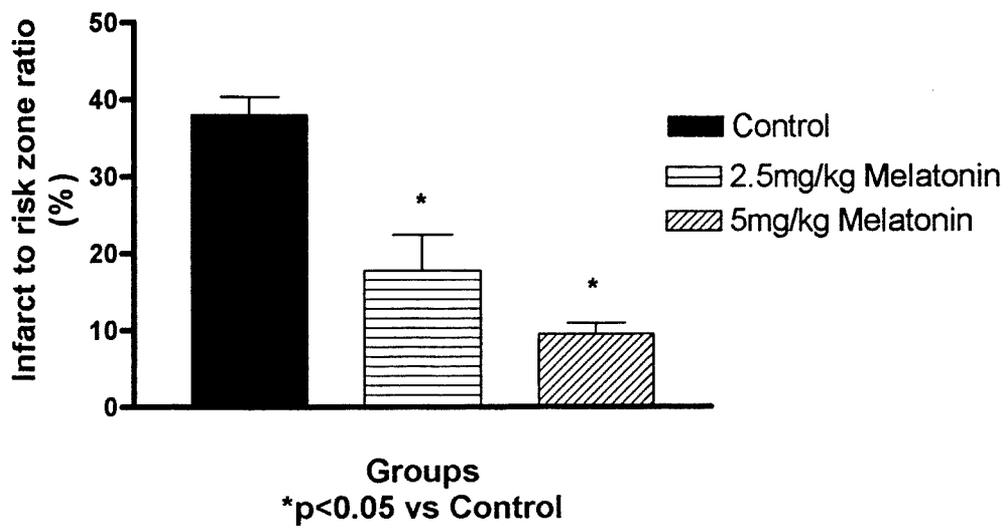
Abbreviations

Group 1 = Control

Group 2 = 2.5 mg/kg melatonin i.p.

Group 3 = 5mg/kg melatonin i.p.

Fig 3.4 The effect of intraperitoneally administration of Melatonin on infarct size



3.5 Effect of orally administrated doses of melatonin in drinking water

3.5.1 Before sustained regional ischaemia

3.5.1.1 Haemodynamics (Table 3.13)

Heart rate, peak systolic pressure, coronary flow, aortic output, cardiac output and total work measured were similar in both 40µg/ml and 20µg/ml pretreated groups as well as the vehicle (0.01% ethanol) treated control group. It is concluded that melatonin pre-treatment at doses 40µg/ml and 20µg/ml in drinking water for seven days as well as vehicle pretreatment had no effect on haemodynamic parameters, before coronary artery ligation.

3.5.2 Haemodynamics after sustained regional ischaemia (Table 3.14)

The post ischaemic heart rates and coronary flow were not statistically different amongs both orally pretreated groups as well as vehicle pretreated group. Peak systolic pressure generation, aortic output, cardiac output and % functional recovery in the high dose melatonin treated group (40µg/ml) were significantly higher than in the vehicle treated control group ($p < 0.05$).

Table 3.13 : Haemodynamic function of orally melatonin treated hearts before regional ischaemia

	Group 1 n=9	Group 2 n=7	Group 3 n=8
HR (beats/min)	218.2±6.8	216.7±18.1	235.8±4.4
PSP(mmHg)	98.0±1.0	101.1±2.2	102.6±1.5
Coronary flow (ml/min)	13.9±0.7	12.6±0.6	14.0±0.7
Aortic output (ml/min)	38.2±0.7	38.9±1.4	39.8±1.3
Cardiac output (ml/min)	52.1±1.4	51.5±2.1	53.8±1.9
Total work (Watt)	12.0±0.5	11.9±0.4	12.7±0.5

All data presented as Mean ± SEM

P>0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group 2 = 2mg/kg Melatonin

Group 3 = 4mg/kg Melatonin

Table 3.14 : Haemodynamic function of orally melatonin treated hearts after regional ischaemia

	Group 1 n=9	Group 2 n=7	Group 3 n=8
HR (beats/min)	228.7±8.5	219.1±11.7	229.8±6.9
PSP(mmHg)	89.6±1.0	91.9±1.2	94.1±1.1.1*
Coronary flow (ml/min)	13.1±0.6	12.6±0.7	14.63±1.01
Aortic output (ml/min)	16.5±1.8	21.0±1.9	24.5±2.2*
%Functional recovery	43.2±4.8	53.8±4.4	62.0±5.5*
Cardiac output (ml/min)	29.6±2.1	33.6±2.5	39.1±1.4*
Total work (Watt)	6.5±0.6	6.9±0.5	8.3±0.32

All data presented as Mean ± SEM

*P<0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group 2 = 2mg/kg Melatonin

Group 3 = 4mg/kg Melatonin

3.5.3 The effect of orally administrated melatonin on infarct size

3.5.3.1 Risk zone (Table 3.15)

Risk zones expressed as a percentage of the left ventricular volume, was similar for all three groups.

3.5.3.2 Infarct size (Fig 3.5)

All rats drank 20- 25 ml per day and there was no difference in total daily water consumption. The infarct areas for group 3, group 2 and group 1 (Fig 3.5) were 13.2 ± 2.6 , 35.4 ± 4.0 and 35.1 ± 4.1 , respectively. Rats receiving low dose melatonin ($20\mu\text{g/ml}$) (Group 2) for seven days had an infarct size similar to control treated rats that were treated with vehicle (0.01% etOH). High dose melatonin ($40\mu\text{g/ml}$) (Group 3) administration for seven days before exposure to sustained regional ischaemia 24 hours later (Group3)($p<0.0003$) resulted in a 62.04% reduction in infarct size in comparison to the vehicle - treated rats.

3.6 The effect of melatonin withdrawal on infarct size (Fig 3.6)

When melatonin was administered in drinking water for 7 days, withdrawal of two days resulted in no loss of protection, as infarct size in this group was similar to the group sacrificed on day 0. Withdrawal of melatonin for up to 4 days resulted in an increase of infarct size ($26.0\pm 4.6\%$) and protection was totally lost after 6 days withdrawal ($37.8\pm 3.9\%$). These results demonstrate that melatonin administered in this mode and

Table 3.15 : Melatonin pre-treated orally : Volume at risk expressed as a percentage of left ventricle volume

	Group 1 n=9	Group 2 n=7	Group 3 n=8
R/L	46.6%±1.1	40.2%±4.5	40.3%±3.8

R = Risk volume

L = Left ventricular volume

All data presented as Mean ± SEM

P >0.05 "vs" Group1

Abbreviations

Group 1 = Control

Group 2 = 2mg/kg Melatonin

Group 3 = 4mg/kg Melatonin

Fig 3.5 The effect of orally supplemented melatonin on infarct size

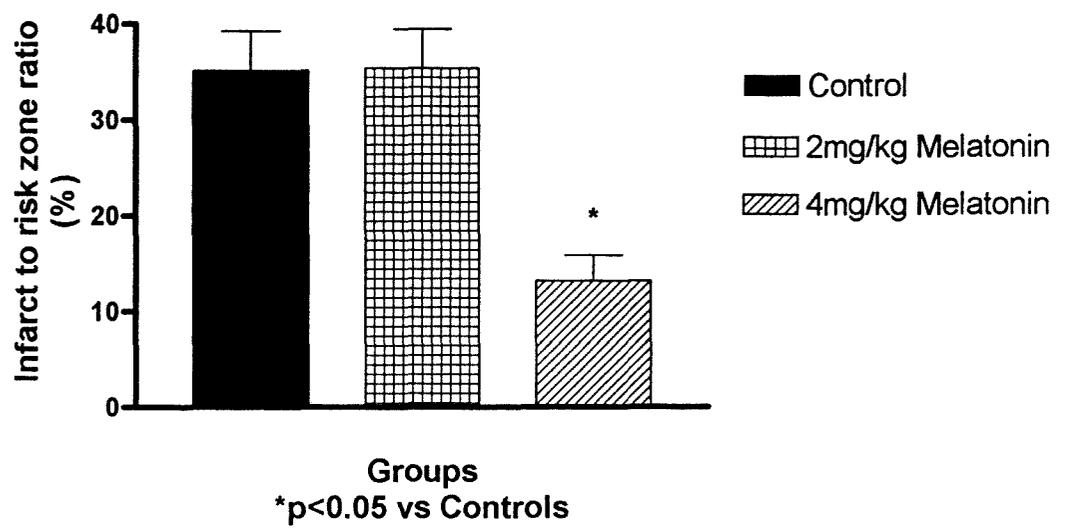
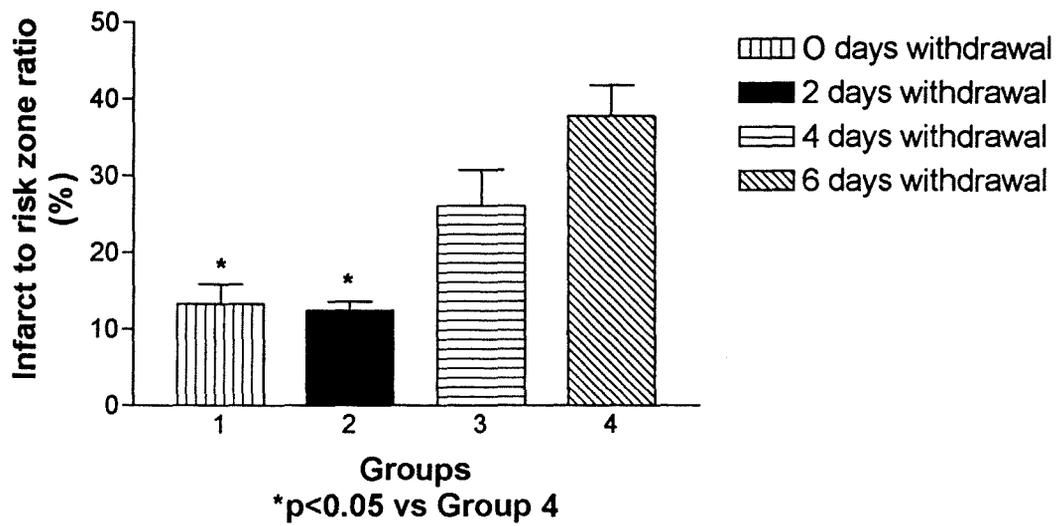


Fig. 3.6 The effect of melatonin withdrawal on infarct size



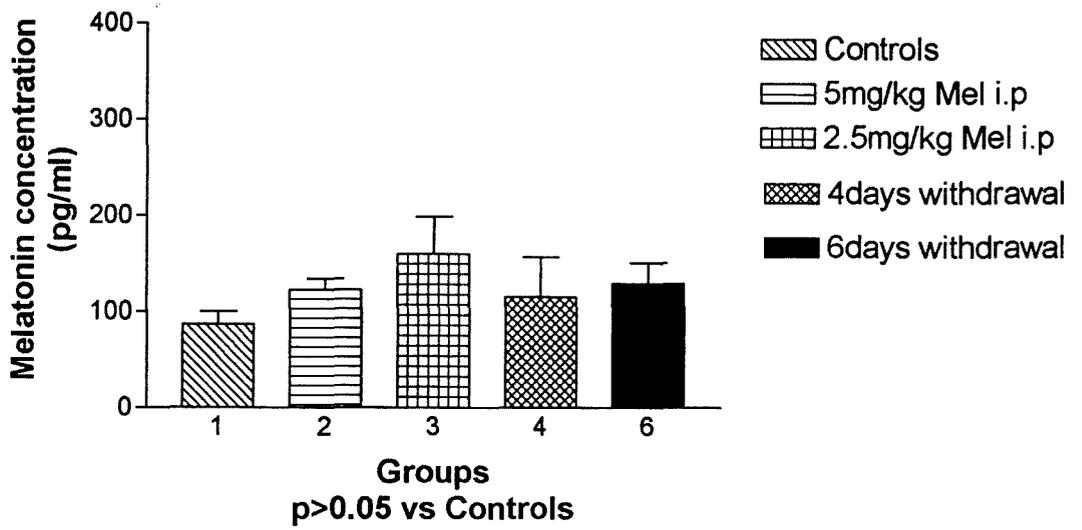
protocols resulted in significant myocardial protection against infarction for up to 48 hours later.

3.7 Biochemical results

3.7.1 Serum melatonin levels (Fig 3.7)

The melatonin assay only allows a certain amount of samples and therefore only selected samples were done in triplicate. The serum melatonin level of both pretreated groups (2.5 mg/kg and 5 mg/kg) was 122.4 pg/ml and 160.0 pg/ml respectively. Although the serum melatonin values were higher than those of the control treated hearts (86.6pg/ml) the difference was not significant. Serum melatonin levels of group 4 and group 5 (melatonin withdrawal groups) did not differ from control serum values.

Fig 3.7 The effect of melatonin treatment on serum values



CHAPTER 4

Discussion

4.1 Introduction

Myocardial protection against the consequences of ischaemia is an important goal to pursue, as many patients are exposed to myocardial ischaemia daily. For this reason exogenous and endogenous agents have been studied over the past decades. One problem that always limited the clinical usefulness of studies on myocardial protection was the fact that protective agents had to be administered in the acute setting, i.e. immediately prior to the occurrence of ischaemia, or during reperfusion. The phenomenon of preconditioning opened a new horizon in the quest for protection of the myocardium. Firstly it was regarded as the most potent form of myocardial protection against ischaemia yet observed. Secondly, the “second window of protection” of preconditioning with ischaemia made it possible to activate the protective mechanism against ischaemia long before ischaemia occurred.

In this study it was attempted to investigate whether SWOP could be activated pharmacologically with beta- adrenergic stimulation, and to elucidate the mechanism of this protection. Furthermore, in a sub-study, the ability of an endogenous agent, melatonin (an endogenous substance produced by the pineal gland), to confer longterm protection against ischaemia was studied. The specific aims of this sub-study was to investigate the effectiveness of systemically and orally administered

melatonin to protect against infarct size, and to investigate the duration of protection conferred by oral administration.

The major findings of this study can be summarised as follows:

1. *In vivo* treatment with isoproterenol elicited a dose dependent infarct limiting effect against 35 minutes of regional ischaemia. The optimal protection was achieved with four repeated intraperitoneal injections at dose 0.0004mg/kg and the infarct size was reduced by 71% in comparison to the control group.
2. This β -adrenergic delayed myocardial protection is NO mediated, as the non-selective NOS inhibitor (L-NA) administered an hour before preconditioning with isoproterenol (4 X 0.0004mg/kg) abolished the protection elicited with repeated β - adrenergic stimulation.
3. This β -adrenergic delayed myocardial protection is also mediated by the generation of reactive oxygen species, as NAC, a ROS scavenger, administered 5 minutes before eliciting delayed preconditioning with isoproterenol (4 X 0.0004mg/kg) completely abrogated the infarct sparing effect.
4. Intraperitoneally and orally administered melatonin both protected against 35 minutes of sustained regional ischaemia induced 24 hours after administration of the drug. The protective effect of orally administered melatonin continued 48h after cessation of oral treatment. Serum levels of melatonin did not correlate with cardiac protection against ischaemia.

4.2 Appropriateness of the end-points - infarct size and functional recovery

There has been a longstanding difference of opinion concerning the best end-point to use to assess myocardial protection – infarct size or functional recovery. The main reason for the disagreement was that a smaller infarct size was not necessarily associated with improved recovery (Cohen et al., 1999), thus generating conflicting interpretations about the validity and meaning of the results obtained with either end-point. For this reason we have systematically evaluated the role of the perfusion model (retrograde vs working), the infarct size and severity of ischaemia (regional vs global) as well as the endpoint (functional recovery vs infarct size) in preconditioning (Lochner et al., 2003). In that study, the isolated perfused rat heart was preconditioned by 3 x 5 min global ischaemia, followed by different periods of regional or global ischaemia and reperfusion. Ischaemic preconditioning of working hearts resulted in increased functional recovery (i.e. aorta flow and cardiac output) after 25-35 min global ischaemia, while retrogradely perfused hearts showed no significant improvement (i.e. developed pressure), except after 30 min global ischaemia. In addition, the percentage reduction in functional performance during reperfusion observed in the latter group was significantly less than in working hearts. Of more relevance to the work presented in this thesis are the findings with regard to hearts subjected to regional ischaemia. Hearts were perfused in either retrograde or working mode followed by infarct size determination. Regionally ischaemic working as well as retrogradely perfused hearts when preconditioned showed a significant

increase in functional recovery after 35 min ischaemia only. In contrast to global ischaemia, the percentage recovery in mechanical performance of regionally ischaemic hearts was not affected by the mode of perfusion. Preconditioning of working hearts caused a significant reduction in infarct size after both 30 and 35 min ischaemia. However, preconditioned retrogradely perfused hearts showed a significant decline in infarct size after 35 min regional ischaemia only. In conclusion, the effect of the perfusion mode on functional recovery was dependent on the size and severity of ischaemia. It also affected the ischaemic time at which infarct size reduction by prior preconditioning occurred in the retrogradely perfused heart.

It can thus be confidently stated that the endpoints used in this study were well validated, and relevant. In our study, animals were treated pharmacologically (in vivo) 24h prior to inflicting the infarct on excised hearts and infarct size was determined on hearts from this *in vitro* study. The next question is therefore whether there are differences in infarct size in *in vivo* and *in vitro* studies. In studies of delayed preconditioning, both *in vivo* and *in vitro* (isolated buffer perfused hearts) studies showed a similar reduction in infarct size. Studies in the dog (Kuzuya et al., 1993) and rabbit (Marber et al., 1993) observed delayed preconditioning against infarct size in vivo, that were similar to the reduction in infarct size observed in *in vitro* studies, such as in rats (Yamashita et al., 1998) and in mice (Zhao et al., 2000). Based on previous studies done at our laboratory we decided to ignore a reperfusion period longer than 30 minutes. Results from these studies indicated that hearts exposed to the same perfusion protocol and

stained with triphenyl tetrazolium irrespective of reperfusion periods, 30 and 120 minutes, did not have different infarct sizes. Preference is given to the shorter reperfusion period as the longer reperfusion period is known to suppress cardiac function (unpublished observations).

4.3 β - stimulation with isoproterenol elicits delayed cardioprotection

The first aim of the study was to investigate whether β -stimulation with isoproterenol could elicit delayed protection. A dose finding study was undertaken, and different doses of isoproterenol at different repeated intervals were administered to animals. Baseline functional characteristics of hearts were similar for all groups with the exception of Group 5, which had a significantly lower aorta output than the other groups. No explanation for this could be discerned, as the ability of this group to generate pressure was in the high range compared to the other groups. The area at risk for infarction was comparable between all groups.

Preconditioning with isoproterenol successfully elicited delayed myocardial protection as determined by measuring infarct size, and the ability to induce protection increased with a decrease in isoproterenol dose and increase in the number of administrations. Hearts preconditioned with isoproterenol 0.04mg/kg or two administrations of isoproterenol 0.04mg/kg three hours apart were least effective. The most effective preconditioning protocol was the administration of isoproterenol at a dose of

(4X 0.0004mg/kg), one hour apart which resulted in a 71% reduction in infarct size compared to controls.

It is important to notice that the isoproterenol dose found to elicit delayed preconditioning was a very low dose. In a previous study from this laboratory (Marais et al., 2001), it was also found that classic preconditioning was elicited by a very low dose of isoproterenol – 10^{-7} M. Indeed, we found at that time that 10^{-8} M was equally effective. Furthermore, it was the single administration of isoproterenol 10^{-7} M that elicited classic preconditioning – repeated administrations of isoproterenol (5 minutes apart) failed to elicit protection (unpublished observations). It therefore seems as if there is a fine line of distinction between beneficial and detrimental effects of beta-adrenergic stimulation. The observations in this thesis were very similar – the most effective protocol was observed with the lowest dose, repeated at hourly intervals.

In this study isoproterenol elicited delayed protection did not cause an improvement in mechanical function of the hearts that showed a significant reduction in infarct size after the regional ischaemic period. The study from our laboratory discussed above (validity of end-point (section 4.2)) concurs with this observation – when regional ischaemia is used, the duration of ischaemia has to exceed 35 minutes before an effect on functional recovery is observed. It must however be noted that in similar studies by Ping et al., (1999), Takano et al., (1998 a), Takano et al., (1998) and Xuan

et al., (1994) it was shown that late PC not only reduced infarct size but also produced a modest improvement of left ventricular systolic wall thickening during the first few days after 30 minutes of coronary occlusion. These experiments were all done *in vivo*, and this may play an important role due to the presence of other hormonal factors that may contribute to functional recovery of the hearts. Another explanation may be the timing post-infarction. In a study on delayed preconditioning with ischaemia as the preconditioning trigger, Takano et al (2000) reported enhanced left ventricular function in rabbits subjected to 30 minutes of coronary artery occlusion followed by 28 days of reperfusion. It is possible that we did not observe improved contractile function due to a too short reperfusion period (30 minutes) - myocardial stunning is known to persist for weeks after reperfusion (Bush et al., 1983 , Lavalee et al., 1983). As will be discussed below, the ROS scavenger melatonin was able to reduce infarct size and improve functional recovery in heart exposed to identical circumstances of regional ischaemia. This invokes the possibility that isoproterenol does not change the way in which the myocardium handles ROS, and that protecting mechanisms against infarction are distinct from protection against functional recovery. In conclusion, due to the short reperfusion period studied in an *in vitro* system, it is not possible to make any meaningful deductions about the ability of isoproterenol elicited delayed preconditioning to improve functional recovery.

This is the first study to demonstrate that in vivo treatment with isoproterenol elicits delayed preconditioning. The finding is exciting, as it implies that this mode of eliciting myocardial protection should be investigated in the clinical setting. A potential group of patients who may benefit from this approach may be those who undergo open heart surgery, and have to go on bypass. Another group who will have to be looked at again is patients with severe intractable heart failure. This latter group of patients is often treated with infusions of dobutamine for periods of 48 – 72 hours. Dobutamine, which has beta-adrenergic actions, has also been shown to elicit classic preconditioning (Asimakis & Conti, 1995). Dobutamine infusion clearly improves symptoms in these patients, but also shortens their survival (Dies et al., 1986). It is clear from our study that high doses of isoproterenol did not elicit protection – indeed, the starting dose (0.04mg/kg) was obtained from a study by Woodiwiss et al. (2001) in which daily administration of isoproterenol in that dose was used to induce cardiomyopathy! The question that arises is: will the molecular changes induced by isoproterenol as shown above be beneficial to patients with heart failure or is it due to a specific protection against ischaemia?. Further can low dose isoproterenol or dobutamine infusions improve symptoms in patients with intractable heart failure without having a deleterious effect on their survival.

The finding that beta-adrenergic stimulation elicits delayed preconditioning is novel and exciting, but should not come as a surprise. All agents shown to elicit classic preconditioning were able to elicit delayed preconditioning.

As explained in detail in the literature review, the ability of isoproterenol to elicit classic preconditioning was known, and it was therefore reasonable to expect it to elicit delayed preconditioning too. A delayed protective effect of transient beta-adrenergic stimulation is also known, albeit not typical delayed preconditioning. Meng et al., (1993) demonstrated that the administration of noradrenaline 24 hours, but not 4 hours earlier prior to ischaemia, resulted in enhanced contractile function. Beckman and colleagues (1981) observed that dogs that developed long term tolerance to intravenously administered adrenaline were also resistant to the ischaemic effects of coronary embolization with micropheres. In this study 1 of 14 tolerant dogs fibrillated on coronary artery occlusion compared with 11 of 32 in the control treated group. Kovanecz and co-workers (1996) reported that intravenous administrations of isoproterenol induced longterm cardiac adaptation 24 to 48 hours after preconditioning using electrocardiographic changes as end-point. In this study isoproterenol was administered intravenously at dose ($5 \times 2 \mu\text{g}/\text{kg}$ at ten minute intervals) in rabbits and resulted a reduced stress induced elevation in the ST segment of endocardial ECG 6, 24 and 48 hours later.

4.4 The role of nitric oxide in β - adrenergic delayed preconditioning

L-NA, a nonselective inhibitor of all three NOS isoforms (Southan and Szabó 1996) was used to investigate whether beta-adrenergic delayed preconditioning was mediated by nitric oxide production. L-NA was administered an hour before preconditioning hearts with four repeated

doses of isoproterenol 0.0004mg/kg, an hour apart. Significant drug effects were observed in the L-NA treated groups – L-NA caused a significant decrease in coronary flow in both the groups treated with L-NA alone, and in the group preconditioned with isoproterenol. This reflects the importance of NO in coronary flow regulation, which is distinct from the role played by adenosine (Suarez & Torres, 1998). However, this drug effect had no significant effect on the ability of hearts to generate pressure, and had no effect on the area at risk for infarction.

L-NA pre-treatment abolished the infarct limiting effect of delayed beta-adrenergic preconditioning completely. The drug effect of L-NA (a decrease in coronary artery flow) was not the reason for this observation, as the control group treated with L-NA alone had a similar (and not larger) infarct size as the untreated control group. This observation indicates that the protective mechanism of delayed isoproterenol induced preconditioning is nitric oxide mediated.

This finding is in agreement with the "NO hypothesis of delayed preconditioning" as advanced by Bolli and co-workers (1997). They postulated that NO acts as both trigger and distal mediator of delayed myocardial protection. Delayed myocardial preconditioning against myocardial stunning and infarction was abolished by treatment with a NOS inhibitor during the preconditioning phase in rabbits (Bolli et al., 1997, Qui et al., 1997). The role of NO as a trigger is further supported by observations that pre-treatment with the NO donors diethylenetriamine-NO

and S-nitroso-N-pencillamine induced protection against both myocardial stunning and infarction 24 hours later (Qui et al.1997a, Shinmura et al. 1997).

We hypothesize that NO generated as a result of beta-adrenergic receptor activation stimulates myocardial adaptation through a cascade of intracellular events similar to those evoked for second window of ischaemic preconditioning (Bolli et al.,1997). The mechanism by which isoproterenol mediated delayed preconditioning involves NO has to be addressed. It is known that endothelial NOS is activated upon beta-adrenergic stimulation (Balligand et al., 1999), and this is therefore the most likely explanation for our results. Endothelial NOS (eNOS) is the most represented constitutive form of NO synthase in the ventricular myocardium. The fact that L-NA abrogated the infarct limiting effect of isoproterenol (β -adrenergic) delayed preconditioning could implicate that the mechanism of cardioprotection could involve eNOS. Balligand et al (1993) reported an increased contractile response of ventricular myocytes to isoproterenol in the presence of NOS inhibitor *N-N* Arg which supports a functional regulatory role of NO on contractility. However, we have not shown endothelial NOS activation, and conclusions concerning the connection between isoproterenol administration and eNOS activation are speculative at this stage. Furthermore, our results do not enable one to identify which isoform(s) is involved, as L-NA is a non-selective inhibitor of all the isoforms of NOS. Thus, the precise isoform that is involved in the

development of delayed isoproterenol induced myocardial adaption is still not clear.

Delayed PC is not affected by pre-treatment with the guanylate cyclase inhibitor ODQ (Kodani 2000a) but is completely abolished by pre-treatment with the antioxidant mercaptopropionyl glycine (Takano et al., 1998, Tang et al., 1997). In view of this, it has been postulated that nitric oxide generates peroxynitrate which then protonates and decomposes to generate the hydroxy radical or some other potent radical similar in activity (Kodani et al., 2002, Beckman et al., 1990). It thus suggests that the generation of ROS is important in the mechanism of delayed preconditioning. In our experiments, the effect of ODQ on isoproterenol induced delayed preconditioning was not investigated. Our results can therefore be criticised as not being able to exclude a role for cGMP in the mechanism of protection. In view of the above findings however, it is highly unlikely that ODQ would abolish isoproterenol induced delayed preconditioning.

One can speculate on the sub cellular mechanisms involved in eliciting delayed protection with isoproterenol. The sub cellular mechanisms of action of isoproterenol have been established. The stimulation of the beta-adrenergic receptor (for example by isoproterenol) couple to G-stimulatory proteins to stimulate the production of cAMP through activation of adenylate cyclase (Fleming et al., 1992). PKA is activated via cAMP and stimulates the L-type voltage channels (Schroder & Herzig, 1999) by

phosphorylation. In our study different protocols and doses employed resulted in a reduction in infarction 24 hours later. It would be reasonable to speculate that in these protocols a threshold of cAMP was reached to trigger changes that resulted in delayed myocardial protection. On the other hand, it seems as if beta-adrenergic stimulation was not excessive, and avoided excessive cAMP increases and their accompanying toxic side-effects. Further biochemical studies to determine the exact level of cAMP needed to trigger this delayed cardioprotective response are needed.

Another possibility is that PKC activation was responsible for activating the protection. Work done by Miyawaki and Ashraf (1997) demonstrated specific PKC inhibitors abolished isoproterenol induced cardioprotection. Evidence for the earliest involvement of PKC in delayed preconditioning was reported by Yamashita et al. (1994) who showed that staurosporine, a PKC inhibitor, prevented delayed preconditioning in myocytes preconditioned with hypoxia. To date the exact mechanism through which PKC results in delayed myocardial preconditioning is not very clear. It has been proposed that PKC could influence many transcription events either by directly phosphorylating transcription factors or indirectly via activation of MAP kinase families (Hug & Sarre, 1993). The involvement of PKC in isoproterenol induced delayed preconditioning was not studied in this thesis.

4.5 **The role of oxygen radicals in mediating delayed β preconditioning**

Oxygen radicals produced in the setting of ischaemic preconditioning contribute to delayed myocardial adaptation (Sun et al., 1996). Beta-adrenergic stimulation also produces free radicals (Opie et al., 1979). To investigate the role of free radicals in our model we utilised three free radical scavengers namely melatonin, mercapto-propionyl glycine and N-acetylcysteine. Animals were pretreated with 5 mg/kg melatonin, MPG (1mg/kg) or N-acetylcysteine 10mg/kg intraperitoneally half an hour before preconditioning with four repeated injections of isoproterenol at dose 0.0004mg/kg an hour apart. None of these drugs had any effect on haemodynamic parameters before induction of regional ischaemia, or on area at risk for infarction. Only N-acetylcysteine abolished the infarct sparing effect of isoproterenol induced delayed preconditioning. The reason for this becomes apparent when one studies the effect of the two other ROS scavengers, melatonin and MPG, on control hearts. From the data it is clear that both these ROS scavengers have an inherent and long-lasting protective effect against myocardial ischaemia, as infarct size was reduced to a similar extent as hearts preconditioned with isoproterenol alone. These results are similar to the findings of Bell and workers (1999), who pretreated hearts with MPG (300 μ mol/l) before treating with a SNAP donor (2 μ mol/l) to elicit myocardial protection. The protection afforded by the SNAP donor was also not abolished by MPG in these experiments.

Chen et al (1995) also reported that classic ischaemic preconditioning is associated with a more oxidized cellular redox state, and the administration of N-acetylcysteine, a glutathione precursor during preconditioning blocked the protective effects of preconditioning. The production of free radicals during ischaemic preconditioning is also involved in the mechanism against myocardial stunning in conscious pigs (Sun et al., 1996).

These results thus confirm that isoproterenol induced delayed preconditioning is also dependent on the generation of ROS, similar to the findings in hearts preconditioned with ischaemia (Takano et al., 1998a, Tang et al., 1997a). It also provides indirect support for the hypothesis that the generation of nitric oxide generates peroxynitrate which somehow induces protection. It is also clear that melatonin and MPG cannot be used to study the effect of ROS in the experimental setting, due to their strongly protective effects. This provided the basis to study one of these drugs, melatonin, in more depth.

4.6 The protective effect of melatonin against ischaemia

In view of the observed effects of melatonin described above, the systemic (intraperitoneal) as well as oral administration of melatonin was investigated in more detail. Haemodynamic parameters at baseline were similar for the different groups treated with intraperitoneal melatonin, with the exception of coronary flow, which was less in melatonin treated groups. An effect of melatonin on coronary circulation has not been

described elsewhere. Also, in Table 3.7, no such effect of any of the other ROS scavengers, was noted, which argues against a general propensity for ROS scavengers to reduce coronary flow. In Table 3.13 no such effect for oral melatonin was documented. This observation remains difficult to explain. However, there was no significant difference in the infarct zone at risk in these hearts.

Intraperitoneal melatonin administration had a pronounced protective effect against an ischaemic insult given 24 h later, both in hearts treated with intraperitoneal and oral melatonin. When melatonin was administered intraperitoneally, both doses (2.5 mg/kg and 5 mg/kg) reduced infarct size (Fig 3.4) significantly in comparison to the control treated hearts. Others have demonstrated a protective action of melatonin against ischaemia, but in all these experiments the ischaemia was studied in an acute setting. For instance, Lagneux et al (2000) showed that hearts pretreated with melatonin (1 or 10mg/kg i.p.) and the analogue 5-MCA-NAT (10mg/kg i.p.) thirty minutes before hearts were exposed to regional ischaemia (30 minutes) provided effective protection against infarction. Similarly, in an *in vivo* study by Lee et al. (2002) a bolus intravenous injection of 0.5 , 1.0 and 5.0 mg/kg melatonin before LAD occlusion protected against ischaemia/reperfusion tachycardia and fibrillation. The results shown above (Figures 3.4, 3.5) are the first to prove that the protective effect of melatonin extended beyond the acute time frame, and was observed 24 hours after administration.

The observation that systemic administration of melatonin had a long lasting protective effect against ischaemia prompted the question whether oral administration shared this property. Our work demonstrated that melatonin administration of 40 μ g/ml (but not 20 μ g/ml) in the drinking water for seven days protected against infarction 24 hours later (Fig 3.5). Furthermore, the haemodynamic parameters (PSP, CO and aortic output post ischaemia) of the high dose melatonin treated group were significantly higher than low dose treated group and vehicle treated controls (Table 3.15). These findings are very significant, as it suggests the possibility that it may be worthwhile to investigate the possibility of using oral melatonin administration to protect against myocardial ischaemia in other species, including humans. A novel finding is that oral melatonin not only protected against infarct size, but also improved functional recovery. As pointed out above, protection against functional recovery was not observed with isoproterenol induced delayed preconditioning. These findings support the idea that ROS generation may play an important role in the pathophysiology of stunning, as has been discussed by Opie (1997). It must however be stated that the issue of free radicals and infarction is not without controversy (Jeroudi et al., 1994), but our results support a role for free radicals in both infarction and stunning. It also suggests that the protective effects against infarction and stunning are distinct – isoproterenol induced delayed protection can activate the anti-infarct protection, but does not seem to be able to protect against stunning. Melatonin seems to be able to protect both against ischaemia and stunning.

The question arises why oral administration of melatonin, but not intraperitoneal melatonin, was accompanied by improved functional recovery. A critical appraisal of the data in Table 3.10 and 3.13 suggest that the answer may lie in the fact that the groups of animal studied during the oral administration were more homogenous in their pre-ischaemia haemodynamic characteristics than was the case in the experiments where melatonin was administered intraperitoneally. It is highly unlikely that melatonin would affect the coronary flow of intraperitoneally treated animals differently than when administered orally. Also, no effect of melatonin on coronary flow has been documented to date.

Once it was established that oral administration of melatonin in a dose of 40 μ g/ml provided protection against myocardial ischaemia, the question was asked how long this protective effect lasted. From our experiments it is clear that the effect was still strongly present 48 hours after cessation of oral intake, virtually lost at 72 hours and totally lost at 96 hours. The time course of loss of protection of oral melatonin has not been described previously.

The question that arises from these observations is whether serum or myocardial tissue levels of melatonin had any relationship to protection. Serum levels of melatonin from selected experimental conditions in which there was a wide variation of infarct protecting effects were therefore measured. Serum melatonin levels had no relationship to protection by

melatonin, which may indicate that it is not of any importance, or that tissue levels are more important. This aspect will have to be studied in future.

4.7 Mechanism of action of melatonin

Ischaemia and reperfusion is associated with rapid reoxygenation of ischaemic tissues and is associated with the generation of highly reactive oxygen species. The efficacy of melatonin to protect against ischaemia is most likely due to its actions as a scavenger of radical oxygen species. The high lipid solubility of melatonin allows this substance to readily enter the cytosol and cellular compartments where it can act as an endogenous antioxidant and a scavenger of toxic free radicals (Hardeland & Rodrigues, 1995). Further proof of the anti-oxidant action of melatonin comes from observations in our laboratory. Isolated adult rat ventricular myocytes were pretreated with 50 and 100 μM melatonin before being subjected to 12.5 min chemical hypoxia. Pretreated cardiomyocytes showed marked morphological improvement, and reduced fluorescence intensity of DCDHF, DHR and Fluo which indicates that melatonin effectively reduced damage induced by chemical hypoxia. The activity of melatonin in vitro has been shown to be superior to that of glutathione (Tan et al., 1993) and of Vitamin E (Pieri et al., 1994). Also, melatonin is able to stimulate antioxidative enzymes, such as glutathione peroxydase and superoxide dismutase (Reiter et al., 1997, Kotler et al., 1998). Melatonin also binds to nuclear binding sites (Kotler et al., 1998). Chen et al (1994) have

demonstrated that melatonin pretreatment protected against adriamycin induced cardiomyopathy, another pathology involving toxic free radicals.

In addition to melatonin, other methoxyindoles may have therapeutic properties – for instance, 5 – methoxytryptamine also has a radioprotective action (Lebkova, 1966 , Feher et al., 1968 , Hara et et al., 1997). 5 – methoxytryptamine and other methoxyindoles are present in the serum, pineal gland and retina of animals (Tsang et al., 1996 , Li et al., 1997a , Li et al., 1997b , Zawilska et al., 1998 , Vivien et al., 1999) – all of these are currently being investigated for their therapeutic effects. The fact that 5 - methoxyindols are potent antioxidants and that melatonin is a relatively safe molecule (Bubenik et al., 1998) with clinical potential justify further examination of its use in animal models and human studies.

4.8 Conclusion

The present study advances our understanding of the mechanism of delayed myocardial preconditioning. Our results support a role for beta-adrenergic stimulation with isoproterenol as a trigger of delayed preconditioning. The optimal dose that successfully elicits delayed preconditioning in the rat was $4 \times 0.0004 \text{ mg/kg}$ given one hour apart. Reduction in infarct size in isoproterenol induced delayed protection is not associated with improvement in functional recovery. Beta-adrenergic delayed preconditioning is mediated through nitric oxide (NOS) activation as well as the generation of reactive oxygen species. Melatonin protects against regional ischaemia and results in reduced infarct size when

administered orally and intraperitoneally and also in improved functional recovery (when administered orally). These findings provide evidence that both these therapeutic strategies are potentially useful in protecting patients against myocardial ischaemia.

References

- Abete P, Bianco S, Calabrese C, Napoli C, Cacciatore F, Ferrera N, Rengo F. Effects of melatonin in isolated rat papillary muscle. *FEBS Lett* 1997; 412:79-85.
- Armstrong S, Ganote CE. Adenosine receptor specificity in preconditioning of isolated rabbit cardiomyocytes: evidence of A₃ receptor involvement. *Cardiovasc Res* 1994; 28:1049-1056
- Armstrong S, Ganote CE. In vitro ischemic preconditioning of isolated rabbit cardiomyocytes: effects of selective adenosine receptor blockade and calphostin C. *Cardiovasc Res* 1995; 29: 647-652
- Armstrong SC, Delacy M, Ganote CE. Phosphorylation state of hsp27 and p38 MAPK during preconditioning and protein phosphatase inhibitor protection of rabbit cardiomyocytes. *J Mol Cell Cardiol* 1999; 31:555 – 567
- Asimakis GK, Conti VR. Preconditioning with dobutamine in the isolated heart. *Life Sciences* 1995; 57:177-187
- Asimakis GK, Inners-McBride K, Conti VR, Yang C. Transient β -adrenergic stimulation can precondition the rat heart against postischemic contractile dysfunction. *Cardiovasc Res* 1994; 28:1726-1734
- Auchampach JA, Grover GJ, Gross GJ. Blockade of ischemic preconditioning in dogs by the novel ATP dependant potassium channel antagonist sodium 5-hydroxydeconoate. *Cardiovasc Res* 1992; 26:1054-1062
- Auchampach JA, Gross GJ. Adenosine A₁ receptors, M_{ATP} channels, and ischemic preconditioning in dogs. *Am J Physiol* 1993; 264:H1327-1336
- Auchampach JA, Maruyama M, Cavero I, Gross GJ. Pharmacological evidence for a role of ATP-dependant potassium channels in myocardial stunning. *Circulation* 1992; 86:311-319
- Auchampach JA, Rizvi A, Qiu Y, Tang XL, Maldonado C, Teschner S, Bolli R. Selective activation of A₃ adenosine receptors with N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide protects against myocardial stunning and infarction without hemodynamic changes in conscious rabbits. *Circ Res* 1997; 80:800-809
- Auchampach JA, Takano H, Yang Z, Tang XL, Bolli R. Activation of A₃ adenosine receptors induces late preconditioning against myocardial infarction but not stunning in conscious rabbits. *J Mol Cell Cardiol* 1999; 31:5 A10.(Abstract)
- Axelrod J, Weisbach H. Enzymatic O-methylaytion of N-acetylserotonin to melatonin. *Science* 1995; 270:1681-1683
- Baines CP, Goto M, Downey JM. Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J Mol Cell Cardiol* 1997; 29:207-216

Balligand JL, Kelly RA, Marsden PA, Smith TW, Michel T. Control of cardiac muscle cell function by exogenous nitric oxide signaling system. *Proc Natl Acad Sci* 1993; 90:347-351

Balligand JL, Smith TW. Molecular regulation of NO synthase in the heart. *Life Sci* 1999; 64:471-477

Banerjee A, Locke-Winter C, Rogers KB, Mitchell MB, Brew EC, Cairns CB, Bensard DD, Harken AH. Preconditioning against myocardial dysfunction after ischemia and reperfusion by an α_1 – adrenergic mechanism. *Circ Res* 1993; 73:656–670

Banerjee S, Tang XL, Qiu Y, Takano H, Manchikalapudi S, Dawn B, Shirk G, Bolli R. Nitroglycerin induces late preconditioning against myocardial stunning via a protein kinase C dependant pathway. *Am J Physiol* 1999; 277:H2488-H2494

Banerjee S, Tang XL, Qiu Y, Takano H, Manchikalapudi S, Takano H, Jadoon AK, Wu WJ, Bolli R. Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits. *Am J Physiol* 1997; 273:H2931-2936

Bankwala Z, Hale SL, Kloner RA. Alpha-adrenoceptor stimulation with exogenous norepinephrine or release of endogenous catecholamines mimics ischaemic preconditioning. *Circulation* 1998; 90:1023–1028

Baracik M, Htun P, Strohm C, Kilian S, Scraper W. Inhibition of cardiac p38 MAPK pathway by SB203580 delays ischemic cell death. *J Cardiovasc Pharmacol* 2000; 35: 474–483

Baxter GF, Goma FM, Yellon DM. Characterization of the infarct-limiting effect of delayed preconditioning : timecourse and dose-dependancy studies in rabbit myocardium. *Basic Res Cardiol* 1997; 92:159-167

Baxter GF, Goma FM, Yellon DM. Involvement of protein kinase C in the delayed cytoprotection following sublethal ischaemia in rabbit myocardium. *Br J Pharmacol*. 1995; 115:222-224.

Baxter GF, Goodwin RW, Wright MJ, Kerac M, Heads RJ, Yellon DM. Myocardial protection after monophosphoryl lipid A: studies of delayed anti-ischaemic properties in rabbit hearts. *Br J Pharmacol*. 1996; 117:1685-1692

Baxter GF, Kerac M, Zaman MJ, Yellon DM. Protection against global ischemia in the rabbit isolated heart 24h after transient adenosine A_1 receptor activation. *Cardiovasc Drugs Ther* 1997; 11:83-85]

Baxter GF, Marber MS, Patel VC, Yellon DM. Adenosine receptor involvement in a delayed phase of myocardial protection 24 hours after ischemic preconditioning. *Circulation* 1994; 90:2993-3000

Baxter GF, Mocanu MM, Yellon DM. Attenuation of myocardial ischaemic injury 24 hours after diacylglycerol treatment in vivo. *J Moll Cell Cardia* 1997; 29:1967-1975.

Baxter GF, Yellon DM. Delayed preconditioning against lethal ischaemic injury. In: Baxter GF, Yellon DM eds. *Delayed Preconditioning and Adaptive Cardioprotection*. Dordrecht, the Netherlands: Kluwer Academic Publishers; 1998:1-28.

Bazzano LA, Munter JP, Vupputuri S, Whelton PK. The Relationship Between Cigarette Smoking and Novel Risk Factors for Cardiovascular Disease in United States. *Annals of Internal Medicine* 2003 ; 138:891-897

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrate: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87:1620-1624

Bell RM, Rees DD, Yellon DM. The protection observed during delayed preconditioning of the heart appears independently of iNOS: a study in iNOS knockout mice. *Circulation* 1999; 100:240-242

Bernado NL, D'Angelo M, Okubo S, Joy A, Kukreja RC. Delayed ischemic preconditioning is mediated by opening of ATP-sensitive potassium channels in rabbit heart. *Am J Physiol Heart Circ Physiol* 1999; 276:H1323-1330

Bernado NL, Okubo S, Maaieh MM, Wood MA, Kukreja RC. Delayed preconditioning with adenosine is mediated by opening of ATP-sensitive K(+) channels in rabbit heart. *Am J Physiol.* 1999; 277:H128-H135

Berne RM. Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. *Am J Physiol.* 1963; 204:317-322

Birnbaum Y, Hale SL, Kloner RA. Ischemic preconditioning at a distance. Reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit. *Circulation* 1997; 96:1641-1646

Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ, Sugden PH. Stimulation of the stress-activated mitogen-activated protein kinase subfamily in the perfused rat heart p38/RK mitogen-activated protein kinase and c-Jun N-terminal kinases are activated by ischaemia/reperfusion. *Circ Res* 1996; 79:162-173

Bolli R, Bhatti ZA, Tang XL, Qiu Y, Zhang Q, Guo Y, Jadoon AK. Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by generation of nitric oxide. *Cir Res* 1997; 81:42-52

Bolli R, Manchikalapudi S, Tang XL, Takano H, Qiu Y, Guo Y, Zhang Q, Jadoon AK. The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase: evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. *Cir Res* 1997a; 81:1094-1107

Bolli R. The early and late phases of preconditioning against myocardial stunning and the essential role of oxyradicals in the late phase: an overview. *Basic Res Cardiol.* 1996; 91:57-63

Bolli R. The late phase of preconditioning. *Circ Res* 2000; 87:972-983

Brown JM, Anderson BO, Repine JE, Shanley PF, White CW, Grosso Ma, Banerjee A, Bensard DD, Harken AH. Neutrophils contribute to TNF myocardial tolerance to ischemia. *J Mol Cell Cardiol.* 1992;24:485-495.

Brown JM, Gross MA, Terada LS, Whitman GJR, Banerjee A, White CW, Harken AH, Repine JE. Endotoxin pretreatment increases endogenous myocardial catalase activity and decreases ischemia-reperfusion injury of isolated rat hearts. *Proc Natl Acad Sci U S A* 1989; 86:2516-2520

Brown JM, White CW, Terada LS, Grosso MA, Shanley PF, Mulvin DW, Banerjee A, Whiteman GJ, Harken AH, Repine JE. Interleukin -1 pre-treatment decreases ischemia/reperfusion injury. *Proc Natl Acad Sci USA* 1990; 87:5026-5030.

Brusco LI, Garcia-Bonacho M, Esquifino AI, Cardinali DP. Diurnal rhythms in norepinephrine and acetylcholine synthesis of sympathetic ganglia, adrenals of ageing rats: effect of melatonin. *J Auton Nerv Syst* 1998; 74:49-61

Bubenik GA, Blask DE, Brown GM, Maestroni GJ, Pang SF, Reiter RJ, Viswanathan M, Zisapel N. Prospects of clinical utilization of melatonin. *Biol Signals Recept* 1998; 7:195-219

Bugge E, Ytrehus K. Ischemic preconditioning is a protein kinase C dependant but not through stimulation of α -adrenergic or adenosine receptors in the isolated rat heart. *Cardiovasc Res* 1995; 29:401-406

Bush LR, Buja LM, Samowitz W, Rude RE, Wathen M, Tilton GD, Willerson JT. Recovery of left ventricular segmental function after long-term reperfusion following temporary coronary occlusion in conscious dogs. Comparison of 2- and 4-hour occlusions. *Circ Res* 1983; 53:248-263

Carr CS, Hill RJ, Masamune et al. Evidence for a role for both the adenosine A_1 and A_3 receptors in protection of isolated human atrial muscle against simulated ischaemia. *Cardiovasc Res* 1997; 36:p52- (Abstract)

Chan CW, Song Y, Alienberg M, Wheeler M, Pang SF, Brown GM, Silverman M. Studies of melatonin effects on epithelia using the human embryonic kidney-293 (HEK-293) cell line. *Endocrinology* 1997; 138:4732-4739.

Chen LD, Kumar P, Reiter RJ, Tan DX, Chamber JP, Manchester LC, Poeggeler, B. Melatonin reduces 3H-nitredipine binding in the heart. *Proc Soc Exp Biol Med* 1994a; 207:34-37

Chen LD, Kumar P, Reiter RJ, Tan LC, Chamber DX, Manchester JP, Poeggeler B, Saarela S. Melatonin prevents the suppression of cardiac Ca^{2+} -stimulated ATPase activity induced by alloxan. *Am J Physiol* 1994b; 267:E57-62

Chen LD, Tan DX, Reiter RJ, Yaga K, Poeggeler B, Kumar P, Manchester LC, Chambers JP. In vivo and in vitro effects of the pineal gland and melatonin on $[Ca^{2+}Mg^{2+}]$ -dependent ATPase in cardiac sarcolemma. *J Pineal Res* 1993; 14:178-183.

Chen W, Gabel S, Steenbergen C, Murphy E. A redox-based mechanism for cardioprotection induced ischaemic preconditioning in perfused rat heart. *Circ Res* 1995; 77:424-429

Chong NW, Sugden D. The ontogeny of 2-iodomelatonin binding sites in chicken brain. *Neurosci Letter* 1992; 138 :37-40.

Cohen MV, Downey JM. Myocardial preconditioning promises to be a novel approach to the treatment of ischaemic heart disease. *Ann Rev Med* 1996; 47:21-29

Cohen MV, Yang XM, Downey JM. Conscious rabbits become tolerant to multiple episodes of ischaemic preconditioning. *Circ Res* 1994; 74:998-1004

Cohen MV, Yang XM, Downey JM. Smaller infarct after preconditioning does not predict extent of early functional improvement of reperfused heart. *Am J Physiol* 1999 ;277:H1754-1761

Cohen MV, Yang XM, Neumann T, Heusch G, Downey JM. Favorable remodeling enhances recovery of regional myocardial infarction in the weeks after infarction in ischemically preconditioned hearts. *Circulation* 2000; 102:579-583

Cole WC, Mc Pherson CD, Sontag D, ATP-regulated K⁺channels protect the myocardium against ischemia/reperfusion damage. *Cir Res* 1991; 69:571-58

Crow JP, Bechman JS. Reactions between nitric oxide, superoxide, and peroxynitrate: footprints of peroxynitrate in vivo. *Adv Pharmacol* 1995; 34:17-43

Currie RW, Karmazyn M, Kloc M, Mailer K. Heat-shock response is associated with enhanced postischemic ventricular recovery. *Circ Res* 1988; 63:543-549

Dana A, Baxter GF, Yellon DM. Both protein kinase C and protein tyrosine kinase mediate adenosine induced delayed cardioprotection in rabbits. *Circulation*. 1997; 96:I-312.(Abstract)

Dana A, Jonassen AK, Yamashita N, Yellon DM. Adenosine A₁ receptor activation induces delayed preconditioning in rats mediated by manganese superoxide dismutase. *Circulation* 2000; 101:2841-2848

Dana A, Skarli M, Papakrivopoulou J, Yellon DM. Adenosine A₁ receptor induced delayed preconditioning in rabbits: induction of p38 MAPK activation and Hsp27 phosphorylation via a tyrosine kinase and protein kinase C-dependent mechanism. *Circ Res*. 2000a; 86:989-997

Dana A, Yamashita N, Baxter GF, Yellon DM. Involvement of protein kinase and manganese-superoxide dismutase in adenosine induced delayed preconditioning. *J Mol Cell Cardiol* 1998; 30:A 75. Abstract

Das M, Parker JE, Halestrap aP. Matrix volume measurements challenge the existence of diazoxide/glibenclamide sensitive K_{ATP} channels in rat mitochondria. *J Physiol* 2003; 547:893-902

Dave R H, Hole S L, R A Kloner. The effect of melatonin on hemodynamics, blood flow and myocardial infarct size in a rabbit model of ischemia-reperfusion. *J Cardiovasc Pharmacol Ther* 1998; 3:153-160.

Dawn B, Xuan Y-T, Qiu Y, Takano H, Tang X-L, Ping P, Banerjee S, Hill M, Bolli R. Bifunctional role of protein tyrosine kinases in late preconditioning against myocardial stunning in conscious rabbits. *Circ Res* 1999; 85:1154-1163.

Dies F, Krell MJ, Whitlow P, Liang C-S, Goldenberg I, Applefield MM, et al. Intermittent dobutamine in ambulatory outpatients with chronic cardiac failure. *Circulation* 1986; 74:11-38 (Abstract)

- Dougherty C, Barucha J, Shcofield PR, Jacobson KA, Liang BT. Cardiac myocytes rendered ischemia resistant by expressing the human adenosine A1 or A3 receptor. *FASEB J* 1998; 12:1758-1792
- Downey JM, Cohen MV, Ytrehus K, Lui Y. Cellular mechanisms in ischemic preconditioning: the role of adenosine and protein kinase C. *Ann N Y Acad Sci* 1994; 723:82-98
- Downey JM, Cohen MV. Mitochondrial KATP channel opening during index ischemia and following myocardial reperfusion in ischaemic rat hearts. *J Mol Cell Cardiol.* 2001; 33:651-653
- Downey JM, Cohen MV. Signal transduction in ischemic preconditioning. *Adv Exp Med Biol.* 1997a; 430:39 - 55.
- Downey JM, Yellon DM. The biology of preconditioning. In: Hyendrickx GR, Vatner SF, Wijns W, eds. *Stunning, Hibernation and preconditioning: Clinical Pathophysiology of Myocardial Ischemia.* Philadelphia, Pa: Lippincott-Raven Publishers; 1997: 105-119
- Ebrahim Z, Yellon DM, Baxter GF. Bradykinin induces delayed cardioprotection in rat heart via a nitric dependant mechanism. *Am J Physiol Heart Circ Physiol* 2001; 281:H1458-H1464
- Edwards RJ, Saurin AT, Roby D, Marber R, Marber MS. Therapeutic potential of ischaemic preconditioning. *Br J Clin Pharmacol* 2000; 50:87-97
- Feher I, Gidali J, Sztanyik L Study of the radioprotective effect of 5-methoxytryptamine of haemopoietic stem cells. *Int J Radiat Biol Relat Stud Phys Chem Med* 1968; 14:257-262
- Fleming JW, Wisler PL, Watanabe AM. Signal transduction by G-protein in cardiac tissues. *Circulation* 1992; 84:420-433
- Fryer RM, Hsu AK, Eells JT, Nagase H, Gross GJ. Opioid- induced second window of cardioprotection: potential role of mitochondrial K_{ATP} channels. *Cir Res* 1999; 84:846-851
- Fryer RM, Hsu AK, Gross GJ. Mitochondrial KATP channel opening is important during index ischemia and following myocardial reperfusion in ischemic preconditioned rat hearts. *J Mol Cell Cardiol* 2001; 33:831-834
- Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA, and Grover GJ. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K⁺ channels. Possible mechanism of cardioprotection. *Circ Res* 1997; 81:1072-1082
- Gho BCG, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in non-cardiac tissue. *Circulation* 1996; 94:219 -2200
- Gross GJ and Fryer RM. Sarcolemmal versus mitochondrial ATP-sensitive K⁺ channels modulate cardiac mitochondrial function. *Am J Physiol Heart Circ Physiol* 1999; 275: H1567-H1576
- Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992; 70:223-233

Grover GJ and Garlid KD. Cardioprotection with the K_{ATP} channel opener. *J Mol Cell Cardiol* 2000; 32:677-695

Grover GJ. Pharmacology of ATP-sensitive potassium channel (K_{ATP}) openers in models of myocardial ischemia and reperfusion. *Can J Physiol Pharmacol* 1997; 75:44-49

Grover GJ. Protective effects of ATP-sensitive potassium channel openers in experimental myocardial ischemia. *J Cardiovasc Pharmacol* 1994 ;24(suppl 4):S18-S27

Gunaydin B, Cakici I, Soncul H, Kalaycioglu S, Cervik C, Sancak B, Kanzik I, Karadenizli. Does remote organ ischaemia trigger cardiac preconditioning during coronary artery surgery. *Pharmacological Research* 2000; 4:493 – 496

Guo Y Jones WK, Bao W, Tang X-L, Wu WJ Bolli R. Targeted disruption of iNOS gene abrogates NO donor-induced late preconditioning. *Circulation*. 1999;100;I-562.(Abstract)

Guo Y, Bao W, Tang XL, Wu WJ, Bolli R. Nitric oxide donors induce late preconditioning against myocardial infarction in mice. *J Mol Cell Cardiol* 1999; 31:A II. (Abstract)

Guo Y, Bao W, Wu W-J, Shinmura K, Tang X-L, Bolli R. Evidence for an essential role of cyclooxygenase-2 as a mediator of the late phase of ischemic preconditioning in mice. *Basic Res Cardiol*. 2000; 95:480-485.

Guo Y, Bolli R, Bao W, Wu WJ, Black RG, Murphree SS, Salvatore CA, Jacobson MA, Auchampach JA. Targeted deletion of the A_3 adenosine receptors confers resistance to myocardial injury and does not prevent early preconditioning. *J Mol Cell Cardio* 2001; 33:825-830

Guo Y, Jones WK, Xuan YT, Tang XL, Bao W, Wu WJ, Han H, Laubach VE, Ping P, Yang Z, Qui Y, Bolli R. The late phase of ischemic preconditioning is abrogated by targeted disruption of the iNOS gene. *Proc Natl Aca Sci U S A* 1999;96: 11507-11512

Hale SL, Kloner RA. Protection of myocardium by transient, preischemic phenylephrine administration in the rabbit. *Coron Artery Dis* 1994; 5:605–610

Halestrap AP. The regulation of the matrix volume of the mitochondria in vivo and in vitro and its role in the control of mitochondrial metabolism. *Biochim Biophys Acta* 1989; 973:355-382

Hara M, Ligo M, Ohtani-Kaneko R, Nakamura N, Suzuki T, Reiter RJ, Hirata K. Administration of melatonin and related indoles prevents exercise-induced cellular oxidative changes in rats. *Biol Signals* 1997; 6:90-100.

Hardeland R, Rodriguez: Versatile melatonin: A pervasive molecule serves various functions in signaling and protection. *Chronobiol Int* 1995; 12(3):157-165

Heads RJ, Baxter GF, Latchman DS, Marber MS, Yellon DM. Hsp and cytoskeletal protein expression and localisation during delayed preconditioning in the rabbit heart. *J Mol Cell Cardiol* 1998;30:A20. (Abstract)

Hill M, Kodani E, Shinmura K, Tang XL. Nitroglycine induces late preconditioning against myocardial infarction despite nitrate tolerance. *Circulation* 2000 ;102 : (suppl):II-25 (Abstract)

Hoshida S, Kuzuya T, Fuji H, Yamashita N, Oe H, Hori M, Suzuki K, Taniguchi N, Tada M. Sublethal ischemia alters myocardial antioxidant activity in canine heart. *Am J Physiol* 1993;264 :H33-H39

Hug H, Sarre TF. Protein kinase C isoenzymes: divergence in signal transduction?. *Biochem J* 1993; 291: 329-343

Imagawa J, Baxter GF, Yellon DM. Genistein, a tyrosine kinase inhibitor, blocks the "second window of protection" 48hours after ischemic preconditioning in the rabbit. *J Mol Cell Cardiol* 1997; 29:1885-1893.

Imagawa J, Yellon DM, Baxter GF. Pharmacological evidence that inducible nitric oxide synthase is a mediator of delayed preconditioning. *Br J Pharmacol* 1999; 126:701-708

Iwamoto T, Muira T, Adachi T, Noto T, Tsuchida A, Iimura O. Myocardial infarct size-limiting effect of ischemic preconditioning was attenuated by oxygen free-radical scavengers in the rabbit. *Circulation*. 1991; 83:1015-1022

Jaberansari MT, Baxter GF, Muller CA, Latouf SE, Roth E, Opie LH, Yellon DM. Angiotensin-converting enzyme inhibition enhances a subthreshold stimulus to elicit delayed preconditioning in pig myocardium. *J Am Coll Cardiol* 2001; 37:1996-2001

Jansen BJ, Van Essen H, Struyker Boudier HA, Smits JF. Hemodynamic effects of activation of renal and mesenteric sensory nerves in rats. *Am J Physiol* 1989; 257:R29 - R36

Jeroudi MO, Hartley CJ, Bolli R. Myocardial reperfusion injury: role of oxygen radicals and potential therapy with antioxidants. *Am J Cardio* 1994; 73(6):2B-7B

Jones WK, Flaherty MP, Tang X-L, Takano H, Qiu Y, Banerjee S, Smith T, Bolli R. Ischemic preconditioning increases iNOS transcript levels in conscious rabbits via a nitric oxide-dependant mechanism. *J Moll Cell Cardiol* 1999; 100:1-562 (Abstract)

Joyeux M, Baxter GF, Thomas DL, Ribouot C, Yellon DM. Protein kinase C is involved in resistance to myocardial infarction induced by heat stress. *J Mol Cell Cardiol* 1997; 29:3311-3319.

Kaeffer N, Richard V, Thuillez C. Delayed coronary endothelial protection 24 hours after preconditioning: role of free radicals. *Circulation* 1997; 96:2311-2316

Kaneko S, Okumura K, Numaguchi Y et al, Melatonin scavenges hydroxyl radical and protects isolated rat heart from ischemic reperfusion injury. *Life Sci* 2000; 67:101-112

Kannengieser GJ, Opie LH, Van der Werff TJ. Impaired cardiac work and oxygen uptake after reperfusion of regionally ischemic myocardium. *J Mol Cell Cardiol* 1979; 11:197-207

Kaszala K, Vegh A, Papp JG, Parratt JR. Time course of the protection against ischaemia and reperfusion-induced ventricular arrhythmias resulting from brief periods of cardiac pacing. *J Mol Cell Cardiol* 1996; 28:2085-2095

Kitikaze M, Hori M, Morioka T, Minamino T, Takashima S, Sato H, Shinozaki Y, Chujo M, Mori H, Inoue M, Kamada T. Alpha₁-adrenoceptor activation mediates the infarct size-limiting effect of ischemic preconditioning through augmentation of 5'-nucleotidase activity. *J Clin Invest* 1994; 93:2197-2205

Knowlton AA, Brecher P, Apstein CS. Rapid expression of heat shock protein in the rabbit after brief cardiac ischemia. *J Clin Invest.* 1991; 87:139-147.

Kodani E, Xuan Y-T, Takano H, Shinmura K, Tang X-L, Bolli R. Role of Cyclic Guanosine Monophosphate in Late Preconditioning in Conscious Rabbits. *Circ* 2002; 105:3046

Kodani E, Tang XL, Takano H, Shinmura K, Hill M, Bolli R. The role of cyclic guanosine monophosphate (cGMP) in late preconditioning against myocardial stunning in conscious rabbits. *J Mol Cell Cardiol* 2000 a; 32:A23(Abstract)

Kodani E, Tang X-L, Xuan Y-T, Shinmura K, Takano H, Bolli.R. Role of cyclic guanosine monophosphate in nitric oxide-dependent late preconditioning in conscious rabbits. *Circulation* 2000b;102(suppl II):II-270(Abstact)

Korthuis R, Granger D. Reactive oxygen metabolites, neutrophils, and the pathogenesis of ischemic-tissue/reperfusion. *Clin Cardiol* 1993; 16:119-126

Kotler M, Rodriguez C, Sainz RM, Autolin I, Menendez-pelaez A. Melatonin increases gene expression for antioxidant enzymes in rat brain cortex. *J Pineal Res* 1998; 24(2):83-89

Kovancz I, Papp GJ, Szekeres L. Long-term ischaemic preconditioning of the heart induced by repeated beta-adrenergic stress. *Acta Physiol Hung* 1996; 84: 297-298

Kukreja RC, Qian YZ, Okubo S, Flaherty EE. Role of protein kinase C and 72 kDa heat shock protein in ischemic tolerance following heat stress in the rat heart. *Mol Cell Biochem.*1999;195:123-131.

Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993; 72:1293 – 1299

Lagneux C, Joyeux M, P Demenge P, Rabant Cand Godin-Ribuot D, Protective effect of melatonin against ischemia-reperfusion injury in the isolated rat heart. *Life Sci* 2000; 66 :503-509

Lamprecht W, Trauthold I. Determination with hexokinase and glucose-6-phosphate dehydrogenase. In: *Methods of enzyme analysis*: 2105. Ed Bergermeyer HU, New York; Academic press

Lavallee M, Cox D, Patrick TA, Vatner SF. Salvage of myocardial function by coronary artery reperfusion 1, 2, 3 hours after occlusion in conscious dogs. *Cir Res* 1983; 53: 235-247

Lebkova, NP. Effect of the separate and combined use of 5-methoxytryptamine and castymine on the myelogram and degeneration of cells in the haemopoietic stem cells. *Int Radiat Biol Relat Stud Phys Chem Med* 1996, 14:257-262

Lee YM, Chen HR, Hsiao et al., . Protective effects of melatonin on myocardial ischemia/reperfusion injury in vivo. *J Pineal Res* 2002; 33:72-80

Leesar MA, Stoddard M, Ahmed M, et al. Preconditioning of human myocardium with adenosine during coronary angioplasty. *Circulation* 1997; 95:2500 – 2507

Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. Isolation of melatonin, the pineal gland factor that lightens melanocytes. *J AM Chem Soc* 1958; 80:2587-2587.

Li P Pang SF, Tsang CW. Retinal 5-methoxytryptamine and 5-methoxyindole-3-acetic acid in the rat and quail: diurnal rhythms and inter-species differences. *Biochem Biophys Res Comm* 1997b; 239:353-356

Li P, Pang SF, Chan CL, Tsang CW. Identification and diurnal studies of pineal and serum 5-methoxytryptamine in the rat and quail. *Neurosci Lett* 1997a; 228:353-356.

Li RCX, Zhang J, Zheng Y, Weed W, Gao J, Cao X, Bolli R, Ping P. PKC ϵ enhances NF- κ B activation via modulation of the IKK complex in cardiac myocytes. *Circulation*. 2000;102(suppl 2):2-120. (Abstract)

Li Y, Kloner RA. The cardioprotective effects of ischemic preconditioning are not mediated by adenosine receptors in rat hearts. *Circulation* 1993; 87:1642–1648

Li Y, Whittaker P, Kloner RA. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *AM Heart J* 1992; 123:346-353.

Light PE, Sabir AA, Allen BG, et al. Protein kinase C-induced changes in the stoichiometry of ATP binding activate cardiac ATP-sensitive K⁺ channels. A possible mechanistic link to ischaemic preconditioning. *Circ Res* 1996; 79:399–406

Lim KHH, Javadov SA, Das M, Clarke SJ, Suleiman MS, Halestrap AP. The effects of ischaemic preconditioning, diazoxide and 5-hydroxydecanoate on rat mitochondrial volume and respiration. *J Physiol* 2002; 545(3):961-974

Liu GS, Thornton J, Van Winkle D.M, Stanley AW, Olsson RA, Downey JM .Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation* 1991 ; 84:350-356

Liu SG, Richards SC, Olsson RA, Mullane K, Walsjh RS, Downey JM. Evidence that the adenosine A₃ receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. *Cardiovasc Res* 1994; 28:1057–1061

Liu Y, Sato T, O'Rourke B, Marban E. Mitochondrial ATP-sensitive potassium channels: novel effectors of cardioprotection? *Circulation* 1998; 97:2463–2469

- Lochner A, Genade S, Marais E, Podzuweit T, Moolman JA. Ischaemic preconditioning and the β -adrenergic signal transduction pathway. *Circulation* 1999; 10 :958-966
- Lochner A, Genade S, Moolman JA. Ischemic preconditioning: infarct size is a more reliable endpoint than functional recovery. *Basic Res Cardiol*. 2003; 98(5):337-46
- Lui Y, Sato T, Seharaseyon J, Szewczyk A, O'Rourke B, Marban E. Mitochondrial ATP-dependant potassium channels. Viable candidate effectors of ischemic preconditioning. *NY Acad Sci* 1999; 874: 27-37
- Ma XL, Kumar S, Gao F, Louden CS, Lopez BL, Christopher TA, Wang C, Lee JC, Feuerstein GZ, Yue T-L. Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. *Circulation* 1999; 99:1685-1691
- Mackay K, Mochly – Rosen D. An inhibitor of p38 mitogen-activated protein kinase protects neonatal cardiac myocytes from ischaemia. *J Biol Chem* 1999; 274: 6272-6279
- Mackay K, Mochly – Rosen D. Involvement of a p38 mitogen-activated protein kinase phosphatase in protecting neonatal rat cardiac myocytes from ischemia. *J Mol Cell Cardiol* 2000; 32: 1585-1588
- Maldonado C, Qiu YM, Tang XL, Cohen MV, Auchampach J, Bolli R. Role of adenosine in late preconditioning against myocardial stunning in conscious rabbits. *Am J Physiol* 1997; 42:H1324-H1332
- Marais E, Genade S, Strijdom H, Moolman JA, Lochner A. p38 MAPK activation triggers pharmacologically-induced beta-adrenergic preconditioning, but not ischaemic preconditioning. *J Mol Cell Cardiol* 2001; 33:2157-77
- Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993; 88:1264-1272
- Marber MS, Mestri R, Chi SH, Sayen MR, Yellon DM, Dillman WH. Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J Clin Invest* 1995; 95:1446-1456.
- Maulik N, Engelman RM, Wei Z, Lu D, Rousou JA, Das DK. Interleukin-1- α preconditioning reduces myocardial ischemia reperfusion injury. *Circulation* 1993; 88:387-394
- Maulik N, Watanabe M, Engelman D, Engelman RM, Kagan VE, Kisin E, Tyurin V, Cordis GA, Das DK. Myocardial adaptation to ischemia by oxidative stress induced by endotoxin. *Am J Physiol*. 1995; 269:C907-C916
- Maulik N, Yoshida T, Zu YL, Sato M, Banerjee A, Das DK. Ischemic preconditioning triggers tyrosine kinase signalling: a potential role for MAPKAP kinase 2. *Am J Physiol* 1998; 275:H1857-H1864
- McCianahan TB, Nao BS, Wolke LJ, Martin BJ, Mertz TE, Galagher KP. Brief renal occlusion and reperfusion reduces myocardial infarct size in rabbits. *FASEB J* 1993; 7:A118

- Mei YA, Pang SF. The L-type Calcium Current in the Chick Embryonic Heart Cells is Increased by Melatonin, 2-Iodomelatonin and 2-Iodo-n-butanol-5-methoxytryptamine. *Biol Signals Recept* 2000; 30:1-13
- Miki T, Cohen MV, Downey JM. Opioid receptor contributes to ischemic preconditioning through protein kinase C activation in rabbits. *Mol Cell Biochem* 1998; 186:3-12
- Miki T, Swafford AN, Cohen MV, Downey JM. Second window of protection against infarction in conscious rabbits: real or artifactual. *J Mol Cell Cardiol* 1999; 31:809-816
- Mitchell MB, Meng X, Ao L, Brown JM, Harken AH, Banerjee A. Preconditioning of isolated rat heart mediated by protein kinase C. *Circ Res* 1995; 76:73- 83
- Miura T. Adenosine and bradykinin: are they independent triggers of preconditioning? *Basic Res Cardiol*. 1996; 91:20–22
- Miyawaki H, Ashraf M. Isoproterenol mimics calcium preconditioning-induced protection against ischemia. *Am J Physiol Heart Circ Physiol* 1997; 272:H927-H936
- Moolman JA, Genade S, Winterbach R, Harper IS, Williams K, Lochner A. Preconditioning with a single episode of ischaemia in the isolated working heart: effect on structure, mechanical function and energy metabolism for various durations of sustained ischaemia. *Cardiovasc Drugs & Therapy* 1995; 9: 103–115
- Morishima I, Matsui H, Mukawa H, Hayashi K, Toki Y, Okumura K, Ito tT, HayakawaT. Melatonin , a pineal hormone with antioxidant property, protects against adriamycin cardiomyopathy in rats. *Life Sci* 1998; 63:511-521
- Muira T, Imura O. Infarct size limitation by preconditioning : its phenomenological features and key role of adenosine. *Cardiovasc Res* 1993; 27:36–42
- Murray CJ, Lopes AD. Alternate projections of mortality and disability by cause 1990-2020: global burden of disease study. *Lancet* 1997; 349:1498-1504
- Murry CE, Jennings RB, Reimer RA. Preconditioning with ischaemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74:1124–1136
- Nagarkatti D, Sha'afi RI. Role of p38 kinase in myocardial stress. *J Mol Cell Cardiol* 1998; 30:1651–1664
- Nakano A, Baines CP, Kim SO, Pelech SL, Downey JM, Cohen MV, Critz SD. Ischemic preconditioning activates MAPKAP2 in the isolated rabbit heart: evidence for involvement of p38 MAPK. *Cir Res* 2000; 86:144-151
- Nasa Y, Yabe K, Takeo S. Beta-adrenoceptor stimulation-mediated preconditioning-like cardioprotection in perfused rat hearts. *J Cardiovasc Pharmacol* 1997; 29(4):436-43
- Natelson BH Ottenweller, JE Tapp, WN Heung, S Beldowicz, D The pineal affects life in hamsters with heart disease. *Physiol behav* 1997 ;62: 1059-1064.

Nelson SK, Wong GH, McCord JM. Leukemia inhibitory factor and tumor necrosis factors induce manganese superoxide dismutase and protect rabbit hearts from reperfusion injury. *J Mol Cell Cardiol* 1995; 27:223-229

Newton AC. Protein kinase C. Structure, function and regulation. *J Biol Chem* 1996; 270:28495-28498

Noda K, Sasaguri M, Ideishi M, et al. Role of locally formed angiotensin II and bradykinin in the reduction of myocardial infarct size in dogs. *Cardiovas Res* 1993; 27:334-340

Ockaili R, Emani VR, Okubo S, Brown M, Krottapalli K, Kukreja RC. Opening of mitochondrial K_{ATP} channel induces early and delayed cardioprotective effect: role of nitric oxide. *Am J Physiol Heart Circ Physiol* 1999; 277:H2425-2434

Opie LH, Thandroyen Ft, Muller C et al. Adrenalin induced "oxygen wastage" and enzyme release from working rat heart: effects of calcium antagonism, beta-blockade, nicotinic acid and coronary artery ligation. *J Moll Cell Cardiol* 1979; 11:1073-1094

Opie LH. Reperfusion injury and its pharmacological modification. *Circulation* 1997; 80:1049-1062

Osborne D, Aw T, Cepinskas G, Kvietys P. Development of ischemia/reperfusion tolerance in the rat small intestine. An epithelium independent event. *J Clin Invest* 1994; 94:1910-1918

Oxman T, Arad M, Klein R, Avazov N, Rabinowitz B. Limb ischemia preconditions the heart against reperfusion tachyarrhythmia. *Am. J. Physiol* 1997; 273:1707-1712

Pablos MI, Agapito MT, Gutierrez R, Reico JM, Reiter RJ, Barlow-Walden L, Acuna-Castroviejo D, Menendez-Pelaez A. Melatonin stimulates the activity of the detoxifying enzyme glutathione peroxidase in several tissues of chicks. *J Pineal Res*; 1995 19:111-115

Pang CS, Ali MA, Reddy PK, Leatherland JF, Brown GM, Pang SF. A comparative study of picomolar affinity 2-[125I]iodomelatonin binding sites of three salmonid species. *Fish Physiol Biochem* 1994;13:371-378.

Pang CS, Brown GM, Tang PL, Cheng KM, Pang SF. 2-[125I]iodomelatonin binding sites in the lung and heart. A link between the photoperiodic signal, melatonin, and the cardio-pulmonary system. *Bio Signals* 1993; 2:228-236

Pang CS, Tang LP, Song Y, Brown GM, Pang SF. 2-[125I]iodomelatonin binding sites in the quail heart: Characteristics, distribution and modulation by guanine nucleotides and cations *Life Sci* 1996 ;58:1047-1057.

Pang CS, Xi SC, Yau MYC, Wang XF, Yang JC, Tang PL, Pang SF. Melatonin receptors in the chick heart: Modulation on isoproterenol stimulated cAMP level. *Biol Signals Recept* 1998; 7:268-279.

Pang CS, Yang JC, Xi SC, Tang PL, Brown GM, Pang SF. Melatonin Receptors in the Chick Heart, Characterization, Ontogeny and Development Studies. *Biol Signals* 2000; 41:871-874

Pangerl B, Pangerl A, Reiter RJ. Circadian variations of adrenergic receptors in the mammalian pineal gland: a review. *J Neural Transm Gen Sect* 1990; 81:17-2

Pappano AJ, Loffelholz K, Ontogenesis of adrenergic and cholinergic neuroeffector transmission in chick embryo heart. *J Pharmacol Exp Ther* 1974; 191(3):468-478

Paul P, Lahaye C, Delagrangé P, Nicholas JP, Canet E, Bounit JA. Characterization of 2-[125I]iodomelatonin binding sites in Syrian hamster peripheral organs. *J Pharmacol Exp Ther* 1999; 290(1):334-40

Pieri C, Marra M, Moroni F, Recchioni R, Marcheselli F. Melatonin: A Peroxyl radical scavenger more effective than Vit E. *Life Sci* 1994; 15:PL271-PL276

Ping P, Guo Y, Zhang J, Li R, Bao W, Wu W, Bolli R. Targeted deletion of Lck tyrosine kinase abrogates the NF- κ B activation and the infarct-sparing effect induced by late preconditioning. *Circulation*. 2000;102(suppl 2):2-211(Abstract)

Ping P, Murphy E. Role of p38 mitogen-activated protein kinases in preconditioning: a detrimental factor or a protective kinase? *Circ Res*. 2000; 86:921-922.

Ping P, Takano H, Zhang J, Tang XL, Qiu Y, Li RC, Banerjee S, Dawn B, Balafonova Z, Bolli R. Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signalling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. *Circ Res* 1999; 84 :587-604

Ping P, Takano H, Zhang J, Tang X-L, Qiu Y, Li RCX, Banerjee S, Dawn B, Balafonova Z, Bolli R. Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide -induced and ischemia -induced preconditioning. *Circ Res* 1999; 84:587-604.

Ping P, Zhang J, Huang S, Cao X, Tang X, Li R, Zheng Y, Qiu Y, Clerk A, Sugden P, Han J, Bolli R. PKC-dependant activation of p46/p54 JNKs during ischemic preconditioning in conscious rabbits. *Am J Physiol* 1999; 277:1771-1785

Ping P, Zhang J, Qiu Y, Tang X-L, Machikalapudi S, Cao X, Bolli R. Ischemic preconditioning induces selective translocation of protein kinase C isoforms ϵ and η in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res*. 1997; 81:404-414.

Ping P, Zhang J, Zheng Y, Li R, Guo Y, Bao W, Bolli R. Cardiac targeted transgenesis of active PKC ϵ renders the heart resistant to infarction. *Circulation* 2000; 102(suppl 2):2-24.(Abstract)

Ping P, Zhang J, Zheng Y-T, Li RC, Dawn B, Tang X-L, Takano H, Balafanova Z, Bolli R. Demonstration of selective, PKC-dependent activation of Src and Lck tyrosine kinases during ischemic preconditioning in conscious rabbits. *Circ Res*. 1999; 85:542-550.

Plumier JCL, Ross BM, Currie RW, Angelidis CE, Kazlaris H, Kollias G, Pagoulatos GN. Transgenic mice expressing human heat-shock protein-70 have improved postischemic myocardial recovery. *J Clin Invest* 1995; 95:1854-1860

Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischaemic preconditioning protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993; 87:893-899

Qian Y-Z, Bernado NL, Nayeem MA, Chieliah J, Kukreja RC. Induction of 72-kDa heat shock protein does not produce second window of ischemic preconditioning in rat heart. *Am J Physiol*. 1999; 276:H224-H234

Qian Y-Z, Shipley JB, Levasseur JE, Kukreja RC. Dissociation of heat shock proteins expression with ischemic tolerance by whole body hyperthermia in rat heart. *J Mol Cell Cardiol* 1998; 30:1163-1172

Qiu Y, Ping P, Tang X-L, Manchikalapudi S, Rizvi A, Zung J, Takano H, Wu W-J, Teschner S, Bolli R. Direct evidence that protein kinase C plays an essential role in the development of late preconditioning against myocardial stunning in conscious rabbits and that ϵ is the isoform involved. *J Clin Invest*. 1998; 01:2182-2198

Qiu Y, Rizvi A, Tang XL, Manchikalapudi s, Takano H, Jadoon AK, Wu WJ, Bolli R. Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits.

Am J Physiol 1997; 273 :H2931-2936

Qiu Y, Tang X-L, Parks S-W, Sun J-Z, Kalya A, Bolli R. The early and late phases of ischemic preconditioning. A comparative analysis of their effects on infarct size, myocardial stunning, and arrhythmias in conscious pigs undergoing a 40- minute coronary occlusion. *Circ Res*. 1997b; 80:730-742.

Radford NB, Fina M, Benjamin IJ, Moreadith RW, Graves KH, Zhao PY, Gavva S, Wiethoff A, Sherry AD, Molly CR, Williams RS. Cardioprotective effects of 70-kDa heat shock protein in transgenic mice. *Proc Natl Acad Sci U S A*. 1996; 93:2339-2342.

Reilly P, Schiller H, Bulkley G. Pharmacologic approach to tissue injury mediated by free radicals and other oxygen metabolites. *Am J Sur* 1991; 161:488-503

Reimer KA, Murray CE, Yamasawa I, Hill ML, Jennings RB. Four brief periods of myocardial ischemia cause no cumulative ATP loss or necrosis. *Am J Physiol* 1986; 251:H1306-H1315

Reiter RJ, Tan DX, Cabera J D,' Arpa D, Sainz RM, Mayo S. The oxidant/antioxidant network: Role of melatonin. *Biol Signals Recpt* 1999, 8:56-63.

Reiter RJ, Tan DX, Qi WB, Manchester LC, Karbownik MM, Calvo JR Pharmacological and physiology of melatonin in the reduction of oxidative stress in vivo. *Biol Signals* 2000; 89:105-116

Reiter RJ, Tan D-X. Melatonin: a novel protective agent against oxidative injury of the ischemic/reperfused heart. *Cardiovascular Research* 2003; 58:10-19

Reiter RJ, Tang JJ, Garcia JJ, Munozhoyos. Pharmacological actions of melatonin in free radical pathophysiology. *Life Sci* 1997; 60:2255-2271

Rizvi A, Tang XL, Qiu Y, Xuan YT, Takano H, Jadoon AK, Bolli R. Increase protein synthesis is necessary for the development of late preconditioning against myocardial stunning in conscious rabbits. *Am J Physiol* 1999; 277:H874-H884

Sahna E, Acet A, Olmez E, Acet A. Effects of physiological and pharmacological concentrations of melatonin on ischaemia/reperfusion arrhythmias in rats: Can the incidence of complete sudden cardiac death be reduced?. *J Pineal Res* 2002; 32:194-198

Sahna E, Acet A, Ozer MK, Olmez E. Myocardial ischaemia-reperfusion in rats: Reduction of infarct size by either supplemental physiological or pharmacological doses of melatonin. *J Pineal Res* 2003: in press

Salie R, Harper I, Allie C et al. Melatonin protects against ischemic-reperfusion myocardial damage. *J Moll Cell Cardiol* 2001; 33:343-357

Salvemini S, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P. Nitric oxide activates cyclooxygenase enzymes. *Proc Nat Acad Sci U S A*. 1993; 90:7240-7244.

Sargent CA, Smith MA, Dzwonczyk S, Sleph PG, Grover GJ. Effect of potassium channel blockade on the anti-ischemic actions of mechanistically diverse agents. *J Pharmacol Exp Ther* 1991; 259: 97-103

Sato M, Cordis GA, Maulik N, Das DK. SAPKs regulation of ischaemic preconditioning. *Am J Physiol* 2000; 279:H901-H907

Saurin A, Zhao J, Mockridge JW. Negative regulation of p38 and p42/44 MAPK by PKC in a surrogate model of ischaemic preconditioning. *Circulation* 1998; 98: 1-72

Saurin AT, Heads RJ, Foley C, Wang Y, Marber MS. Inhibition of p38 α activation may underlay protection in a surrogate model of ischemic preconditioning. *Circulation* 1999; 10 (suppl I):I-492. (Abstract)

Saurin AT, Martin JC, Heads RJ, Foley C, Mockridge JW, Wright MJ, Wang Y, Marber S. The role of differential activation of p38 mitogen activated protein kinase in preconditioned ventricular myocytes. *Faseb J* 2000; 14:2237-2246

Saurin AT, Pennington DJ, Raat NJ, Latchman DS, Owen MJ, Marber MS. Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischemic preconditioning of isolated buffer-perfused mouse hearts. *Cardiovasc Res*. 2002; 55(3):672-80.

Schott RJ, Rohmann S, Braun. Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res* 1990; 66:1133-1142

Schroder F, Hertzig S. Effects of beta2-adrenergic stimulation on single channel gating of rat cardiac L-type Ca²⁺ channels. *Am J Physiol* 1999; 276:H834-43

Schultz JJ, Hsu AK, Gross GJ. Ischemic preconditioning mediated by peripheral opioid receptor mechanism in the rat heart. *J Moll Cell Cardiol* 1997; 29: 1355-1362

Schultz R, Rose J, Heusch G. Involvement of activation of ATP-dependent potassium channels in ischemic preconditioning in swine. *Am J Physiol* 1994; 267:H1341-H135

Schulz R, Post H, Vahlhaus C, Heusch G. Ischemic preconditioning in pigs: a graded phenomenon. Its relation to adenosine and bradykinin. *Circulation* 1998; 98:1022-1029

Shiki K, Hearse DJ. Preconditioning of ischaemic myocardium:reperfusion-induced arrythmias. *Am. J. Physiol* 1987; 253: H1470-H1476

Shinmura K, Liu S-Q, Tang X-L, Kodani E, Xuan Y-T, Bhatnagar A, Bolli R. Aldose reductase is an obligatory mediator of the late phase of ischemic preconditioning. *Circulation*. 2000; 102(suppl II):11-12

Shinmura K, Tang XL, Wang Y, Xuan YT, Liu SQ, Takano H, Bhatnagar A, Bolli R. Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci USA* 2000; 97:10197-10202

Sies H. Oxidative stress: From basic research to clinical application. *Am J Med* 1991; 91:31S-38

Song C, Zhang J, Gao J, Zheng Y, Cao X, Wead W, Bolli R, Ping P. Lck tyrosine kinase is a direct substrate of PKC ϵ in cardiac myocytes. *Circulation*. 2000;102(suppl 2):2-221(Abstract)

Song Y Chan, CW Brown, GM Pang, SF Silverman, M. Studies of the renal action of melatonin: evidence that effects are mediated by 37 kDa receptors of the Mel1a subtype localized primarily to the basolateral membrane of the proximal tubule. *FASEB J* 1997; 11(1):93-100

Song, Y Poon, AM Brown, GM Pang, SF Ontogeny of 2-iodomelatonin binding sites in the chicken (*Gallus domesticus*) kidney and spleen. *Can J Physiol Pharmacol* 1995, 73(6):685-692

Southan GJ, Szabo C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem Pharmacol* 1996; 51(4):383-94

Srivastava S, Chandra A, Wang LF, Seifert WE, DaGue BB, Ansari NH, Srivastava SK, Bhatnagar A. Metabolism of the lipid peroxidation product, 4-hydroxy-trans-2-nonenal, in isolated perfused rat heart. *J Biol Chem* 1998; 273:10893-10900

Stearse S, Yellon D. The potential for endogenous myocardial antioxidants to protect the myocardium against ischemia-reperfusion injury: Refreshing the parts exogenous antioxidants cannot reach?. *J Mol Cell Cardiol* 1995; 27:65-74

Suarez J, Torres JC. Modulatory Role of Endothelium-Derived Relaxing Factors on the Flow-Regulated Coronary Vascular resistance. *Proc West Pharmacol Soc* 1998; 41:15-16

Sugden PH, Bogoyevitch MA. Intracellular signalling through protein kinases in the heart. *Cardiovasc Res* 1995; 30:478-492

Sugden PH, Clerk A. "Stress responsive" mitogen-activated protein kinases(c-Jun N- terminal kinases and p38 mitogen -activated protein kinases) in the myocardium. *Circ Res* 1998; 83: 345-352

Sugden PH. Clerk A. "Stress-responsive" mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. *Circ Res* 1998; 83:345-352

Sumeray MS, Yellon DM. Myocardial preconditioning. What have we learned? *Eur Heart J* 1997; 18(Suppl A):A8-A14

Sun JZ, Kaur H, Halliwell B, Li XY, Bolli R. Use of aromatic hydroxylation of phenylalanine to measure production of hydroxyl radicals after myocardial ischemia in vivo: direct evidence for a pathogenetic role of the hydroxyl radical in myocardial stunning. *Cir Res* 1993; 73:534-549

Sun JZ, Tang XL, Knowlton AA, Park SW, Qiu Y, Bolli R. Late preconditioning against myocardial stunning: an endogenous protective mechanism that confers resistance to postischemic dysfunction 24 hours after brief ischemia in conscious pigs. *J Clin Invest* 1995; 95:388-403

Sun JZ, Tang XL, Park SW, Qiu Y, Turrens JF, Bolli R. Evidence for an essential role of reactive oxygen species in the genesis of late preconditioning against myocardial stunning in conscious pigs. *J Clin Invest* 1996; 97:562-572

Takano H, Bolli R, Black RG, Kodani E, Tang XL, Yang Z, Bhattacharya S, Auchampach JA. A1 or A3 receptors induce late preconditioning against infarction in conscious rabbits by different mechanisms. *Cir Res* 2001; 88:520-528

Takano H, Bolli R, Tang XL, Yang Z, Black R, Auchampach J. Activation of A₁ or A₃ receptors produces delayed protection against infarction in conscious rabbits by a mechanism involving KATP channels. *Circulation* 1999; 100:I-56 Abstract

Takano H, Manchikalapudi S, Tang XL, Qiu Y, Rizvi A, Jadoon A, Zhang Q, Bolli R. Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. *Circulation* 1998b; 98:441-449

Takano H, Tang XL, Bolli R. Different roles of KATP channels in late preconditioning against myocardial stunning and infarction. *Am J Physiol Heart Circ Physiol* 2000a; 279:H2350-H2359

Takano H, Tang XL, Kodani E, Bolli R. Late preconditioning enhances recovery of myocardial function after infarction in conscious rabbits. *Am J Physiol Heart Circ Physiol* 2000; 279: H2373-H2381

Takano H, Tang X-L, Qiu Y, Guo Y, French B, Bolli R. Nitric oxide donors induce late preconditioning against myocardial stunning in conscious rabbits via an antioxidant-sensitive mechanism. *Circ Res* 1998a; 83:73-84

Takano H, Tang XL, Qiu Y, Manchikalapudi S, Wu WJ, French B, Bolli R. Intracoronary administration of oxygen radicals induces late preconditioning against stunning in conscious rabbits. *Circulation* 1997; 96(suppl-1):I-256-I-257

Takashi EN, Wang Y, Ashraf M. Activation of Mitochondrial K_{ATP} Channel Elicits Late Preconditioning Against Myocardial Infarction via Protein Kinase C Signalling Pathway. *Circulation Research* 1999; 85:1146

Tan D X, Manchester L C, Reiter R J, et al ,Ischemia/reperfusion-induced arrhythmias in the isolated rat heart: Prevention by melatonin. *J Pineal Res* 1998; :25:184-191

- Tan DX, Manchester LC, Reiter RJ, Plummer BF. Cyclic 3- hydroxymelatonin: a melatonin metabolite generated as a result of hydroxyl radical scavenging. *Biol Signals Recept* 1999; 253:614-620
- Tan DX, Manchester LC, Reiter,Qi RJ, WB Karbowink M, Calvo JR. Significance of melatonin in anitoxidative defense system: rections and products. *Biol Signals Recept* 2000; 30:2294-2296
- Tan DX, Manchester LC, Poeggeler B, Reiter RJ. Melatonin, hydroxyl radical-mediated oxidative damage, and aging: a hypothesis. *J Pineal Res* 1993; 14(4):151-168
- Tan DX, Manchester LC, Reiter RJ, Plummer BF, Hardies LJ, Weintraub ST, Vijayalaxmi A, Sheperd AM. A novel melatonin metabolite , cyclic 3-hydroxymelatonin: a biomarker of in vivo hydroxyl radical generation *Biochem Biophys Res Commun* 1998; 253:614-620
- Tan DX, Manchester LC, Reiter RJ, Qi W, Kim SJ, EL-Sokkary GH. Ischemia/reperfusion-induced arrhythmias in the isolated rat heart: prevention by melatonin. *J Pineal Res* 1998; 25:184-194
- Tanaka M, Fujiwara H, Yamasaki K. Ischemic preconditioning elevates cardiac stress Protein but does not limit infarct size 24 or 48 h later in rabbits. *Am J Physiol* 1994; 267:H1476-H1482
- Tang XL, Qiu Y, Park SW, Sun JZ, Kalya A, Bolli R. Time couese of late preconditioning against myocardial stunning in conscious pigs. *Cir Res* 1996;79:424-434
- Tang XL, Qiu Y, Turrens JF, Sun JZ, Bolli R. Late preconditioning against stunning is not mediated by increased antioxidant defences in conscious pigs. *Am J Physiol.* 1997; 273:H1651-H1657
- Tang XL, QiuY, Turrens JF, Bolli R. Ischemic preconditioning does not upregulate antioxidant enzymes in conscious rabbits. *J Mol Cell Cardiol* 2000; 32:A54(Abstract)
- Tang XL, Rizvi AN, Qiu Y, Takano H, Zhang Q, Guo Y, Bolli R. Evidence that the hydroxyl radical triggers late preconditioning against myocardial stunning in conscious rabbits. *Circulation* 1997a; 96: I-255
- Tang XL, Xu M, Qian ZM Differential behaviour of cell membranes towards iron-induced oxidative damage and the effect of melatonin. *Biol Signals* 1997; 6:291-300.
- Thornton J, Striplin S, Liu GS et al. Inhibition of protein synthesis does not block myocardial protection afforded by preconditioning. *Am J Physiol* 1990; 259:H1822-H1825
- Thornton JD, Liu RA, Olsson RA, Downey JM. Intravenous pretreatment with A₁-selective adenosine analogues protects the heart against infarction. *Circulation* 1992; 85:659-665
- Thornton JD, Thornton CS, Downey JM. Effect of adenosine receptor blockade: Preventing protective preconditioning depends on the time of initiation. *Am J Physiol* 1993; 265:H504-H508
- Thorton JD, Thornton CS, Sterling L, Downey JM. Blockade of ATP-sensitive potassium channels does not prevent preconditioning in the rabbit heart. *Cir Res* 1993; 72:44-49

Toombs CF, Moore TL, Shebuski RJ. Limitation of infarct size in the rabbit by ischemic preconditioning is reversible with glibenclamide. *Cardiovasc Res*. 1993; 27:617-622

Tracey WR, Magee W, Masamune H, Kennedy SP, Knight DR, Bucholz RA, Hill RJ. Selective adenosine A₃ receptor stimulation reduces ischaemic myocardial injury in rabbit heart. *Cardiovasc Res* 1997; 33:410-415

Trapp S, Ashcroft FM. A metabolic sensor in action: news from the ATP-sensitive K⁺-channel. *News Physiol Sci* 1997; 12:255-63

Tsang CW, Chan CL, Li P, Pang SF. Analysis of 5-methoxytryptamine at the femtomole level in the rat an quail brain by gas chromatography-electron-capture negative-ion chemical ionization mass spectrometry. *J Chromatogr B Biomed Appl* 1996; 682:185-94

Tsuchida A, Liu Y, Liu GS, Cohen MV, Downey JM. α 1-Adrenergic agonists precondition rabbit ischaemic myocardium independent of adenosine by direct activation of protein kinase C. *Cir Res* 1994; 75:576-585

Tsuchida A, Miura T, Miki T, Shimamoto K, Imura O. Role of adenosine receptor activation in myocardial infarct size limitation by ischaemic preconditioning. *Cardiovasc Res* 1992; 26:456-461

Van Winkle DM, Downey JM, Cohen MV. Cardioprotection from ischaemic preconditioning is lost following prolonged reperfusion in the rabbit. *Cor Art Dis* 1991; 2:613-619

Van Wylen D, Willis J, Shodi J. Cardiac microdialysis to estimate interstitial adenosine and coronary blood flow. *Am J Physiol* 1990; 258: H1642-H1649

Vane JR, Bakhle YS, Botting RM. Cyclooxygenase 1 and 2. *Annu Rev Pharmacol Toxicol*. 1998; 38:97-120

Vegh A, Papp JG, Parratt JR. Prevention by dexamethasone of the marked antiarrhythmic effects of preconditioning induced 20h after rapid cardiac pacing. 1994; 113:1081-1082

Vegh A, Papp JG, Szekeres L, et al. Prevention by an inhibitor of the L-arginine-nitric oxide pathway of the antiarrhythmic effects of bradykinin in anaesthetized dogs. *Br J Pharmacol* 1993; 110:18-19

Victor T, Bester AJ, Lochner A. A sensitive and rapid method for separating adenine nucleotides and creatine phosphate by ion-pair-reversed-phase high-performance liquid chromatography. *J Chromatogr* 1987; 389:339-344

Vivien-Roels Pevet B, Zarazaga P, Malpoux L, Chemineau P () Daily and light-at-night induced variations of circulating 5-methoxytryptohol (5-ML) in ewes with respectively high and low nocturnal melatonin secretion. *J Pineal Res* 1999; 27:230-236

Wall TM, Sheedy R, Hartman JC. Role of bradykinin in myocardial preconditioning. *J Pharmacol Exp Ther* 1996; 78: 443-454

Walsh RS, Tsuchida A, Daly JJ, Thorton JD, Cohen MV, Downey JM. Ketamine-xylazine anaesthesia permits a K_{ATP} channel antagonist to attenuate preconditioning in rabbit myocardium. *Cardiovasc Res* 1994; 28(9):1337-41

Wang Y, Hirai K, Ashraf M. Activation of mitochondrial ATP-sensitive K^+ channel for cardiac protection against ischemic injury is dependant on protein kinase C activity. *Circ Res* 1999; 85:731-741

Wang Y, Zhang S, Guo Y, Bao W, Wu J, Bolli R. Ischemic preconditioning(PC) upregulates inducible nitric oxide synthase (iNOS) , but not eNOS, in cardiac myocytes in vivo. *Circulation* 2000; 102(suppl-II):II-93(Abstract)

Weinbrenner C, Liu GS, Cohen MV, Downey JM. Phosphorylation of tyrosine 182 of p38 mitogen activated protein kinase correlates with the protection of preconditioning in the rabbit heart. *J Mol Cell Cardiol* 1997; 29:2383-2391

Witte K, Grebmer W, Scalbert E, Delagrang P, Guardiola-Lemaitre B, Lemmer B. Effects of melatoninergic agonists on light-suppressed circadian rhythms in rats. *Physiol Behav* 1998; 65:219-224.

Woodiwiss AJ, Tsotetsi OJ, Sprott S, Lancaster EJ, Mela T, Chung ES, Meyer TE, Norton GR. Reduction in Myocardial Collagen Cross-Linking Parallels Left Ventricular Dilatation in Rat Models of Systolic Chamber Dysfunction. *Circulation* 2001; 103:155

Wu WJ, Bolli R. Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits. *Am J Physiol* 1997;273:H2931-2936

Xi L, Chelliah J, Nayeem MA, Levasseur JE, Hess ML, Kukreja RC. Whole body heat shock fails to protect mouse heart against ischemia/reperfusion injury: role of 72 kDa heat shock protein and antioxidant enzymes. *J Mol Cell Cardiol* 1998; 30:2213-2227

Xi L, Jarret NC, Hess ML, Kukreja RC. Essential role of inducible nitric oxide synthase in monophosphoryl lipid A-induced late cardio-protection: evidence from pharmacological inhibition and gene knock out mice. *Circulation* 1999; 99:2157-2163.

Xi L, Salloum F, Tekin D, Jarret NC, Kukreja RC. Glycolicid RC-552 induces delayed preconditioning-like effect via iNOS-dependant pathway in mice. *Am J Physiol* 1999; 277:H2418-H2424.

Xuan Y-I, Tang X-L, Banerjee S, Takano H, Li J-J, Qiu Y, Han H, Li R, Bolli R. Nuclear factor κ B plays an essential role in the genesis of the late phase of ischemic preconditioning in conscious rabbits. *Circ Res* 1999; 84:1095-1109.

Xuan YT, Tang XL, Banerjee S, Takano H, Li RC, Han H, Qiu Y, Li JJ, Bolli R. Nuclear factor-KB plays an essential role in the late phase of ischemic preconditioning in conscious rabbits. *Circ Res* 1999; 84:1095-1099

Xuan YT, Tang XL, Qiu Y, Banerjee S, Takano H, Han H, Bolli R. Biphasic response of cardiac NO synthase to ischemic preconditioning in conscious rabbits. *Am J Physiol* 2000; 279:H2360-H2371

Yamashita N, Hoshida S, Nishida M, Igarashi J, Aoki K, Hori M, Kuzuya T, Tada M. Time course of tolerance to ischemia-reperfusion injury and induction of heat shock protein 72 by heat stress in the rat heart. *J Moll Cell Cardiol* .1997; 29:1815-1821

Yamashita N, Hoshida S, Otsu K, Asahi M, Kuzuya T, Hori M. Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation. *J Exp Med* 1999; 189:1699-1706

Yamashita N, Hoshida S, Otsu K, Taniguchi N, Kuzuya T, Hori M. Involvement of cytokines in the mechanism of whole-body hyperthermia-induced cardioprotection. *Circulation* 2000; 102:452-457

Yamashita n, Hoshida S, Otsu K, Taniguchi N, Kuzuya T, Hori M. The involvement of cytokines in the second window of ischemic preconditioning. *Br J Pharmacol* 2000; 131:415-422

Yamashita N, Hoshida S, Taniguchi N, Kuzuya T, Hori M. "A second window of protection" occurs 24h after ischemic preconditioning in the rat heart. *J Mol Cell Cardiol* 1998; 30:1181-1189

Yamashita N, Hoshida S, Taniguchi N, Kuzuya T, Hori M. Whole-body hyperthermia provides biphasic cardioprotection against ischemia/reperfusion injury in rat . *Circulation* 1998a; 98: 1414-1421

Yamashita N, Nishida M, Hoshida S, Kuzuya T, Hori M, Taniguchi N, Kamada T, Tada M. Induction of manganese superoxide dismutase in rat cardiac myocytes increase tolerance to hypoxia 24 hours after preconditioning. *J Clin Invest* 1994 :94:2193-2199

Yao Z, Auchampach JA, Pieper GM, Gross GJ. Cardioprotective effects of monophosphoryl lipid A, a novel endotoxin analogue, in the dog. *Cardiovasc Res* 1993; 27:832-838

Yellon D , Baxter G. A 'second window of protection' or delayed preconditioning phenomenon: Future horizons for myocardial protection. *J Moll Cardiol* 1995; 27:1023-1034

Yoshida KI, Maaieh MM, Shipley JB, Doloresco M, Bernado NL, Qian YZ, Elliott GT, Kukreja RC. Monophosphoryl lipid A induces pharmacologic preconditioning in rabbit hearts without concomitant expression of 70-kDa heat shock protein. *Mol Cell Biochem* 1996;159:73-80

Yoshioka T, Bills T, Moore-Jarrett H, Greene I, Burr I, Ichikawa. Role of intrinsic antioxidant enzymes in renal oxidant injury. *Kidney Int* 1990; 38: 282-288

Zawilska JB, Skene DJ, Nowak JZ. 5-Methoxytryptohol rhythms in the chick pineal gland: effect of enviromental lighting conditions. *Neurosci Lett* 1998; 251:33-36

Zeman M, Illnerova H. Ontogeny of n-acetyltransferase activity rhythm in pineal gland of chick embryo. *Comp Biochem Physiol* 1990; A 97:175-178.

Zhang J, Bolli R, Tang X-L, Jones K, Ping P. Direct demonstration of both tyrosine and serine phosphorylation of I κ B- α during ischemic preconditioning in conscious rabbits. *Circulation*. 1999; 100:1-56. Abstract.

Zhao L, Weber PA, Smith JR, Comerford ML, Elliot GT. Role of inducible nitric oxide synthase in pharmacological "preconditioning" with monophosphoryl lipid A. *J Moll Cell Cardiol* 1997; 29:1567-1576

Zhao T, Xi L, Chelliah J, Levassuer JE, Kukreja RC. Inducible nitric oxide synthase mediates delayed myocardial protection induced by activation of adenosine A₁ receptors: evidence from gene knockout mice. *Circulation* 2000; 102:902-907

Zhao T.C Hines D.S, Kukreeja R.C. Adenosine-induced late preconditioning in mouse hearts: role of p38 MAP kinase and mitochondrial K_{ATP} channels. *Am. J. Physiol.*2000; 280: 989-997

Zhou X, Zhai X, Ashraf M. Direct evidence that initial oxidative stress triggered by preconditioning contributes to second window of protection by antioxidant enzyme in myocytes. *Circulation* 1996; 93:1177-1184