

# The role of p38 MAPK activation in preconditioning mediated protection against ischaemia/reperfusion injury

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

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December 2002

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## **Declaration**

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

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## Summary

The ultimate consequence of the interruption of blood flow to the myocardium is necrosis. In view of the prevalence of coronary artery disease in the general population, and the deleterious effects of myocardial ischaemia on myocardial tissue, it is important to develop new strategies to protect the myocardium against ischaemia. Necrosis of myocardial tissue has for a long time been considered to be the main component of the damage incurred by myocardial infarction. Recently the importance of the contribution of apoptotic cell death in the context of myocardial ischaemia/reperfusion injury has become apparent.

There is a general agreement that early reperfusion is necessary to salvage myocardial tissue from cell death. Preconditioning is the phenomenon whereby brief episodes of ischaemia and reperfusion protect the heart against a subsequent longer period of ischaemia. This endogenous mechanism is the strongest form of protection against myocardial infarction that has yet been described. Apart from ischaemic preconditioning (IPC), protection can also be elicited with pharmacologic agents, such as activation of the beta-adrenergic receptor with isoproterenol. Ischaemic preconditioning protects the myocardium against necrosis, arrhythmias and apoptosis, and increases functional recovery upon reperfusion. Beta-adrenergic receptor stimulated preconditioning ( $\beta$ PC) has been shown to improve post-ischaemic functional recovery, but it is not known whether it also protects against myocardial infarction and apoptosis.

The signaling pathways involved in preconditioning have been extensively studied. A distinction is usually made between factors that act as triggers, or as mediators of protection. Triggers activate cellular responses before the onset of sustained ischaemia, and its involvement is demonstrated by showing that inhibitors of the trigger bracketing the preconditioning protocol can block its protective effect, or that transient administration with washout before sustained ischaemia can activate a protective effect. A mediator operates during sustained ischaemia, and its involvement is demonstrated by showing that infusion of an inhibitor of its action immediately prior to sustained ischaemia (without washout) can block its protective effect. Another approach to demonstrate a mediator role is to attempt to activate signal transduction pathways during sustained ischaemia. As it is not possible to infuse substances during ischaemia, activators are infused immediately prior to ischaemia without washout of the agent and subsequently its effect on protection is observed.

It is clear that the evolutionary conserved stress activated pathways are involved in preconditioning. There are three pathways i.e., the extracellular receptor activated pathways (ERK), c-jun terminal activated kinases (JNK) and p38 mitogen-activated protein kinases (MAPK). The precise role of the p38 MAPK pathway has not been elucidated. Experimental evidence has suggested a role for the activation of p38 MAPK as a trigger, as well as a mediator of the protective effect of preconditioning. There is however also strong evidence that the attenuation of p38 MAPK activation during sustained ischaemia, rather than its activation, is responsible for the

protection that is observed. Furthermore, the role of p38 MAPK has only been investigated in relation to its protection against necrosis, but not apoptosis.

### **AIMS:**

The aim of this study was to:

- (I) Establish a model of preconditioning in neonatal cardiomyocyte cell culture.  
The reason was that such a model could potentially enable one to rapidly elucidate the signal transduction pathways in an environment without the influence of non-cardiac cells.
- (II) Investigate whether IPC and  $\beta$ PC protect against necrosis and apoptosis.
- (III) Elucidate the role of the stress-activated kinase, p38 MAPK, in preconditioning.

### **METHODS:**

#### 1. Neonatal rat cardiomyocyte cell culture model

A viability assay with 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was first developed using different concentrations - a concentration of 0.25% was found to be optimal to determine viability. Neonatal cardiomyocyte cell cultures were subjected to sustained simulated "ischaemia" by using either 5 mM KCN plus deoxyglucose (DOG) for 5 min or potassium cyanide (KCN) for 45 min. Some cell cultures were preconditioned with either chemical ischaemia (5 mM KCN for 5 min) or isoproterenol ( $10^{-7}$  M) for 5 min and 60 min reoxygenation before being exposed to sustained simulated ischaemia.

## 2. Isolated adult rat cardiomyocyte model

Isolated cardiac myocytes were exposed to 2 hours of hypoxia, which was induced by pelleting the cells by centrifugation, and covering them with a thin layer of mineral oil. Some groups were preconditioned with either hypoxia for 10 min at 37° C or isoproterenol ( $10^{-7}$  M) for 5 min, followed by reoxygenation for 20 minutes. The trypan blue exclusion method and MTT method developed in the neonatal cardiomyocytes were used to assess viability.

## 3. Isolated perfused rat heart model

3.1 Infarct size was determined in a model of regional ischaemia by using tetrazolium staining and determining the area of necrosis (exclusion of tetrazolium) as a percentage of area at risk. These hearts were subjected to 35 min global ischaemia and 30 min reperfusion. Some groups were preconditioned by three cycles of 5 min global ischaemia or addition of isoproterenol ( $10^{-7}$  M) for 5 min, followed by 5 min reperfusion before the onset of sustained regional ischaemia.

3.2 p38 MAPK activation and markers of apoptosis: p38 MAPK activation was determined using antibodies against dual phosphorylated p38 MAPK (i.e. activated p38 MAPK). Apoptosis was measured by using antibodies against activated caspase-3, and against a fragment of PARP (PARP cleavage). For these experiments isolated rat hearts were exposed to global ischaemia for 25 min followed by 30 min reperfusion. Some groups were preconditioned

with three cycles of 5 min global ischaemia. A global ischaemia model was used in order to have sufficient tissue available for the Western blot determinations. This necessitated a shorter period of sustained ischaemia, as the globally ischaemic heart does not recover sufficiently after a longer period of ischaemia such as is necessary in regional ischaemia experiments.

- 3.3 The role of p38 MAPK in ischaemic preconditioning was investigated by administration of SB 203580 (1 $\mu$ M), a selective inhibitor of p38 MAPK, either bracketing the preconditioning (i.e. to determine its role as a trigger) or for 10 min immediately prior to sustained ischaemia (i.e. to determine its role as a mediator). The second approach was to use anisomycin, an activator of p38 MAPK, as a trigger (infusion for 10 min followed by wash out) or as a mediator (10 min immediately prior to sustained ischaemia) in the same model as used for determination of p38 MAPK activity. The infusion of anisomycin for 10 min has been shown to elicit activation of p38 MAPK to a similar extent as has been observed with an ischaemic preconditioning protocol. The endpoints used were infarct size and markers of apoptosis.

## RESULTS:

### 1. Neonatal rat cardiomyocyte cell culture model

It was not possible to establish a model of preconditioning of neonatal cardiomyocytes that was consistently successful. It was therefore decided to abandon the attempts and to use a different cell model.

### 2. Isolated adult rat cardiomyocyte model

Isolated adult cardiomyocytes were preconditioned successfully, but produced too little material to perform simultaneous determinations of cell viability and Western blots (p38 MAPK activation and markers of apoptosis). It was therefore decided to use the isolated perfused adult rat heart.

### 3. Isolated perfused adult rat heart model

#### 3.1 Both IPC and $\beta$ PC protect against infarction and apoptosis:

Using two models of preconditioning i.e., IPC and  $\beta$ PC, the protective effects of preconditioning were demonstrated convincingly against infarction (necrosis). IPC and  $\beta$ PC both caused a significant reduction in infarct size ( $12.2\pm 1.4$  and  $15.2\pm 2.6\%$ ) versus Non-PC hearts ( $29.6\pm 2.9\%$ ) ( $p < 0.001$ ). Both forms of preconditioning also protected against apoptosis, by significantly reducing the markers of apoptosis, caspase-3 activation and PARP cleavage. The protection afforded by both forms of preconditioning was accompanied by a marked decrease in activation of p38 MAPK upon reperfusion. The relationship between p38 MAPK and the protection that was elicited by preconditioning was then investigated,

namely whether p38 MAPK acted as a trigger, or as a mediator of protection. To investigate the role of p38 MAPK as a mediator or a trigger in preconditioning, use was made of (i) a specific inhibitor of p38 MAPK activation i.e., SB 203580 and (ii) a known activator of p38 MAPK i.e., anisomycin.

### 3.2 p38 MAPK as a trigger of protection:

Administration of SB 203580 during the IPC protocol and washed out before sustained ischaemia did not abolish the protective effect of ischaemic preconditioning, and resulted in a small, but significant increase in caspase-3 activation and PARP cleavage. On the other hand, activation of p38 MAPK with anisomycin for 10 min followed by washout also resulted in a significant reduction in necrosis (infarct size  $14.9 \pm 2.2$  versus  $29.6 \pm 2.9\%$  in Non-PC hearts) ( $p < 0.001$ ) and both markers of apoptosis. The latter results suggested that p38 MAPK was a trigger of preconditioning. If this was the case, why didn't SB 203580 abolish the protection of IPC? The most likely explanation was that multiple protective mechanisms were activated during a multi-cycle protocol of ischaemic preconditioning, of which activation of p38 MAPK was only one. Inhibition of p38 MAPK with SB 203580 would therefore not be expected to block the activation of those mechanisms that were independent of p38 MAPK, but were still capable of protecting against necrosis or apoptosis. It is very interesting that a small increase in apoptosis was observed when SB 203580 was used in this situation, as it may indicate that the protection against apoptosis was more dependent on the activation of p38 MAPK than the protection against necrosis, as no effect was seen

on infarct size. Another explanation could be that infarct size determination was not sensitive enough to detect such small effects.

### 3.3 p38 MAPK as a mediator of protection:

Inhibition of p38 MAPK activation with SB 203580 administered 10 min before sustained ischaemia caused a significant decrease in infarct size compared to Non-PC hearts ( $12.6 \pm 1.9$  vs  $29.6 \pm 2.9\%$ ) ( $p < 0.001$ ) equivalent to that of hearts preconditioned with ischaemia. This was accompanied by a similar pattern of protection against apoptosis, with significantly reduced activation of caspase-3 activation and PARP cleavage.

These results strongly supported a role for the *attenuation* of p38 MAPK activation as a mediator of preconditioning against ischaemia/reperfusion-mediated necrosis and apoptosis. However, the results of the experiments with anisomycin were at first glance not compatible with such a conclusion. The administration of the activator of p38 MAPK, anisomycin, for 10 min immediately prior to sustained ischaemia resulted in significant *protection* against necrosis (infarct size  $16.6 \pm 2.4\%$  vs  $29.6 \pm 2.9\%$  in Non-PC hearts) ( $p < 0.01$ ) and reduced caspase-3 activation and PARP cleavage indicating less apoptosis. The reason for these findings were probably that this method of administration of anisomycin did in fact not activate p38 MAPK during sustained ischaemia, but actually served as a trigger to protect against ischaemia - similarly as if it had been infused with washout of the drug. Support for this notion was found in the fact that p38 MAPK activation was

decreased upon reperfusion. These results suggested that the logistical problem of not being able to infuse a drug into the myocardium during ischaemia could not be overcome by immediate prior infusion, and that the administration of anisomycin in this way had activated downstream effectors of the p38 MAPK signal transduction pathway. An important contender for such an effector would be heat shock protein 27 (HSP27), which has been shown to play an important role in protection against apoptosis, and stabilisation of actin, and thus the cytoskeleton. Another possibility was that anisomycin had activated the JNK stress activated kinases. The elucidation of a role of this signal transduction pathway would necessitate the use of anisomycin in the presence of an agent such as curcumin, an inhibitor of JNK.

Final conclusion:

The work in this thesis showed that the stress activated kinase, p38 MAPK, was involved in the protective effect of ischaemic preconditioning. The results suggested a role for the activation of p38 MAPK as a trigger of protection, and the attenuation of p38 MAPK as a mediator of protection, which was observed in the reduction of both necrosis (infarct size) and apoptosis as determined with caspase-3 activation and PARP cleavage.

## Opsomming

Die afsluiting van bloedvloei na die miokardium gee aanleiding tot nekrose. In die lig van die voorkoms van koronêre bloedvatsiekte onder die algemene populasie, en die nadelige effekte van miokardiale isgemie op miokardiale weefsel, is dit belangrik om nuwe strategieë te ontwikkel wat die miokardium teen isgemie beskerm. Nekrose van miokardiale weefsel word tradisioneel as die belangrikste komponent van die skade aangerig deur miokardiale infarksie beskou. Die belang van apoptotiese seldood in die konteks van miokardiale isgemie/herperfusie (I/R) het onlangs na vore getree.

Dit word algemeen aanvaar dat vroeë vroegtydige herperfusie noodsaaklik is om miokardiale weefsel te beskerm teen seldood. Prekondisionering is 'n verskynsel waartydens kort episodes van I/R die hart teen 'n daaropvolgende langer periode van isgemie beskerm. Hierdie endogene meganisme is die kragtigste vorm van beskerming teen miokardiale infarksie tot dusver beskryf. Afgesien van isgemiese prekondisionering (IPC), kan beskerming ook deur farmakologiese middels, soos byvoorbeeld die aktivering van die beta-adrenerge reseptore met isoproterenol, ontlok word. IPC beskerm die miokardium teen nekrose, arritmieë en apoptose, en verhoog funksionele herstel na herperfusie. Daar is reeds aangetoon dat beta-adrenerge prekondisionering ( $\beta$ PC) post-isgemiese funksionele herstel verbeter, maar dit is nog onbekend of beskerming ook teen miokardiale infarksie en apoptose verleen word.

Die seintransduksie paaie betrokke tydens prekondisionering is reeds in detail bestudeer. Daar word gewoonlik tussen faktore wat optree as snellers, of as mediators van beskerming, onderskei. Snellers aktiveer sellulêre response voor die aanvang van volgehoue isgemie, en hul betrokkenheid word aangetoon deurdat inhibisie van snellers tydens die prekondisionering protokol, beskerming ophef. Snellers se effekte kan ook ontlok word deur hulle tydelike toe te dien en dan net voor volgehoue isgemie weer uit te was. Mediators oefen hulle effek tydens volgehoue isgemie uit, en hulle betrokkenheid word gedemonstreer deurdat toediening van inhibitors net voor volgehoue isgemie (sonder uitwas) hulle beskermende effekte ophef. Mediators se rol kan ook aangetoon word deur te poog om seintransduksie paaie tydens volgehoue isgemie te aktiveer. Aangesien dit ontmoontlik is om middels tydens isgemie te infuseer, word aktiveerders onmiddelik voor die aanvang van isgemie toegedien sonder om hulle uit te was, sodat hulle effekte op beskerming vervolgens bestudeer kan word.

Dit is duidelik dat die evolusionêr-behoue stres geaktiveerde paaie tydens prekondisionering betrokke is. Daar is drie paaie nl. die ekstrasellulêre reseptor geaktiveerde pad (ERK), c-jun terminaal geaktiveerde kinases (JNK) en p38 mitogeen geaktiveerde proteïen kinases (MAPK). Die spesifieke rol van die p38 MAPK pad is nog nie ontrafel nie. Eksperimentele bewyse stel 'n rol vir die aktivering van p38 MAPK as 'n sneller, sowel as 'n mediator van die beskermende effek van prekondisionering, voor. Daar is egter ook sterk bewyse dat 'n afname in p38 MAPK aktivering tydens volgehoue isgemie, eerder as sy aktivering,

verantwoordelik is vir die waargenome beskermende effek. Verder is die rol van p38 MAPK slegs in die konteks van beskerming teen nekrose, maar nie teen apoptose nie, bestudeer.

#### DOELWITTE:

Die doelwit van hierdie studie was :

- (I) Die vestiging van 'n prekondisionering model in neonatale kardiomyosiet in selkultuur. Hierdie model sou potensieel 'n spoedige ontrafeling van die seintransduksie paaie sonder die invloed van nie-kardiale selle bewerkstellig.
- (II) Om ondersoek in te stel of IPC en  $\beta$ PC teen nekrose en apoptose beskerm.
- (III) Die ontrafeling van die rol van die stres geaktiveerde kinase, p38 MAPK, tydens prekondisionering.

#### METODES:

##### 1. Neonatale rot kardiomyosiet weefselkultuur model

'n Lewensvatbaarheids essai is ontwikkel deur van verskillende konsentrasies van 3-[4,5-dimetielthiazol-2-yl]-2,5-difeniel-tetrazolium bromied (MTT) gebruik te maak – 'n konsentrasie van 0.25% was optimaal om lewensvatbaarheid te bepaal. Neonatale kardiomyosiet weefselkulture is onderwerp aan volgehoue gesimuleerde "isgemie" deur gebruik te maak van 5 mM KCN plus deoksiglukose (DOG) vir 5 minute of 45 min KCN. Sommige weefselkulture is geprekondisioneer deur middel van chemiese isgemie (5 mM KCN vir 5 min) of van isoproterenol ( $10^{-7}$  M) vir 5

minute en 60 minute reoksigenasie alvorens dit bloot gestel is aan volgehoue gesimuleerde isgemie.

## 2. Geïsoleerde volwasse rot kardiomiosiet model

Geïsoleerde kardiomiosiete is aan twee uur hipoksie blootgestel deur selle in 'n pellet te sentrifugeer en met 'n dun lagie mineraalolie te bedek. Sommige groepe is geprekondisioneer deur middel van 10 minute hipoksie by 37°C, of toediening van isoproterenol ( $10^{-7}$  M) vir 5 minute gevolg deur 20 minute reoksigenasie. Die tripaanblou uitsluitings metode en MTT metode soos ontwikkel in die neonatale kardiomiosiet model is gebruik om lewensvatbaarheid te bepaal.

## 3. Geïsoleerde geperfuseerde volwasse rot hart model

3.1 Infarkgrootte is bepaal met 'n model van streeks isgemie deur van tetrazolium kleuring gebruik te maak, waarna die area van nekrose (uitsluiting van tetrazolium) as 'n presentasie van die risiko area bepaal is. Hierdie harte was onderwerp aan 35 minute globale isgemie en 30 minute herperfusie. Sommige groepe is geprekondisioneer met 3 siklusse van 5 minute globale isgemie, of die toevoeging van isoproterenol ( $10^{-7}$  M) vir 5 minute, gevolg deur 5 minute herperfusie voor die aanvang van volgehoue streeks isgemie.

3.2 p38 MAPK aktivering en merkers van apoptose: p38 MAPK aktivering is bepaal deur gebruik te maak van anti-liggame teen tweeledige gefosforileerde p38 MAPK (d.w.s. geaktiveerde p38 MAPK). Apoptose is bepaal deur gebruik te maak

van anti-liggame teen geaktiveerde kaspase-3, en teen 'n fragment van PARP (PARP kliewing). Tydens hierdie eksperimente is geïsoleerde rotharte bloot gestel aan 25 minute globale isgemie gevolg deur 30 minute herperfusie. Sommige groepe is geprekondisioneer met drie siklusse van 5 minute globale isgemie. Om voldoende weefsel vir Westerse klad tegnieke te verkry, is gebruik gemaak van 'n globale isgemie model. As gevolg hiervan was 'n kort periode van volgehoue isgemie genoodsaak, aangesien die globale isgemiese hart nie voldoende herstel na 'n langer periode van isgemie nie, soos wat benodig word in streeks isgemiese eksperimente.

3.3 Die rol van p38 MAPK tydens IPC is bepaal deur die toediening van 'n 1 $\mu$ M konsentrasie van SB 203580, 'n selektiewe inhibitor van p38 MAPK, hetsy tydens prekondisionering (d.w.s. om die rol as 'n sneller te bepaal), óf vir 10 minute direk voor die aanvang van volgehoue isgemie (d.w.s. om dus sy rol as mediator te bepaal). Die tweede benadering was om anisomisien, 'n aktiveerder van p38 MAPK, as sneller (toediening vir 10 minute gevolg deur uitwassing) of as mediator (10 minute direk voor aanvang van volgehoue isgemie) in dieselfde model as in die geval van p38 MAPK aktiviteit bepaling, te gebruik. Die toediening van anisomisien vir 10 minute het aangetoon dat dit p38 MAPK aktivering kan ontlok tot dieselfde maate as die IPC protokol. Die eindpunte was infarkgrootte en merkers van apoptose.

## RESULTATE:

### 1. Neonatale rot kardiomiosiet weefselkultuur model

Dit was nie moontlik om 'n suksesvolle model met konsekwente resultate vir die prekondisionering van neonatale kardiomiosiete te vestig nie. Daar is dus besluit om af te sien van hierdie pogings en eerder 'n alternatiewe selmodel te gebruik.

### 2. Geïsoleerde volwasse rot kardiomiosiet model

Geïsoleerde volwasse kardiomiosiete is suksesvol geprekondisioneer, maar het te min materiaal opgelewer vir die gelyktydige bepaling van sellewensvatbaarheid, p38 MAPK aktivering en merkers vir apoptose. Daar is dus besluit om die geïsoleerde geperfuseerde volwasse rothart te gebruik.

### 3. Geïsoleerde geperfuseerde volwasse rothart model

#### 3.1 Beide IPC en $\beta$ PC beskerm teen infarksie en apoptose:

Deur gebruik te maak van twee prekondisionering modelle d.w.s. IPC en  $\beta$ PC, is die beskermende effekte van prekondisionering teen infraksie (nekrose) oortuigend gedemonstreer. Beide IPC en  $\beta$ PC het 'n betekenisvolle afname in infarkgrootte veroorsaak ( $12.2 \pm 1.4$  en  $15.2 \pm 2.6\%$  respektiewelik), vs Nie-PC harte ( $29.6 \pm 2.9\%$ )( $p < 0.001$ ). Beide vorme van prekondisionering het ook teen apoptose beskerm deur die apoptose merkers, kaspase-3 aktivering en PARP kliewing te verlaag. Die beskerming verkry deur beide vorms van prekondisionering is geassosieer met 'n merkbare afname in die aktivering van p38 MAPK na herperfusie. Die verband tussen p38 MAPK en die beskerming ontlok deur

prekondisionering is gevolglik ondersoek, naamlik of p38 MAPK optree as 'n sneller of as 'n mediator van beskerming. Om die rol van p38 MAPK as 'n mediator of sneller tydens prekondisionering te ondersoek is daar gebruik gemaak van (I) 'n spesifieke inhibitor van p38 MAPK aktivering nl. SB 203580 en (II) 'n bekende aktiveerder van p38 MAPK nl. anisomisien.

### 3.2 p38 MAPK as 'n sneller vir beskerming:

Toediening van SB 203580 tydens die IPC protokol en uitwassing daarvan voor die aanvang van volgehoue isgemie het nie die beskermende effek van IPC opgehef nie, en het gelei tot 'n klein maar betekenisvolle verhoging in kaspase-3 aktivering en PARP kliewing. Andersins het die aktivering van p38 MAPK met anisomisien vir 10 minute gevolg deur 'n uitwas ook tot 'n betekenisvolle afname in nekrose (infarkgrootte  $14.9 \pm 2.2$  vs  $29.6 \pm 2.9\%$  in Nie-PC harte) ( $p < 0.001$ ) in beide merkers van apoptose gelei. Laasgenoemde resultate dui daarop dat p38 MAPK inderdaad 'n mediator van prekondisionering is. Indien dit die geval is, waarom het SB 203580 nie die beskermende effek van IPC opgehef nie? Die mees waarskynlike verklaring is dat veelvuldige beskermingsmeganismes tydens 'n multi-siklus protokol van IPC geaktiveer word, waarvan p38 MAPK aktivering slegs een is. Dit is dus onwaarskynlik dat die inhibisie van p38 MAPK met SB 203580 die aktivering van daardie meganismes onafhanklik van p38 MAPK sal blokkeer en steeds in staat sal wees tot beskerming teen nekrose en apoptose. Dit is interessant dat 'n klein verhoging in apoptose waargeneem is toe SB 203580 gebruik is onder hierdie toestande, aangesien dit daarop kan dui dat die

beskerming teen apoptose meer afhanklik was van die aktivering van p38 MAPK as die beskerming teen nekrose, siende dat geen effek op infarkgrootte waargeneem is nie. 'n Verdere verklaring kan wees dat die bepaling van infarkgrootte nie sensitief genoeg is om sulke klein effekte waar te neem nie.

### 3.3 p38 MAPK as 'n mediator vir beskerming:

Inhibisie van p38 MAPK aktivering deur SB 203580 toediening 10 minute voor volgehoue isgemie het 'n betekenisvolle verlaging in infarkgrootte in vergelyking met Nie-PC harte veroorsaak ( $12.6 \pm 1.9$  vs  $29.6 \pm 2.9\%$ ) ( $p < 0.001$ ) soortgelyk aan dié van harte geprekondisioneer met isgemie. Dit is geassosieer met 'n soortgelyke patroon van beskerming teen apoptose, met betekenisvolle verlaagde kaspase-3 aktivering en PARP kliewing.

Hierdie resultate ondersteun die rol van die afname van p38 MAPK aktivering as 'n mediator van prekondisionering teen I/R-gemedieerde nekrose en apoptose. Die resultate van die anisomisien eksperimente was met die eerste oogopslag nie in oorstemming met hierdie gevolgtrekking nie. Die toediening van die p38 MAPK aktiveerder, anisomisien, vir 10 minute voor volgehoue isgemie het tot 'n betekenisvolle beskerming teen nekrose aanleiding gegee (infarkgrootte  $16.6 \pm 2.4$  vs  $29.6 \pm 2.9\%$  in Nie-PC harte) ( $p < 0.01$ ) en verlaagde kaspase-3 aktivering en PARP kliewing wat dui op verlaagde apoptose. Die rede vir hierdie bevindings is moontlik dat die metode van anisomisien toediening nie p38 MAPK geaktiveer het tydens volgehoue isgemie nie, maar eintlik gedien het as 'n sneller vir beskerming

teen isgemie - amper asof dit toegedien sou word sonder om uitgewas te word. Ondersteuning vir hierdie aanname word gevind in die feit dat p38 MAPK aktivering verlaag is na herperfusie. Hierdie resultate stel voor dat die logistiese probleem dat 'n middel nie tydens isgemie toegedien kan word nie, nie oorkom kan word deur onmiddellike voortydige infusie nie, en dat die toediening van anisomisien op hierdie manier gelei het tot die aktivering van stroom-af effectors van die p38 MAPK seintransduksie pad. 'n Belangrike kandidaat vir so 'n effektor is "heat shock protein 27" (HSP27), wat reeds aangetoon is om 'n belangrike rol in die beskerming teen apoptose en destabilisering, en dus die sitoskelet, te speel. 'n Ander moontlikheid is dat anisomisien die JNK stres geaktiveerde kinases geaktiveer het. Die ontrafeling van die rol van hierdie seintransduksie pad noodsaak die gebruik van anisomisien in die teenwoordigheid van 'n agent soos curcumin, 'n JNK inhibitor.

Finale gevolgtrekking:

Die werk soos vervat in hierdie tesis toon aan dat die stres geaktiveerde kinase, p38 MAPK, betrokke is in die beskermings effek van isgemiese prekondisionering. Die resultate dui op 'n rol vir die aktivering van p38 MAPK as 'n sneller vir beskerming, en die afname in p38 MAPK as 'n mediator vir beskerming, soos waargeneem in die vermindering van veranderlikes van beide nekrose (infarkgrootte) en apoptose soos bepaal deur kaspase-3 aktivering en PARP kliewing.

## Acknowledgements

In the name of Allah, Most Beneficent, Most Merciful

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

My parents, **Uthmaan and Yasmina Hartley**, and family for their love and support.

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

**Professor Johan Moolman** for his critical analysis and supervision in the formulation and completion of this manuscript.

**Prof. Johan Koeslag and everyone at the Department of Medical Physiology** (Tygerberg medical campus) who have all contributed to making my stay here an enjoyable and fruitful one.

**Dr. Hans Strijdom** for being a great colleague and an even better friend.

**Mrs. Natalie Auerbach**, with whom I've shared an office for two years, for being my sounding board and having a great sense of humour.

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## Alphabetical list of abbreviations

### Units of measurement

%	percentage
°C	degrees Celsius
μl	microlitre
μg	microgram
μm	micrometre
μM	micromolar
cm	centimetre
cm <sup>3</sup>	cubic centimetre
cm H <sub>2</sub> O	centimetres water
g	gram
h	hour/s
kDa	kilodalton
l/L	litre
M	molar
mg	milligram
min	minute
ml	millilitres
mm	millimetre
mM	millimolar
mm Hg	millimetre mercury

N	normal
nm	nanometre
OD	optical density
rpm	revolutions per minute
v/v	volume per volume
w	weight
w/v	weight per volume
N	normal
nm	nanometre

#### Chemical compounds

APNEA	N <sup>6</sup> -[2-(4-aminophenyl)ethyl]adenosine
BDM	2,3- butanedione monoxime
BSA	bovine serum albumin
CaCl <sub>2</sub>	calcium chloride
CCPA	2-chloro-N <sup>6</sup> -cyclopentyladenosine
CO <sub>2</sub>	carbon dioxide
DADLE	[D-ala (2), D-leu (5)] enkaphalin
DAG	diacylglycerol
DMEM	Dulbecco's modified eagle's medium
DOG	2-deoxy-D-glucose
DTT	dithiotretiol
EGTA	Ethylene-bis(β-Aminoethylether)- N,N,N',N'-tetra-Acetic acid

HCl	hydrochloric acid
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
H <sub>2</sub> O	water
Iso	isoproterenol
KCl	potassium chloride
KCN	potassium cyanide
KH <sub>2</sub> PO <sub>4</sub>	potassium dihydrogenphosphate
KHB	Krebs-Henseleit buffer
LDH	lactate dehydrogenase
MgSO <sub>4</sub>	magnesium sulphate
MgSO <sub>4</sub> ·7H <sub>2</sub> O	magnesium sulphate 7-hydrate
MPG	N-2-mercaptopropionylglycine
MTT	(3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
NaCl	sodium chloride
NaHCO <sub>3</sub>	sodium hydrocarbonate
NaH <sub>2</sub> PO <sub>4</sub>	sodium dihydrophosphate
Na <sub>2</sub> HPO <sub>4</sub>	di-sodium hydrogen phosphate
Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O	di- sodium hydrogen phosphate 7 hydrate
NaHCO <sub>3</sub>	sodium hydrogen carbonate
NaSO <sub>4</sub>	sodium sulphate
NBCS	newborn calf serum
O <sub>2</sub>	oxygen

Ponceau Red	3 Hydroxy-4-[2-sulfo-4-(4-sulfo-phenylazo)phenylazo]- 2.7-naphtalene disulphonic acid
PBS	phosphate buffered solution
PIA	N6-(phenyl-2R-isopropyl)-adenosine
PMA	phorbol 12-myristate 13 acetate
PMSF	phenylmethyl sulphonyl flouride
PVDF	poly vinylidene fluoride
SDS	sodium dodecyl sulphate
SNAP	S-nitroso-N-pencillamine
SNP	sodium nitroprusside

#### Other abbreviations

AIF	apoptosis inducing factor
APAF-1	apoptotic protease activation factor 1
ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	base pairs
cGMP	cyclic 3',5'- guanosine monophosphate
DNA	deoxyribonucleic acid
cNOS	constitutive nitric oxide synthase
ERK	extracellular-regulated kinases
FasL	Fas ligand
G-protein	guanine nucleotide-binding protein

G <sub>i</sub>	inhibitory G-protein
G <sub>s</sub>	stimulatory G-protein
HSP 27	heat shock protein 27
iNOS	inducible nitric oxide synthase
IPC	ischaemic preconditioning
I/R	ischaemia and reperfusion
ISEL	in situ end labeling
Iso-PC	β-adrenergic receptor stimulated preconditioning with isoproterenol
JNK	c-Jun NH <sub>2</sub> -terminal kinase
K <sub>ATP</sub> channel	ATP dependent potassium channel
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK
MI	myocardial infarction
MKK	MAPK kinase
MKKK	MAPK kinase kinase
n	number of experiments
NFKB	nuclear factor-kappa B
NO	nitric oxide
Non-PC	non-preconditioned
NOS	nitric oxide synthase
PARP	poly (ADP-ribose) polymerase
PC	preconditioning

PLB	phospholipase B
PLC	phospholipase C
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
R	reperfusion
ROS	reactive oxygen species
SI	sustained ischaemia
SWOP	second window of preconditioning
TNF- $\alpha$	tumour necrosis factor alpha
TTC	triphenyl tetrazolium chloride
TUNEL	TdT- mediated dUTP nick end-labelling

# Chapter 1

## Literature Review

### 1.1 Introduction

The inevitable consequence of the abrupt deprivation of oxygen supply to the myocardium is myocardial infarction. Living systems have the ability to respond to stresses that endanger the survival of the organism to minimize the damage that is incurred. An example of such cytoprotection is ischaemic preconditioning, the phenomenon whereby brief episodes of ischaemia and reperfusion protects the heart against a subsequent period of sustained ischaemia (Murray et al 1986). Ischaemic preconditioning has been shown to be a potent endogenous protective mechanism to reduce infarct size during ischaemia/reperfusion.

Myocardial ischaemia/reperfusion injury-induced tissue loss has long been considered to only involve necrosis of myocardial cells. There is however growing evidence that apoptosis also makes an important contribution to ischaemia/reperfusion damage (Bardales et al 1996, Colucci et al 1996, Haunstetter et al 1998, Itoh et al 1995, Saraste et al 1997, Yeh et al 1997). In fact, Anversa et al (1998) has shown that apoptosis is the predominant mode of cell death in ischaemia/reperfusion. A critical role for apoptosis has also been suggested in several other cardiovascular diseases e.g., in myocardial ischaemia/reperfusion both in vitro (Umansky et al 1995) and in vivo (Gottlieb et al 1994), acute myocardial infarction (Olivetti et al 1996, Saratse et al 1997), myocarditis (Bachmaier et al 1997), heart failure (Bing et al 1994, Narula et al 1996, Sharov et al 1996), and ischaemic cardiomyopathy (Olivetti et al 1997).

Much is known concerning the signal transduction pathways involved in ischaemic preconditioning with infarct size as end-point. With the emergence of an increased understanding of the important role of apoptosis in cell death during ischaemia/reperfusion, it has become important to elucidate the role of these signaling pathways in apoptosis in order to develop new therapeutic strategies.

This review of the literature will summarize the known data concerning (i) the biological phenomenon of preconditioning, (ii) the signal transduction pathways involved in eliciting and mediating the protection, (iii) the ability of preconditioning to protect against apoptosis and (iv) the signal transduction pathways implied in the protection against apoptosis.

## **1.2 Preconditioning (PC)**

Preconditioning is the phenomenon whereby exposure to one or more brief episodes of ischaemia renders the heart more resistant to a subsequent sustained period of ischaemia. This phenomenon known as ischaemic preconditioning (IPC), was first described by Murry et al (1986) in the canine heart where it protected against the damaging consequences of ischaemia/reperfusion (I/R) injuries. Subsequently this phenomenon has been shown in all animal species studied (Barbosa et al 1996, Li et al 1992a, Liu Y et al 1992, Schott et al 1990, Wolfe et al 1993,) including humans (Deutsch et al 1990, Kloner et al 1994, Ottani et al 1995, Yellon et al 1993, Yellon et al 2000). The protection of IPC is manifested on different end-points: functional recovery, arrhythmias and the most widely studied, necrosis. Recently the protection of IPC has also been shown in apoptosis (see later).

There is a general agreement that other than expedited reperfusion, preconditioning is the strongest form of in vivo protection against myocardial ischaemic injury (National Heart, Lung and Blood Institute (NHLBI) workshop- Kloner et al 1998).

The phenomenon described by Murry et al is now referred to as “classical” or “early” ischaemic preconditioning which, while powerful, lasts only 1-2 hours (Bolli et al 2000). A second phase of preconditioning, called the Second Window Of Preconditioning (SWOP), appears 12-24 hours after preconditioning and although less potent, can last for up to 3 days (Bolli et al 2000).

### **1.2.1 Triggers and mediators of preconditioning**

In addition to brief episodes of ischaemia-reperfusion a variety of preconditioning stimuli such as hypoxia (Cohen et al 1995), rapid cardiac pacing (Vegh et al 1991, Koning et al 1996), thermal stress (Cumming et al 1996), stretch (Gysembergh et al 1998, Ovize et al 1994), and pharmacological agents (Yellon et al 1998, Cohen et al 2000) are known. These triggers put into motion a series of events that eventually mediate the cardioprotection characteristic of PC. In other words, the myocardium “remembers” that it has been preconditioned. Unravelling the mechanism(s) of this “memory” remains to be established.

Triggers can be tested experimentally by administering the agent to the heart for a limited period followed by a wash-out period before sustained ischaemia, while mediators of protection are evaluated by administration just prior to the sustained ischaemia (without the washout period). Norepinephrine, endothelin, and angiotensin are examples of neurohumoral agonists that can precondition the heart when

administered exogenously but which are not released in sufficient quantities by the ischaemic myocardium to trigger protection endogenously (Schulz et al 2001b).

The term pharmacological preconditioning (Cohen et al 1993, Jennings et al 1996) refers to the condition where pharmacological agents given before coronary occlusion reduces myocardial infarct size by stimulating second messenger pathways thought to be involved in preconditioning. Examples of this kind of preconditioning include administration of adenosine  $A_1$  agonists,  $K_{ATP}$  channel openers, and  $\beta$ -adrenergic receptor agonists. These agents are referred to as “preconditioning mimetics” (Kloner et al 1994).

### **1.2.2 Signal transduction in ischaemic preconditioning**

Endogenous triggers eliciting ischaemic preconditioning may be either receptor-dependent or receptor-independent. Downey and colleagues (Cohen et al 1996, Goto et al 1995, Liu et al 1991, Ytrehus et al 1994) have proposed that in the rabbit, the mechanism of preconditioning involves the stimulation and action of the receptor-dependent endogenous triggers. These include amongst others adenosine, bradykinin, opioids, endogenous catecholamines, prostaglandins, angiotensin, and endothelin. The roles of these triggers were evaluated by using either specific agonists or antagonists of the particular receptor being studied.

### 1.2.2.1 Receptor-dependent triggers

#### 1.2.2.1.1 Adenosine

The first signal transduction element to be identified as part of preconditioning's mechanism was the adenosine receptor (Liu et al 1991). An increase in the interstitial adenosine concentration during the preconditioning ischaemia occurs in rats (Kuzmin et al 2000), rabbits (Lasley et al 1995, Miura 1996, Mortimer et al 2000), dogs (Mei et al 1998), and pigs (Martin et al 1997, Schulz et al 1995, Wikström et al 1995).

Adenosine receptors exist as at least four different subtypes: A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, A<sub>3</sub> (Aucampach et al 1999, Feoktistov et al 1997, Olah et al 1995). Stimulation of the A<sub>1</sub> receptor with N<sup>6</sup>- (phenyl-2R-isopropyl)-adenosine (PIA) or 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA) mimicked IPC in the rabbit, whilst stimulation of the A<sub>2</sub> receptor with CGS 21688, had no protective effect (Thornton et al 1992). Stimulation of the A<sub>3</sub> receptor with N<sup>6</sup>- [2-(4-aminophenyl) ethyl] adenosine (APNEA) was also able to induce protection (Liu GS et al 1994). Therefore, at least in the rabbit, both the A<sub>1</sub> and A<sub>3</sub> receptors are thought to be involved in triggering cardioprotection. In the review by Ganote et al (2000) growing evidence suggests that the A<sub>1</sub> receptor is not required for IPC in the rat heart. This, coupled with a low tissue density of A<sub>3</sub>-receptors, coupled with adenosine's very low A<sub>3</sub> ligand affinity in rats (Van Wylen 1994) may limit the ability of adenosine to precondition via this receptor in this particular animal.

#### 1.2.2.1.2 Bradykinin

Stimulation of bradykinin B<sub>2</sub> receptors results in enhanced formation of prostacyclin and nitric oxide (NO) in cultured human and bovine endothelial cells (Linz et al 1992). Both prostaglandins (Farber et al 1988, Farber et al 1990) and NO (Gelvan et al 1991) have been demonstrated to attenuate ischaemia/reperfusion injury. Bradykinin also acts as a trigger in ischaemic preconditioning (Goto et al 1995, Wall et al 1994). From their experiments in rabbits, Goto et al (1995) found that infusion of the selective bradykinin B<sub>2</sub> receptor antagonist HOE 140 before a single 5 min ischaemic event completely blocked protection in anaesthetised rabbits. Conversely, brief infusion of bradykinin reduced infarction. The response to bradykinin was similar to that of adenosine. Presumably the ischaemic heart releases both adenosine and bradykinin in amounts that are ineffective alone, but when combined are able to reach the threshold for cardioprotection (Cohen et al 2000). It was therefore proposed that additive interactions of triggers contribute to the initiation of ischaemic preconditioning.

#### 1.2.2.1.3 Opioids

Both adenosine and the release of endogenous opioids appear to trigger ischaemic preconditioning in an interactive fashion in several species (Fryer et al 1999b, Schulz et al 1995, Schultz et al 1997). It has been found that naloxone, an opioid antagonist, can block the PC protection in rats (Fryer et al 1999b, Schultz et al 1995, Schultz et al 1997), rabbits (Chien et al 1999, Miki et al 1998), and pigs (Schulz et al 2001a)

suggesting a trigger action. It has been shown by Aitchinson et al (2000) that pharmacological preconditioning with DADLE [D-ala (2), D-leu(5)] enkephalin] (10 nmol/L), a synthetic delta-opioid receptor agonist, resulted in similar infarct size reduction as was seen with IPC (2 cycles of 5 min global ischaemia).

Furthermore, since opiates are known to have anti-adrenergic effects, which hypothetically may help to mediate IPC, cyclic AMP levels were measured in DADLE-treated hearts. Decreased levels of cyclic AMP were found at the start of the regional ischaemic period ( $p < 0.001$  vs controls). This group thus suggested that delta-opioid receptor activity mediated protection may be associated with a reduction of tissue cyclic AMP levels. Recently Genade et al (2001) also showed that DADLE elicited cardioprotection in rats. In contrast to Aitchison et al (2000), DADLE administered as a trigger i.e., 3 x 5 minutes and washed out before sustained ischaemia had no effect. However, if administered 10 minutes before sustained ischaemia, it increased functional recovery during reperfusion, suggesting a mediator effect for opioids. The differences in experimental findings were attributed to the use of different models (regional ischaemia in the work by Aitchison et al, working heart model in the case of Genade et al).

#### 1.2.2.1.4 Endogenous catecholamines and prostaglandins

Asimakis et al (1994) were the first to show that preconditioning with noradrenaline (an  $\alpha$  and  $\beta$ -adrenergic receptor agonist) and isoproterenol (a  $\beta$ -adrenergic receptor agonist) afforded significant protection in the isolated rat heart. They also showed that propranolol, a non-selective  $\beta$ -adrenergic receptor blocker, prevented the

cardioprotective effects of both these agents. This was confirmed by Miyawaki et al (1997a) who showed that isoproterenol provided significant protection against ischaemic myocardial injury by increasing functional recovery and tissue ATP together with a decrease in lactate dehydrogenase (LDH) release in isolated rat hearts. Yabe et al (1998) also showed that preconditioning elicited by  $\beta$ -adrenergic receptor stimulation with isoproterenol increased functional recovery and decreased creatine kinase release during sustained ischaemia. Furthermore, since cardioprotection was abolished by the treatment of polymixin, a protein kinase C (PKC) inhibitor, they suggested that  $\beta$ -adrenergic receptor stimulated preconditioning was PKC mediated. Lochner et al (1999) have provided proof that the protection elicited by a multi-cycle PC protocol may be partially dependent on the activation of the  $\beta$ -adrenergic signaling pathway, since  $\beta$ -adrenergic receptor stimulated preconditioning could be partially blocked by alprenolol, a  $\beta$ -adrenergic receptor blocker.

Blockade of cyclooxygenase by aspirin in rats (Li et al 1992b) or rabbits (Liu GS et al 1992) did not interfere with the infarct size reduction achieved by IPC, suggesting that endogenous prostaglandins were not involved in preconditioning's protection.

#### 1.2.2.2 Receptor-independent triggers

These include (i) free radicals, (ii) nitric oxide (NO), and (iii) calcium.

#### 1.2.2.2.1 Free radicals

Mitochondrial respiration and oxidative phosphorylation are gradually uncoupled during hypoxia or ischaemia/reperfusion (Lesnefsky et al 1997, Van den Hoek et al 1998). An immediate consequence of such gradual impairment of respiratory function is the increase in the production of reactive oxygen species and free radicals in the mitochondria (Van den Hoek et al 1998), and at high concentrations these alter the integrity and increase the fluidity and permeability of cell membranes. Free radicals can act as triggers of ischaemic preconditioning.

Infusion of N-2-mercaptpropionyl glycine (MPG), a cell-permeable radical scavenger, completely abolished protection by IPC in rabbit hearts (Baines et al 1997). The mechanism by which oxygen radicals precondition the myocardium is less clear, but free radicals can activate G-proteins (Nishida et al 2000), protein kinases (Das et al 1999b), and ATP-dependent potassium channels (Bhatnagar et al 1990, Jabr et al 1993, Tokube et al 1996).

In a review by Das et al (1999a), a vital role for the reactive oxygen species generated during preconditioning in mediating cellular responses through diverse intracellular and extracellular ligands was suggested. The following sequence of events was proposed: Stimulation of protein phosphorylation by oxygen free radicals appears to be a feasible mechanism for subsequent signal transduction leading to the activation of the transcription factor NF $\kappa$ B. Activation of NF $\kappa$ B seems to be regulated by the mitogen-activated protein kinases (MAPK). However, the exact mechanism whereby oxygen radicals participate in signal transduction pathways is not yet clear.

#### 1.2.2.2.2 Nitric Oxide (NO)

Nakano et al (2000a) and Post et al (2000) in rabbits and pigs respectively, have provided evidence that NO is neither a trigger nor a mediator of the early phase of ischaemic preconditioning against infarction. NO does however play an important role in the second window of preconditioning (see later). Lochner et al (2000) however, found evidence for a role for NO as a trigger in early/classical preconditioning in rat hearts. In the latter work, pharmacological elevation of tissue cGMP levels by the NO donors S-nitroso-N-pencillamine (SNAP) or sodium nitroprusside (SNP) before sustained ischaemia i.e., as a trigger, elicited functional improvement during reperfusion comparable to that achieved by PC. Administration of the NO synthase inhibitors e.g., (N(omega)-nitro-L-arginine methyl ester or N-nitro-L-arginine) before and during the PC protocol partially attenuated functional recovery, while they had no effect when given after the ischaemic PC protocol or before sustained ischaemia only. Therefore, it was concluded by this group that NO released during an ischaemic preconditioning protocol acted as a trigger of classic preconditioning.

#### 1.2.2.2.3 Calcium

Ashraf et al (1994) showed that calcium preconditioning, i.e., repetitive calcium depletion and repletion for short durations, conferred cardioprotection against ischaemia/reperfusion injury via the activation of PKC (Miyawaki et al 1996). Miyawaki et al (1997b) showed, in Langendorff-perfused rat hearts, that a transient increase in intracellular free calcium during IPC was an important trigger for the activation of PKC, which was responsible for cardioprotection. The method employed

to raise the intracellular calcium as a trigger was called high- $\text{Ca}^{2+}$  preconditioning (HCPC) i.e., 5 min perfusion of higher  $\text{Ca}^{2+}$  perfusate (2.3 mM  $\text{Ca}^{2+}$ ) followed by 10 min perfusion of normal perfusate (1.8 mM  $\text{Ca}^{2+}$ ). The IPC protocol consisted of a single cycle of 5 min ischaemia and 10 minutes reperfusion. This was followed by exposure to 40 min global ischaemia and 30 min reperfusion.

Furthermore the administration of chelerythrine, a PKC inhibitor, for 10 min before and after the preconditioning ischaemia completely abolished the cardioprotection induced by both IPC and HCPC. A significant increase in functional recovery and a decrease in lactate dehydrogenase release in both IPC and HCPC hearts compared with the Non-PC hearts were observed. This group concluded that a transient increase in intracellular calcium during IPC was an important trigger for the activation of PKC, which was responsible for cardioprotection. Work done by Cain et al (2000) suggested that the flux of calcium through the L-type calcium channels acted as a trigger of IPC, since atrial muscle taken from patients with chronic blockade of L-type calcium channels could not be preconditioned by ischaemia (Cain et al 2000). However, the calcium antagonist nisoldipine does not decrease IPC in an in situ model of regional ischaemia in anaesthetised pigs, thereby challenging a major role for endogenous calcium as a trigger of ischaemic preconditioning in vivo (Wallbridge et al 1996). The role of endogenous calcium as a trigger of ischaemic preconditioning requires further study.

### 1.2.3 Signal transduction pathways

#### 1.2.3.1 G proteins

Guanine nucleotide-binding regulatory proteins (G proteins) play a major role in the regulation of a number of physiological processes. Myocardial ischaemia could induce the changes in the receptor-G protein signal transduction system in the heart. The activity of the Gs protein has been found to decrease with ischaemia and reperfusion (Ohyanagi et al 1996).

Ravingerova et al (1995) showed, in isolated Langendorff-perfused rat hearts, that PC led to a reduction of stimulatory Gs proteins, whereas inhibitory Gi proteins were increased. Furthermore, Thornton et al (1993b) found that chronic treatment with pertussis toxin blocks Gi proteins and abolishes preconditioning's protection in the rabbit. Adenosine (Downey et al 1993) and opioid receptors (Schultz et al 1998) are coupled to Gi proteins. The adenosine receptor is coupled through Gi to, amongst others, phospholipases C (PLC) and D (PLD). PLC is responsible for the formation of diacylglycerol (DAG), which in turns stimulates the translocation and activation of PKC. Increased PLD activity accompanies preconditioning and PLD blockade with high-dose propranolol attenuates its protection (Cohen et al 1996). Thus both pathways are thought to be critical components of PKC activation which is involved in PC.

### 1.2.3.2 Protein kinase C (PKC)

The activation of PKC is regarded as very important in ischaemic preconditioning (Speechly-Dick 1994, Ytrehus et al 1994). PKC catalyses the transfer of the  $\gamma$ -terminal phosphate from ATP to the hydroxyl group of serine and/or threonine residues in various protein substrates and this process is considered to be a fundamental regulatory mechanism involved in cellular growth, differentiation and immediate regulation of effector functions (Sugden et al 1995). The PKC family consists of 12 serine/threonine kinases and can be classified into three distinctive subfamilies. The subfamily of classical PKC isoforms includes PKC $\alpha$ ,  $\beta_1$ ,  $\beta_2$  and  $\gamma$  and requires both calcium ions and lipids (i.e., phosphatidylserine, PMA and/or diacylglycerol (DAG)) for their activation.

The subfamily of novel PKC isoforms which includes PKC $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\phi$  and  $\mu$  do not require calcium but still require lipids for their activation. The subfamily of atypical PKC isoforms includes PKC $\zeta$ ,  $\iota$  and  $\lambda$  requires phosphatidylserine for its activity, but neither calcium, nor DAG and/or PMA are required for its activation. In adult rat hearts, novel PKC $\epsilon$  and  $\delta$  are the two major isoforms while the minor PKC isoforms are represented by novel PKC $\eta$  and atypical PKC $\iota$  and  $\zeta$  (Bogoyevitch et al 1993, Mitchell et al 1995).

PKC activation and subsequent translocation to membrane compartments is a key event in the PKC-dependent signaling cascade and is preceded by agonist-mediated stimulation of receptor-associated phospholipase C (PLC). PLC, upon stimulation, cleaves inositol-1,4,5-triphosphate and DAG from the membrane-bound phosphatidylinositol moiety. DAG acts as a hydrophobic factor and increases the

affinity of PKC to the membrane-integrated structures within the subcellular compartments (Newton 1995).

PKC has been implicated as a key signal transduction step in one of the most popular hypotheses (the so called 'PKC hypothesis' Simkhovich et al (1998)) which requires the translocation of PKC from the cytosol to the sarcolemma and subsequent phosphorylation of an as-yet unknown protein target as key events in cardioprotection. Speechly-Dick and colleagues (1994) showed that in the in vivo ischaemia model of the rat heart, the PKC inhibitor chelerythrine, when administered after a preconditioning stimulus (i.e., prior to sustained ischaemia), abolished protection conferred by IPC and caused an increase in infarct size.

Li et al (1995) found that in the rat heart the PKC inhibitor, calphostin C, also abolished the protective effects when administered before the IPC protocol and before the onset of sustained coronary occlusion. These studies may be considered as positive or consistent with the 'PKC hypothesis' of preconditioning. In the isolated rat heart model of regional ischaemia, confirmatory results have been reported by Bugge and colleagues (1995) who found that the PKC antagonists polymyxin B and chelerythrine blocked the protective effects of IPC. There appears to be data obtained in the isolated rat heart subjected to sustained ischaemia and reperfusion supporting the PKC hypothesis. Mitchell et al (1995), suggested that PKC activation is central to protection by IPC by showing that PKC inhibitors, chelerythrine and staurosporine, block the protection of IPC when administered before the IPC protocol and washed out before sustained ischaemia. A study performed by Hu and colleagues (1995) showed that pre-treatment with PKC inhibitors H-7 and

bisindolylmaleimide blocked IPC while the PKC activator, PMA, mimicked its protective effect.

While the studies mentioned above are considered positive, there are also negative studies using the isolated rat heart model. Galinanes et al (1995), using immunoblotting, were not able to demonstrate translocation of PKC $\delta$  and PKC $\epsilon$  in the isolated rat heart preconditioned with two brief episodes of ischaemia and reperfusion. Moolman et al (1996a) reported that PKC inhibitors, chelerythrine and bisindolylmaleimide, given either during the IPC protocol or before the onset of ischaemia, did not abolish the protective effect of IPC in the globally ischaemic rat heart. Moreover, the PKC inhibitors were also unable to block the reduction in lactate accumulation caused by IPC. Thus the conclusion reached by these authors is that PKC may not be involved in the mechanism of IPC in the rat.

The protective phenomenon of IPC has been unequivocally demonstrated in rats, rabbits, dogs and pigs. In contrast, the role of PKC in this protection is controversial in all of these species. It is important to remember that most of the results in support of the PKC hypothesis were obtained using pharmacological approaches i.e., by administration of PKC inhibitors and/or activators. The disadvantages of this strategy (see review by Brooks et al 1996) include amongst other things, that almost every inhibitor has ancillary effects and, for some, their specificity and selectivity are questionable. Unfortunately, the same objection is also applicable to all currently available inhibitors.

Recently the isoforms of PKC have been receiving more attention. Several isoforms of PKC i.e., epsilon, delta, and eta have been suggested as mediators of preconditioning (Kawamura et al 1998, Ping et al 1997). Activation of PKC epsilon has been shown to play a critical role in preconditioning to limit necrosis in cultured neonatal rat ventricular myocytes (Gray et al 1997) and in anaesthetised rats (Mitchell et al 1995, Mochly-Rosen et al 2000). Ping et al (1997) also showed that the activation and translocation of PKC epsilon isoform-mediated preconditioning reduces cardiomyocyte necrosis in conscious rabbits. Lui et al (2001) found that PKC epsilon was selectively activated in IPC and suggested that it was this isoform that attenuates necrosis and apoptosis in isolated cultured chick cardiomyocytes.

The emergence of a role for certain PKC isoforms in preconditioning offers more selective avenues to explore the role of PKC within a particular species and model. It also gives rise to a need for biochemical isoform-specific assays, which would allow quantification of selective PKC isoforms in different subcellular compartments. These assays would also shed more light on the differences in isoform activity and subcellular distribution within different species, models and protocols and the role they play in cardioprotection elicited by preconditioning.

#### 1.2.3.3 cAMP

As discussed above, Asimakis et al (1994) and Miyawaki et al (1997b) earlier showed that  $\beta$ -adrenergic receptor stimulation could elicit pharmacological preconditioning. The signal transduction pathway of  $\beta$ -adrenergic receptor activation is known, namely that stimulation of the  $\beta$ -adrenergic receptor, coupled to  $G_s$  protein, by isoproterenol

activates  $G_s\alpha$  to stimulate the production of cAMP via activation of adenylate cyclase (Fleming et al 1992) which in turn activates cAMP to activate PKA (Campbell et al 1995). The question is what the relative role of each of the components of the  $\beta$ -adrenergic receptor signal transduction pathway is in eliciting protection. Furthermore, it was not known whether the  $\beta$ -adrenergic receptor pathway was involved in ischaemic preconditioning.

Ischaemia releases catecholamines (specifically noradrenaline) from the nerve terminals of the sympathetic nervous system in the myocardium (Schömig et al 1984). Upon its release, noradrenaline could activate the  $\alpha_1$ -adrenergic receptor, which is coupled to the PKC pathway. The involvement of PKC activation in IPC was discussed earlier. Lochner et al (1999) investigated the role of endogenous release of catecholamines and activation of the  $\beta$ -adrenergic receptor in IPC. First they showed that a multi-cycle IPC protocol is characterised by cyclic increases in tissue cAMP levels. By using reserpine (injected intraperitoneally), that depletes endogenous catecholamine stores, they were able to abolish cAMP generation during the IPC protocol. These findings suggest that the release of endogenous catecholamines is mainly responsible for the cyclic increases in tissue cAMP and thus PKA activation during preconditioning.

The question whether this endogenous activation of the  $\beta$ -adrenergic receptor and concomitant increase in cAMP played a role in the triggering of PC was then investigated. The same group (Lochner et al 2000) found that administration of the  $\beta$ -adrenergic receptor alprenolol during the triggering phase partially abolished the protection of a multi-cycle (3 x 5 min ischaemia) preconditioning protocol, thereby

proving the involvement of  $\beta$ -adrenergic stimulation in the triggering pathway of ischaemic preconditioning.

It was also observed by Moolman et al (1995) and Sandhu et al (1997) that preconditioned hearts had a significantly lower cAMP content at the end of sustained ischaemia. The next question was whether this had mechanistic importance. Sandhu et al (1996) found that infusion of NKH477 (which increases cAMP) immediately prior to sustained ischaemia did not have an effect on infarct size in multi-cycle preconditioning, but partially abolished the protection of a single cycle ischaemic preconditioning. Moolman et al (1996b) also could not demonstrate any reduction in functional recovery when attempting to increase cAMP in preconditioned hearts by perfusion with forskolin immediately prior to sustained ischaemia.

These experimental findings indicate that the activation of the  $\beta$ -adrenergic receptor during the trigger phase of IPC is important for the mechanism of preconditioning, but that cAMP has a role as mediator only under specific experimental conditions. The role of PKA in this cascade is not yet clear.

#### 1.2.3.4 Tyrosine kinases

The potential role of protein tyrosine kinases in PC were first suggested by Maulik et al (1996), who showed that IPC induced an alteration in the tyrosine phosphorylation profile of several cardiac proteins. The receptor-dependent endogenous triggers, such as adenosine, bradykinin, and endogenous opioids all couple through phospholipases to activate protein kinase C, which in turn activates a tyrosine kinase (Downey et al 1997). Baines et al (1998) have also suggested that the tyrosine

kinase is downstream of PKC, but in addition, that the tyrosine kinase may be a MAP kinase kinase. Fryer et al (1998) suggested that activation of a tyrosine kinase is involved as a trigger and not as a mediator of IPC in the rat myocardium as assessed by infarct size. Further work by this group (Fryer et al 1999a) suggested that the efficacy of tyrosine kinase and PKC inhibition to block IPC depends on the intensity of the preconditioning stimulus and that these kinases may work through parallel pathways.

Kitakaze et al (2000) however by using genistein, a tyrosine kinase inhibitor, have shown that tyrosine kinase does not trigger the infarct size-limiting effect of the early phase of IPC in canine hearts. Conflicting data and various pathways make the role of tyrosine kinases in preconditioning unclear. Further investigation is required to fully understand its role in preconditioning.

#### 1.2.3.5 Mitogen-activated protein kinases (MAPK)

Potential downstream targets of PKC, PKA, and tyrosine kinases are the mitogen-activated protein kinases (MAPK). Three major divisions of the MAP kinase family exist in the heart: extracellular signal-regulated kinases (ERK's); c-Jun N-terminal kinases (JNK's), and p38 MAPK's (Sugden et al 1998). MAPK's are considered to be primary mediators of the myocyte stress response (Sugden et al 1998), thus implicating a role for MAP kinases in IPC. Available data suggest a role for all these MAP kinases in preconditioning (see below).

#### 1.2.3.5.1 ERK

The role of ERK in the stress response to ischaemia and in PC is still under investigation. Ping et al (1999a) showed in conscious rabbits that ischaemic preconditioning induces a rapid activation of ERK1 and ERK2. An inhibitor of ERK (PD 98059) when administered during ischaemia i.e., as a mediator, fails to dose-dependently block the infarct size reduction seen after IPC in the isolated rabbit heart (Kim et al 1999), and its intramyocardial infusion abolishes IPC's protection in pigs in vivo (Ströhm et al 2000). Mocanu and colleagues (2002) showed, in the isolated perfused rat heart, that when PD 98059 was administered during a multi-cycle IPC protocol, it had no significant effect upon infarct size reduction when compared to the IPC hearts, thus suggesting no role for ERK as a trigger.

The cytoprotective property of ERK was also demonstrated outside the field of ischaemic preconditioning. Baxter et al (2001) examined the effects of transforming growth factor-beta 1 (TGF-beta 1) on reperfusion injury and assessed the role of ERK1 and ERK2 in rat ventricular myocytes subjected to hypoxia and reoxygenation. Incubation with TGF-beta 1 transiently activated ERK1 and ERK2 in normoxic myocytes. Intact hearts that received TGF-beta 1 during reperfusion showed a significantly smaller infarct size ( $p < 0.01$ ). PD 98059 abrogated this protective effect and these studies suggest that TGF-beta 1 decreases cardiac myocyte apoptosis during early reperfusion and limits infarct size through ERK1 and ERK2 activation. This has led to the formulation of the so-called "growth factor hypothesis of cardiac protection", which states that growth factors acting through ERK activation serve as general protective agents against myocardial stress.

#### 1.2.3.5.2 JNK

The JNK family consists of at least 2 isoforms, the 46-kDa JNK1 and the 54-kDa JNK2, both of which are present in the heart and are strongly activated during reperfusion following ischaemia (Clerk et al 1998). Ping et al (1999a) showed that JNK1 and JNK2 activities are significantly increased after the IPC protocol.

JNK1 activation during no-flow ischaemia is most likely mediated by PKC, since activation of JNK1 is completely inhibited by chelerythrine, a PKC inhibitor (Ping et al 1999a). In isolated rat hearts, inhibition of JNK2 with curcumin abolishes the infarct size reduction of IPC to a similar extent as blockade of p38 MAPK using SB 203580 (both agents were administered before the IPC protocol) (Sato et al 2000). This would suggest that JNK has a role to play in PC. Further experimental evidence to support such a triggering role for JNK is however still lacking at this stage.

#### 1.2.3.5.3 p38 MAPK

The role of the p38 MAPK signalling pathway has been extensively investigated but remains controversial. Whether an IPC protocol causes p38 MAPK activation, and whether inhibition of p38 MAPK blocks the cardioprotective effects of PC, lie at the heart of the controversy. At least 4 isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) of p38 MAPK have been identified (Sugden et al 1998) and it is known that different isoforms of p38 MAPK mediate different biological functions (Michel et al 2001). However it would appear that only the p38 $\alpha$  and p38 $\beta$  isoforms are expressed to any degree in the heart (Sugden et al 1998). In neonatal rat cardiomyocytes, p38 $\alpha$  mediates apoptosis

whereas p38 $\beta$  is anti-apoptotic (Wang et al 1998). These different isoforms with different actions may contribute to the controversies surrounding the role of p38 MAPK.

Weinbrenner et al (1997) showed that a short period of ischaemia (5 minutes) did not activate p38 MAPK in rabbit heart. In contrast, others (Bolli et al 1988, Marais et al 2001a, Przyklenk et al 1988) have found that ischaemia causes p38 MAPK activation within 5 minutes. However, its activation during a multi-cycle PC protocol is transient and wanes after exposure to 3 episodes of ischaemia. This does not preclude a role as a trigger, since its activation may set into motion a series of signal transduction events that may eventually lead to protection.

SB 203580, a recently developed selective inhibitor of p38 MAPK (Cuenda et al 1995) has been used extensively. Unfortunately the results obtained have been confusing, partly due to the fact that its effect is dose-dependent – at doses below 10  $\mu$ M it selectively inhibits p38 MAPK, but at higher doses, it also inhibits JNKs.

#### 1.2.3.5.3.1 p38 MAPK as a mediator of protection

Armstrong et al (1999) reported in isolated rabbit cardiomyocytes that SB 203580 promoted injury in non-preconditioned cells. SB 203580 did not affect infarct size in isolated rat hearts (Mocanu et al 2000) or rabbit hearts (Nakano et al 2000b). In contrast, data from our own laboratory shows that administration of SB 203580 protected the heart against damage (Marais et al 2001a).

#### 1.2.3.5.3.2 p38 MAPK as a trigger of protection

When SB 203580 was administered before the IPC protocol, it blocked the protection of IPC observed as an abolishment of reduced fragility (Weinbrenner et al 1997) in isolated rabbit cardiomyocytes. Similarly, Mocanu et al (2000) showed in isolated rabbit hearts that SB 203580 completely blocked the protection afforded by preconditioning. In contrast, Sanada et al (2001) demonstrated a reduction in infarct size in dog hearts, while Schneider et al (2001) (in isolated rat hearts) and Barancik et al (2000) (using pig hearts in vivo) demonstrated that p38 MAPK inhibition did not abolish the infarct size reduction of IPC, and even reduced infarct per se.

Reasons for conflicting results may be due to differing experimental models and animal species. These range from isolated perfused rabbit hearts (Nakano et al 2000c), rat heart (Maulik et al 1998, Schneider et al 1999), and pig hearts (Barancik et al 2000, Behrends et al 2000), to cultured cardiac cells (Armstrong et al 1999, Saurin et al 1999) and biopsy tissue samples from human hearts. It could also be due to the differences in p38 MAPK isoforms and also the selectivity of inhibitors for these isoforms. Different protocols, not distinguishing between triggers and mediators add to the confusion.

Further downstream events from p38 MAPK have received little attention thus far, except for heat shock protein 27 (HSP27). The primary substrate of p38 MAPK is MAPK-activated protein kinase 2 (MAPKAPK-2) which phosphorylates the small (27-kDa) heat shock protein HSP27 (Freshney et al 1994), an important regulator of actin dynamics. The phosphorylated HSP27 promotes polymerisation of actin filaments,

thus increasing the stability of the cytoskeleton (Guay et al 1997). Over expression of HSP27 in isolated rat myocytes confers protection against simulated ischaemia, whereas depletion exacerbates injury (Martin et al 1997). Because prolonged ischaemia is known to cause cytoskeletal disruption (Ganote et al 1993b), activation of the p38 MAPK/HSP27 pathway could well contribute to the protective action of ischaemic preconditioning by maintaining the integrity of the actin cytoskeleton.

#### **1.2.4 Potential end-effectors of ischaemic preconditioning**

These include energy and substrate metabolism, protein synthesis, the sodium-proton exchanger, the cytoskeleton, volume regulation, tumour necrosis factor (TNF)- $\alpha$ , and the most popular one, the  $K_{ATP}$  channels (Schulz et al 2001b). In the following section the focus will be on the  $K_{ATP}$  channels.

##### **1.2.4.1 $K_{ATP}$ channels**

The nature of the end-effector(s) ultimately activated by ischaemic preconditioning has been very elusive. Considerable evidence suggests that opening of  $K_{ATP}$  channels represents the final step in protective signal transduction processes. The  $K_{ATP}$  channel is composed of two subunits: the channel protein itself, which belongs to the inward-rectifier  $K^+$  channel family, and the sulfonylurea receptor, a member of the ATP-binding cassette superfamily, which is involved in the binding of ATP and sulfonyl compounds (Trapp et al 1997, Aguilar-Bryan et al 1998). Both subunits are absolutely required for channel activity and are associated with a 1:1 stoichiometry.

It is important to point out that there two kinds of  $K_{ATP}$  channels i.e., sarcolemmal and mitochondrial. The initial studies focussed on sarcolemmal  $K_{ATP}$  channels.

Much of the evidence supporting the  $K_{ATP}$  hypothesis is based on the blockade of the protective effects of both IPC and pharmacological PC by inhibitors of the  $K_{ATP}$  channels (Grover et al 1997). Auchampach and colleagues (1992), and Gross and colleagues (1992) have shown that the  $K_{ATP}$  channel is important for preconditioning in the canine model. Intravenous glibenclamide, a  $K_{ATP}$  channel antagonist, abolished the protective effect afforded by preconditioning in reducing infarct size in the canine model (Gross et al 1992). However, there have also been discrepancies in the results obtained. In studies with dog hearts (Baxter et al 1997, Yao et al 1993), pre-treatment with diazoxide, a mitochondrial  $K_{ATP}$  channel agonist protected the myocardium from ischaemia, while in another study in rabbits, diazoxide failed to reduce the infarct size (Baxter et al 1997). In addition, Grover et al (1995) and Yao et al (1994) found that direct activation of the  $K_{ATP}$  channels with bimakalim and cromakalin respectively before ischaemia reduced infarct size. In rabbit hearts, isolated rabbit cardiomyocytes and rats, the importance of  $K_{ATP}$  channels in preconditioning has not been confirmed (Liu Y et al 1992, Thornton et al 1993a). To explain the salutatory effect of these  $K_{ATP}$  channels, it was originally proposed that opening of the sarcolemmal channels caused shortening of the myocardial action potential duration, which could in turn exert a cardioplegic action (Grover 1997).

The discovery that  $K_{ATP}$  channels are present in mitochondrial membranes (Garlid et al 1996, Inoue et al 1991) opened a debate as to the relative contribution of sarcolemmal vs mitochondrial  $K_{ATP}$  ( $mK_{ATP}$ ) channels in the myocardial protection

afforded by PC (Baines et al 1999, Gross et al 1999). Recent evidence, however, suggests that the mitochondrial  $K_{ATP}$  channel, rather than its sarcolemmal counterpart, mediate protection. Garlid et al (1997) demonstrated that diazoxide was 2000-fold more selective for opening mitochondrial channels than sarcolemmal  $K_{ATP}$  channels in the heart. Furthermore, 5-hydroxydecanoate (5-HD), a specific inhibitor of  $mK_{ATP}$  channel, inhibited the protection afforded with diazoxide. A similar protective effect with diazoxide, and reversal with 5-HD, was found in a myocyte model of hypoxic cell injury (Liu et al 1998). Diazoxide was subsequently used extensively in studies to elucidate the significance of the  $mK_{ATP}$  channel in preconditioning.

Part of unravelling the PC phenomenon is to establish how preceding signal transduction pathways can be linked to the  $K_{ATP}$  channels. Many studies suggest a role for PKC: Armstrong et al (1995) observed in isolated rabbit cardiomyocytes that the phorbol ester PMA potentiates the opening of the  $K_{ATP}$  channels by pinacidil when ATP levels are reduced by metabolic inhibition with potassium cyanide. They also reported that adenosine has little effect on myocytes in the basal state but it opens  $K_{ATP}$  channels after pre-treatment with PMA, which suggests that PKC participates in opening of the channels. Therefore, opening of the  $K_{ATP}$  channels during preconditioning may require both the phosphorylation by PKC or another kinase and the presence of adenosine or another G protein-coupled agonist. Kirsch et al (1990) reported that  $G_i$  released by stimulated adenosine  $A_1$  receptors opens  $K_{ATP}$  channels directly. In that study, other G protein subunits also affected  $K_{ATP}$  channel conductance. The direct gating of a  $K_{ATP}$  channel has previously been demonstrated in heart tissue (Ito et al 1992) and may contribute to the mechanism of protection.

It is not clear why opening of the ATP-sensitive K<sup>+</sup> channels is cardioprotective. In addition it is still not conclusively proven that the K<sub>ATP</sub> channel is indeed the end effector. Further investigation should therefore continue until the end effector has been identified.

### **1.2.5 Second Window of Preconditioning (SWOP)**

Ischaemic preconditioning has been found to be biphasic, with an early or classical phase and a second window of preconditioning (SWOP), or late/delayed phase of protection. The early phase of PC develops from the initial ischaemic insults and is relatively short-lived (2-3 hours), while SWOP appears 12-24 hours later and lasts for 3-4 days (Kuzuya et al 1993, Marber et al 1993). SWOP's cardioprotective effects have been observed in open chest rabbit and dog and chronically instrumented conscious rabbit and rat (Kuzuya et al 1993, Marber et al 1993, Speechly-Dick et al 1994, Yellon et al 1995). Although early/classical PC and SWOP share many of the same signal transduction pathways, there are differences between both phenomena e.g., both early PC and SWOP limit infarct size but the infarct-reducing effects of early PC are more robust (Cohen et al 2000, Kuzuya et al 1993, Marber et al 1993, Murry et al 1986). SWOP however attenuates myocardial stunning, whereas early PC does not (Bolli 1996). The signalling pathway whereby NO (either endogenous or exogenous) induces late PC involves the generation of reactive oxygen species (Takano et al 1998) and the activation of the epsilon isotype of PKC (Ping et al 1999b, Vondriska et al 2001). It also involves the subsequent recruitment of Src and Lck tyrosine kinases (Dawn et al 1999, Vondriska et al 2001) and the activation of transcription factor NFκB (Xuan et al 1999), culminating in the transcriptional activation of iNOS gene (Jones et al 1999).

The most important difference is that SWOP's cardioprotection results from activation of genes and expression of new protective proteins. It is unlikely that expression of new proteins play a role in the early protection after IPC, and therefore it is possible that an entirely different set of triggers and signalling pathways are important to SWOP (Kloner et al 1998). Salutatory effects can be achieved with exogenous NO, either by NO-releasing agents or NO precursors (Bolli et al 2001). In addition, NO has been found to play a critical bifunctional role (as a trigger and a mediator) in late PC, a paradigm referred to as the NO hypothesis of late PC (Bolli et al 1998). Specifically, enhanced generation of NO by eNOS is essential to trigger the late phase of ischaemia-induced and exercise-induced PC. Enhanced production of NO by iNOS, recently suggested to be a hypoxia-inducible protein, is obligatorily required to mediate the anti-stunning and anti-infarct actions of late PC induced by five different stimuli (ischaemia, adenosine A<sub>1</sub> agonists, opioid  $\alpha_1$  agonists, endotoxin derivatives, and exercise). The NO hypothesis of late PC has thus revealed a previously unrecognised cytoprotective function of iNOS in the cardiovascular system (Bolli 2001).

The identification of the cellular basis of PC should provide a conceptual framework for developing new therapeutic strategies aimed at mimicking the cardioprotective effects of late PC. This can be achieved with pharmacological agents (e.g. PC-mimetic agents), or genetic approaches (e.g. transfer of cardioprotective genes), that can maintain the heart in a sustained or chronic defensive (preconditioned) state (Bolli et al 2000).

## 1.2.6 The endpoints of preconditioning

### 1.2.6.1 Contractile function

Contractile recovery is often used as an endpoint in the study of ischaemic preconditioning. Although it appears logical that a reduction in infarct size should result in improved contractile function during reperfusion, regional function is often not improved within the first hours of reperfusion (Cohen et al 1999, Jenkins et al 1995). However with longer periods of reperfusion, functional recovery may ensue. Therefore a lack of functional recovery does not necessarily refute the morphological protection induced by prior preconditioning, while caution should also be exercised when extrapolating from functional recovery to a reduction in infarct size. In addition to the above, it appears as if the perfusion model may also influence the outcome of the results - retrogradely perfused hearts (without afterload) recover functionally to a larger extent than working hearts that have to work against an afterload (Lochner, unpublished observations).

### 1.2.6.2 Oncosis/necrosis or infarct size

Cell death in ischaemia may occur by two different mechanisms: oncosis or “accidental” cell death, and apoptosis or “suicidal” cell death. Necrosis is commonly used but is not appropriate, because it does not indicate a form of cell death but refers to changes secondary to cell death by any mechanism, including apoptosis (Majno et al 1995). Because ischaemic cell death (in known models) is accompanied by swelling, the name oncosis was proposed for this condition. Oncosis is derived

from onkos, meaning swelling, which leads to necrosis with karyolysis. To avoid confusion and for the sake of convenience, it was decided to make use of the conventional term “necrosis” as the form of cell death opposed to apoptosis.

Necrosis is characterized by depletion of intracellular ATP, cellular swelling, disruption of organelles, and plasma membrane disruption, followed by an inflammatory reaction which removes the injured cells (Dipsersyn et al 2001). Although cleavage of DNA strands may occur late in the terminal evolution of cell necrosis (Gold et al 1994), the afore-mentioned cellular damage characteristically precedes it (Arends et al 1990). Necrosis develops only minimally during ischaemia and is detected mainly during reperfusion (Ganote et al 1993a, Hu et al 1995, Li et al 1993, Van den Hoek et al 1996, Yao et al 1999, Zhang et al 2000).

#### 1.2.6.2.1 Techniques for necrosis detection

Triphenyl tetrazolium chloride staining (TTC) has facilitated the macroscopic identification of myocardial infarction. This method has been shown to be sensitive and specific for myocardial necrosis (Gallagher et al 1980) and thus it is possible to distinguish necrotic from viable myocardium (Vivaldi et al 1985). Tetrazolium dyes form coloured precipitates in the presence of intact dehydrogenase enzyme systems. Areas of necrosis lack dehydrogenase activity and therefore fail to stain. Areas of necrosis are therefore distinguishable and thus quantifiable by using computerised planimetry. At the end of the experiments, hearts are injected with Evan's Blue dye to colour the in vivo area at risk. They are then excised, cut into transverse slices and stained with TTC. The method of TTC staining for the determination of infarct size

and area at risk has been used extensively in rats (Wolfe et al 1993), dogs (Fishbein et al 1981), pigs (Freeman et al 1990), and cats (Brunvand et al 1995). Also in the study of both classic and late PC, infarct size measurement has been the preferred endpoint by many workers (for example Downey and colleagues, Yellon et al).

#### 1.2.6.2.2 Measure of viability

In the intact heart, the effect of myocardial injury and the protective actions of endogenous or exogenous agents and mechanisms have primarily been investigated with regard to physiological changes e.g., functional recovery during reperfusion after ischaemia. In isolated cardiomyocytes, the effects of cell injury on the viability of these cells, and the effect of cardioprotective agents on cellular survival have only been partially addressed (Buerke et al 1994, Cheirif et al 1995, Ohata et al 1994).

Various assays exist to investigate cellular viability. "Lytic" viability assays, such as the Trypan blue exclusion method or the lactate dehydrogenase release assay, rely upon the integrity of the plasmalemma as indicator of cell viability (Isowa et al 1996, Tollefson et al 1996). The trypan blue exclusion method works on the principle that cells with intact plasma membranes will not take up the dye and thus be stained yellow and often appear rod-shaped. Cells with compromised/lysed plasma membranes will take up the dye and be stained blue. An assay, which reflects not only cell membrane integrity but also cellular metabolic status, may be more appropriate for cells with high-energy requirements (Gomez et al 1997).

The MTT assay is such an assay, based upon the reduction of (3,4,5 dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium (MTT) bromide into formazan pigments (Buttke et al 1993, Mosmann 1983). Intracellularly, this reduction is predominantly dependent upon mitochondrial reductase activity, and is carried out by catalytic reactions coupled to the NADPH- and NADH-dependant redox systems (Ferrara et al 1993). Changes in MTT reductase activity are detectable even before membrane lysis (Ni et al 1996). Decline in enzyme activity (essential for electron transfer) as well as the depletion of cofactors needed for redox reactions correlate with loss of cellular viability. This makes the MTT reductase activity a refined marker of cell viability (Carmichael et al 1987, Roehm et al 1991, Tollefson et al 1996).

### **1.3 Apoptosis**

#### **1.3.1 General**

For many years it has been believed that necrosis was the predominant form of cardiomyocyte death following ischaemia and reperfusion. However, during the past few years it has become clear that apoptosis plays a significant role in myocardial ischaemia/reperfusion injury (Gottlieb et al 1994, Kajstura et al 1998). Ischaemic preconditioning has also been shown to protect against apoptosis (see below).

#### **1.3.2 Characteristics of cellular changes in apoptosis (Table 1.1)**

Apoptosis is characterized by distinct morphologic alterations. There is profound shrinkage and membrane blebbing, which is associated with externalization of phosphatidylserine. Cytoskeletal architecture is altered by the proteolytic cleavage of fodrin, actin, and of one or more G-proteins that regulate the state of assembly of the

cytoskeleton (Rudel et al 1997). One of the most important aspects of apoptosis is the preservation of an intact plasma membrane with expression of cell surface markers for ingestion of a neighbouring cell or professional phagocyte to ingest the cell. This serves to limit inflammation. It should be noted that these external features are distinct from those seen in necrosis in which cell swelling results in rupture of the plasma membrane which in turn causes an inflammatory reaction leading to removal of these injured cells (Dispersyn et al 2001). Other features of apoptosis include shrinkage of nuclei, condensed chromatin, intact mitochondria, the cleavage of nucleosomal DNA into fragments of 185 base pairs whereas necrotic cells exhibit irregular clumping of the chromatin, mitochondrial swelling and irregular nucleosomal DNA. Another important difference is that apoptosis is energy-dependent (Eguchi et al 1997) while necrosis is not. Energy is needed in apoptosis to start and keep the apoptotic machinery operative while if the energy is depleted the cell will die by necrosis.

**Table 1.1: Cell death in ischaemia**

	<u>Apoptosis</u>	<u>Oncosis/ necrosis</u>
<b>Cell volume</b>	<b>shrinkage</b>	<b>oedema</b>
<b>Chromatin</b>	<b>condensation</b>	<b>clumping</b>
<b>Mitochondria</b>	<b>intact</b>	<b>swelling</b>
<b>Cell membrane</b>	<b>intact</b>	<b>ruptured</b>
<b>Nucleosomal DNA</b>	<b>cleaved 185 kB</b>	<b>irregular</b>
<b>Inflammation</b>	<b>no</b>	<b>yes</b>
<b>ATP-dependent</b>	<b>yes</b>	<b>no</b>

### 1.3.3 Biochemical events associated with apoptosis (Figure 1.1)

One of the most central biochemical features of apoptosis is the activation of a class of cysteine proteases known as caspases. Caspases are a group of cysteine proteases that cleave protein substrates at the carboxyl side of aspartate residues (Nicholson et al 1997). Cells possess multiple caspases, which may work in a cascade fashion. They have a common structure, in which there is a prodomain of variable length, followed by a region of ~20 kD and a carboxyl terminal region of ~10 kD. The three domains are separated by consensus caspase cleavage sites and the inactive zymogens (pro-caspases) must be cleaved to between the 20 and 10 kD to become active. The two fragments associate and form a heterotetramer that represents the fully active enzyme.

Two pathways of caspase activation have been identified: one activated through a cell surface signal leading to caspase activation, also known as the extrinsic pathway or "death receptor pathway". The other pathway involves the mitochondria and is hence known as the "mitochondrial pathway" (Borgers et al 2000, Haunstetter et al 1998). The mitochondrial pathway seems to be more important in ischaemia/reperfusion induced apoptosis. Both these pathways ultimately activate the downstream (effector) caspases such as caspase-3, which leads to cleavage of death substrates such as PARP, which in turn leads to DNA fragmentation and cell death.

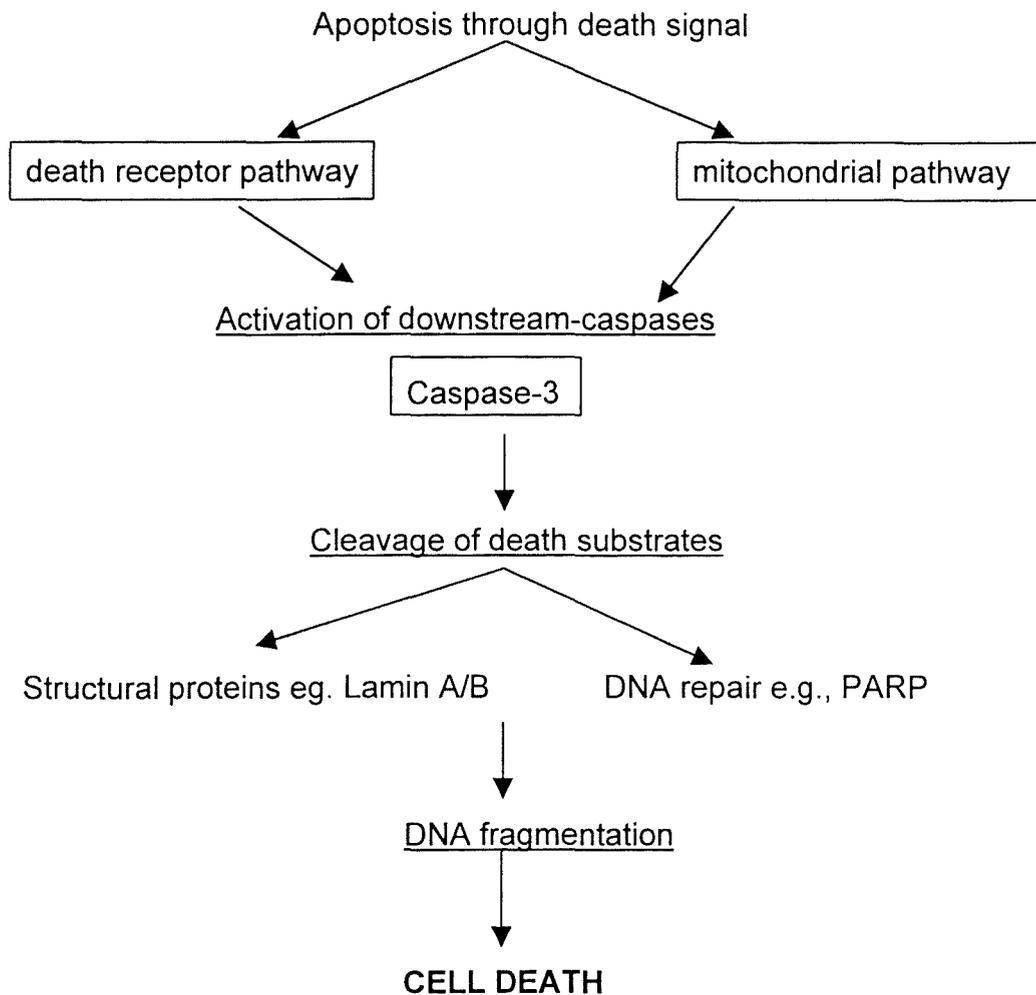


Fig 1.1 Simplified version of the major events in the apoptotic cascade.

(Elsässer et al 2001)

#### 1.3.3.1 The extrinsic/death receptor pathway

Cell-surface receptor-mediated mechanisms that control apoptosis act through a signal transduction system that is the same for all activated receptor processes. It involves the stimulation of the receptor, the activation of protein kinase/phosphatase cascades, and the release of second messengers to upregulate or suppress the transcription of specific genes.

The best characterized pathways involve binding of a Fas ligand (FasL) and TNF- $\alpha$  to their respective receptors (Fas and TNFR-1 respectively) (Nagata 1999). Binding of the ligand to the receptor leads to intracellular association of proteins that interact through a conserved region known as the death domain, one example of which is FADD (Fas- associated death domain). FADD then recruits a specialized caspase (caspase 8, also known as FLICE) through a second domain (the death effector domain), and the aggregated complex results in autoprocessing of caspase-8 molecules. The fully processed caspase-8 is then free to interact with downstream caspases such as caspase-3, as well as other targets such as the mitochondria (Kuwana et al 1998). Many cellular stressors, such as DNA-damaging agents, gamma irradiation, and pro-apoptotic cytokines such as TNF- $\alpha$  also activate acidic and/or neutral sphingomyelinases to generate ceramide. Ceramide acts as an intracellular signaling molecule to activate a kinase pathway that leads to apoptosis in some settings and proliferation in others (Obeid et al 1993). Ceramide has also been shown to play a role in TNF- $\alpha$ -mediated apoptosis in cultured cardiomyocytes (Krown et al 1996).

Ultraviolet light, DNA-damaging agents, and oxidative stressors (e.g., hydrogen peroxide) have also been shown to play a role in apoptosis via the JNK pathway in a variety of systems (Johnson et al 1996). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in particular has been shown to induce TUNEL positivity, cytochrome c translocation, caspase-3 activation, PARP cleavage, and dissipation of the mitochondrial membrane potential in cultured neonatal rat cardiac ventricular myocytes making it a particularly good trigger of apoptosis as it activated all the relevant markers of apoptosis in this model.

### 1.3.3.2 The intrinsic/mitochondrial pathway

It has become evident that multiple proteins of the huge Bcl-2 family regulate mitochondrial function. Pro-apoptotic factors such as Bax, Bak and Bid counteract anti-apoptotic components such as Bcl-X<sub>L</sub> resulting under physiological circumstances in a balance in favour of protection from cell death and in preservation of the mitochondrial permeability pore complex (PTP). Under stress conditions such as ischaemia/reperfusion the PTP is disturbed and cytochrome c, AIF (apoptosis inducing factor) and procaspase-9 are released from the intermembrane mitochondrial space into the cytoplasm where Apaf-1 (apoptotic protease activation factor) is activated. Apaf-1 interacts with cytochrome c and dATP to activate caspase-9 (Li et al 1997). Caspase-9 then activates downstream caspases such as caspase-3 and also protein kinases and phosphatases, which promote fragmentation of DNA.

Once activated, caspase cleaves multiple targets. A widely recognized target is the enzyme poly (ADP-ribose) polymerase (PARP). PARP is involved in DNA repair and in the supervision of genome structure and integrity in stressed cells (Nicholson et al 1995). This enzyme recognizes single-strand DNA breaks and activates repair enzymes by poly-ADP-ribosylating them. However, in the face of extensive DNA damage, this enzyme can consume all the available NAD, which will lead to depletion of cellular energy currency. Apoptosis requires energy (Eguchi et al 1997) and thus it has been suggested that PARP cleavage, and thus inactivation, is essential for the cell to retain enough energy to complete the apoptotic process. PARP cleavage can therefore be utilized as a marker of apoptosis. Other targets include DFF (DNA

fragmentation factor), an endonuclease responsible for degradation of the genome during apoptosis (Liu et al 1997). DNase I and II are other endonucleases which have been implicated. DNase I has been shown to cleave DNA into oligonucleosomal multiples as seen in apoptosis (Peitsch 1993) and DNase II, as the intracellular pH drops below 6.8, becomes active and can generate oligonucleosomal fragments. These oligonucleosomal fragments are used as markers of apoptosis as detected with the ELISA method or DNA laddering.

It can be seen that apoptosis activates a variety of enzymatic processes that serve to degrade the genomic DNA, inactivate cellular enzymes that might facilitate cell survival, deplete intracellular energy, and fragment the cell into smaller pieces for easy removal.

#### **1.3.4 Techniques for apoptosis detection**

There are several techniques available to verify apoptosis in tissue samples each technique however with its own problems. Apoptotic bodies are best viewed by electron microscopy but apoptotic bodies, are however phagocytosed within hours after cell death and it may therefore be difficult to verify whether apoptosis affects only a limited number of cells or occurs transiently. Nuclear staining with the fluorescent dye bisbenzamide (Hoechst 22358) allows for the visualization of nuclear condensation and fragmentation in both cell culture and tissue sections. Separation of cellular DNA in agarose gels shows a characteristic ladder-like pattern of fragments with multiples of 200 bp in length. The disadvantage is assigning the apoptotic process to one cell type whenever different cell populations are analyzed as in whole tissue.

The TUNEL (TdT-mediated dUTP nick end-labelling) technique is the most widely used but it can also label non-apoptotic DNA fragmentation, and is therefore also the most criticized method (Onoh et al 1998, Saraste et al 1997). ISEL (in situ end labeling) is also commonly used. A technique, which employs the binding of Annexin V to phosphatidylserine exposed on the cell membrane during apoptosis, has recently been extensively employed as a reliable and quantifiable marker of apoptosis (Clodi et al 2000, Hreniuk et al 2001, Rucker-Martin et al 1999). Detection of protein fragments by Western blotting has also been used to verify cell death in cell cultures of apoptosis.

Activation of execution caspases, such as caspase-3 (Kang et al 2000, Narayan et al 2001, Schaper 1999, Suzuki et al 2001) and specific cleavage of cellular target proteins, such as PARP, are considered to be hallmarks of apoptosis (Lazebnik et al 1994, Nicholson et al 1995).

### **1.3.5 Role of MAP kinases in apoptosis**

MAPK cascades contain at least three protein kinases that work in series and these three enzymes comprise a module. The MAPK kinase kinase (MAPKKK) is activated by a variety of stimuli, which commonly include mitogenic stimuli such as growth factors and PMA in the case of the ERKs, or physiological stress such as ischaemia/reperfusion and hypoxia in the case of JNK and p38 MAPK. These kinases then phosphorylate and activate the MAPK kinase (MAPKK) or MAPK/ ERK kinases (MEKs) which in turn selectively activates ERK 1/2, p38, or JNK respectively.

It is well established that ERK, JNK, and p38 MAPKs are activated during cellular stress. Whereas ERK activation by stress seems to protect against most stressors, the exact role of JNK and p38 MAPK remains elusive. There are at least two possibilities. These activated protein kinases may contribute to signal transduction pathways that culminate in the cell death (i.e., necrosis and apoptosis) following stress. Alternatively these MAPKs may also act to prevent widespread cell death, limit damage, apoptosis or mediate adaptation.

#### 1.3.5.1 Initiating cell death

It is well recognized that activation of p38 MAPK and JNK/SAPK are pro-apoptotic in many situations (Obata et al 2000, Park et al 2000). Specific assessment of the role of stress-activated MAPKs requires the use of specific chemical inhibitors, overexpression of MAPKs, or transgenic approaches.

Specific inhibitors of p38 $\alpha$  and p38 $\beta$  MAPKs, SB 203580 and SB 202190 have been extensively used. Although SB 203580 has a potent effect on the activity of the p38 $\alpha$  and p38 $\beta$  MAPK, its efficacy for p38 $\gamma$  and p38 $\delta$  isoforms is low (Lee et al 1999). Moreover, at a higher concentration (IC<sub>50</sub> 5  $\mu$ mol/L), this compound will inhibit JNK and phosphatidylinositol 3 (PI3) kinase/PKB (Lali et al 2000) both of which have been shown to be recruited by IPC (Ping et al 1999a). The use of chemical inhibitors suggests that the p38 MAPKs promote cardiac myocyte death during extended periods of ischaemia or exposure to hydrogen peroxide (Mackay et al 1999, Meldrum et al 1999). Studies using isolated perfused hearts conclude that p38 MAPK plays a pivotal role in promoting myocardial apoptosis (Ma et al 1999). Cardiac injury in

response to oxidative stress induced by hydrogen peroxide is apparently mediated by a p38 MAPK-dependant production of TNF- $\alpha$  (Meldrum et al 1999). In view of this detrimental role for p38 MAPK in apoptosis, cardioprotection may be correlated with the ability of a compound or intervention to inhibit p38 MAPK activation.

The transgenic overexpression of MAPK pathway members has yielded more insight into the relative importance of the p38 isoforms. Overexpression of their specific activators, MKK3 and MKK6, indicates that this pathway also plays a complex role in the cardiac myocyte. Whilst the overexpression of MKK3 or MKK6 increased cell surface area, sarcomeric organisation and ANF expression, the co-expression of MKK3 with p38 $\alpha$  prevented these changes and induced cell death. In contrast, p38 $\beta$  expression in the presence of MKK3 augmented hypertrophy and promoted cell survival (Wang et al 1998). Thus p38 $\alpha$  apparently promotes cell death whilst p38 $\beta$  promotes cell hypertrophy.

Further evidence which links JNK and p38 MAPK to activation of apoptosis is the finding that activation of the JNK and p38 MAPK pathways is associated with activation of effector caspases, including caspase-3 (Chaudhary et al 1999). Caspase-3 itself can also amplify the activation of the JNK pathway, as it is able to cleave MEKK1 (Cardone et al 1997), a kinase upstream of JNK. Caspase-3 reinforces the inhibition of ERK by cleaving and inactivating Raf-1, an upstream effector of the ERK signaling pathway (Windham et al 1998).

Strong evidence therefore exists for a connection between apoptosis, especially the downstream effector caspase-3, and the MAPK family. The elements downstream of

these MAP kinases leading to caspase activation and ultimately to apoptosis are still not clearly defined.

#### 1.3.5.2 Initiating a survival response

Incubation with SB 202190 alters the responses to endothelin-1, LIF or phenylephrine (Nemoto et al 1998). Thus the p38 MAPKs have been proposed as regulators of cardiac hypertrophy. Studies with SB 203580 have demonstrated its lack of effect on the early changes in cell size stimulated by endothelin-1 or phenylephrine, implying that the role of p38 MAPKs may actually be in the maintenance of the hypertrophic response/survival over a long period of time (Clerk et al 1998). There seems to be little doubt regarding the role of ERKs in the survival response. Activation of ERK predominantly produces anti-apoptotic effects (Park et al 2000, Wang et al 1998). Despite the suggestion that JNK and p38 MAPKs mediate cell death, ample evidence also supports their role in cell survival. TNF- $\alpha$  induced activation of JNK and p38 MAPK enhances fibroblast survival (Roulston et al 1998), JNK and p38 MAPKs protect during photodynamic therapy (Assefa et al 1999), and one JNK isoform maintains survival following hypertonic shock (Wojtaszek et al 1998).

Evidence also supports these pro-survival signaling cascades. Anisomycin, an activator of JNK and p38 MAPK, protected myocytes from ischaemic injury (Mackay et al 1999) and phosphorylation of p38 MAPK correlated with preconditioning protection (Weinbrenner et al 1997). Contradictory findings of anisomycin-initiated cell death have however also been reported. Specific activation of p38 MAPKs by

overexpression of pathway components may also mediate a survival response in cardiac myocytes (Zechner et al 1998). This is supported by p38 $\beta$  MAPK mediating cell survival, sarcomeric organisation, and gene expression (Wang et al 1998).

The effects of different p38 MAPK and JNK isoforms are therefore likely to be complex and dependent on the stimulus and cellular context. Duration and intensity of the stress may ultimately determine the regulation of MAPKs and the final cellular outcome. Also, the extent of p38 MAPK activation may dictate whether a cardiac myocyte is committed to death or survival (Nagarkatti et al 1998).

### **1.3.6 Effect of preconditioning on apoptosis**

Preconditioning has been extensively shown to reduce infarct size in different animal species. Little is however known about the effect that IPC has on apoptosis. What is known is that IPC does indeed protect the ischaemic heart against apoptosis although the mechanism is as yet unknown.

A summary of publications on preconditioning and apoptosis is shown in table 1.2. These provide evidence that cardioprotection elicited by preconditioning does protect against apoptosis in the ischaemic heart. The relationship between these two phenomena has been tested in various animal models, which include the rat, mouse, dog, and cultured chick ventricular myocytes. A multi-cycle IPC protocol, as opposed to a single cycle IPC protocol, followed by ischaemia and reperfusion and not just ischaemia alone, appears to be the protocol of choice for many researchers investigating how IPC affects apoptosis. Electrophoresis and the TUNEL assay, that

**Table 1.2: Literature review of preconditioning and apoptosis**

Author	Species	Model	Protocol	Endpoint	Technique
Piot et al (1997)	Adult rat	In vivo model	IPC (5 min LCA occlusion +5 min R) X 5 20/30 min ischaemia +3h reperfusion	-DNA fragmentation -DNA laddering	-TUNEL assay -ELISA -electrophoresis
Maulik et al (1998)	Adult rat	Perfused heart	IPC (5min I + 10 min R) X 4 15 min ischaemia + 2h reperfusion	-DNA laddering -DNA fragmentation -Left ventricular performance	-electrophoresis -TUNEL assay -Transducer
Okamura et al (1999)	Adult rat	Perfused heart	IPC (3 min I + 3 min R) X 3 30 min ischaemia + 6h reperfusion	-DNA fragmentation	-TUNEL assay
Piot et al (1999)	Adult rat	Perfused heart	IPC (5 min LCA occlusion +5 min R) X 5 20/30 min LCA occlusion +3h reperfusion	-Internucleosomal DNA -Caspase-1 -Caspase-3 -PARP	-ELISA -Western blotting
Yadav et al (1999)	Mouse	Liver	IPC 10 min I + 15 min R 75 ischaemia	-DNA fragmentation -Caspase-3 -PARP	-TUNEL assay -electrophoresis -caspase-3 fluorimetric assay -PARP proteolysis
Wang et al (1999)	Dog	In vivo model	IPC 5 min I + 5 min R 30 min ischaemia + 3h reperfusion	-DNA fragmentation	-TUNEL assay -electrophoresis
Liu et al (2002)	Chick embryos	Cultured cells	PC (1 min SI + 5 min reperfusion) X 3 10h simulated ischaemia + 12 h reoxygenation	-DNA fragmentation -DNA laddering	-TUNEL assay -electrophoresis

KEY: LCA left coronary artery      I ischaemia  
R reperfusion      SI simulated ischaemia

quantifies DNA laddering and DNA fragmentation respectively, also appear to be the endpoint and technique mostly used.

Piot et al (1997) showed that hearts exposed to a multi-cycle IPC protocol of [ (5 min ischaemia + 5 min reperfusion) x 5] before 30 min of ischaemia followed by 3h of reperfusion exhibited a reduction in DNA fragmentation and DNA laddering, used as markers of apoptosis, compared to the Non-PC hearts. Maulik et al (1998) showed similar results but in addition suggested that the lack of apoptotic cells and DNA fragmentation resulted in better left ventricular performance in the preconditioned myocardium compared to the non-preconditioned myocardium after reperfusion.

Recently, there has been a shift away from DNA damage to caspase activation as a marker of apoptosis. In 1999, Piot et al showed that caspase-1, but more importantly caspase-3 activation and PARP cleavage, widely regarded as key components in the effector phase of apoptosis, were significantly reduced in IPC hearts compared to the non-preconditioned hearts at the end of reperfusion using Western blotting. Yadav et al (1999), using the mouse liver with a one-cycle IPC protocol but with no reperfusion after ischaemia, showed similar significant decreases in both caspase-3 activation and PARP cleavage after 75 min of ischaemia. They suggested that apoptosis is decreased through downregulation of caspase-3. The role of caspase-3 as an important marker and player in the apoptotic cascade has been mentioned previously.

The mechanism(s) by which ischaemic preconditioning prevents apoptosis remains unknown. Gottlieb et al (1995) and Caceres-Cortes et al (1994) have demonstrated that apoptosis could be delayed in neutrophils and in haematopoietic stem cells respectively by preservation of intracellular pH homeostasis. On the basis of that finding Gottlieb et al (1996) suggested that activation of the vacuolar proton ATPase by PKC during preconditioning may attenuate intracellular acidification during metabolic inhibition and thereby protect myocytes from apoptosis in vitro. Consistent with these findings, Kida et al (1991), Schaefer et al (1995) and Wolfe et al (1993) have demonstrated that IPC was accompanied by a reduction in intracellular acidosis in rat hearts. In addition to increased proton export through vacuolar proton ATPase by PKC activation, reduced proton production from anaerobic glycolysis may also contribute to prevent myocyte apoptosis in rat hearts in vivo.

PKC and the MAPK stress kinases have also been implicated in the regulation of apoptosis via IPC. Maulik et al (1996) using a multi-cycle IPC protocol [5 X (5 min ischaemic + 10 min reperfusion)] before 30 min ischaemia and 30 min reperfusion showed that IPC stimulated PKC, MAPK, and MAPKAP kinase 2 activities which were inhibited by genistein, a tyrosine kinase inhibitor, when administered before the IPC protocol. This group suggested that IPC may activate the tyrosine kinase-phospholipase D signaling pathway in rat hearts, resulting in the activation of the MAPK pathway. Studies by Han et al (2001) using oxidative stress with H<sub>2</sub>O<sub>2</sub> as a preconditioning stimulus in L (fibroblast) cells, showed that transient increases in p38 MAPK and JNK accompanied by drastically reduced caspase-3 activity was seen after preconditioning. Furthermore SB 203580 abolished the protection provided by PC. Their results point to a central role for p38 MAPK in mediating both the protective

effects of oxidative preconditioning and the pro-apoptotic effects of prolonged oxidative stress.

The data above provides strong evidence that preconditioning does protect the ischaemic heart against apoptosis and the mechanism, although not yet fully understood, seems to involve PKC and the p38 MAPK family. Further investigations are needed to fully elucidate the mechanisms and implications of these mechanisms in the ischaemically preconditioned hearts in reducing apoptosis.

## **Aim of the study**

The aim of the study was:

1. To establish a cell culture model of preconditioning using neonatal cardiomyocytes. The reason for this is to enable one to elucidate the cardiac specific signal transduction pathways more accurately, as a cardiomyocyte cell culture would exclude the influence of non-cardiac cells. Furthermore, it potentially offers a way of doing multiple experiments (i.e. looking at different signal transduction components, and even different end-points) using a single cell culture preparation, and thus enable rapid progression of the investigation.
2. To investigate whether IPC and  $\beta$ PC protect against ischaemia/reperfusion damage as determined by measurements of both infarct size and apoptosis.
3. To determine the importance of the stress kinase, p38 MAPK, in the protective effect of preconditioning against necrosis and apoptosis.

## Chapter 2

### Materials and Methods

#### 2.1 Neonatal cardiomyocytes in culture

##### 2.1.1 Preparation of the cell culture

For the preparation of neonatal cardiomyocytes in culture, Wistar rats (2-4 days old) were used. The neonatal rats were culled by intraperitoneal administration of 2.5% Intraval sodium (thiopentone sodium 2.5mg/rat) and upon death, immersed in 70% alcohol. Neonatal cardiomyocytes were prepared by a modification of the technique of Pinson (1990). Sterile conditions were employed throughout. The chest was opened by a transverse cut with sterile scissors and the hearts removed. The atria were cut off and discarded. The excised ventricles were placed in a petri dish with a buffer containing (in mM): NaCl 120, KCl 5, D-glucose 6, Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O 1, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.3, HEPES 19, pH 7.4

The excised ventricles were transferred to a second petri dish containing the above buffer, cut into smaller fragments with a pair of scissors and washed twice with buffer. After washing, the buffer was replaced by the same buffer containing 0.3% collagenase and 0.06% pancreatine. The digestion process was performed at 37 °C in a shaking waterbath at a stirring rate of 50 strokes/min for 20 minutes.

Remaining ventricular fragments were allowed to settle and the supernatant transferred to a conical centrifuge tube. Newborn calf serum (NBCS) (20% v/v) was added to the supernatant to stop the digestion process. The supernatant was then centrifuged at 300 g for 5 minutes at room temperature. After centrifuging, the supernatant was discarded and the cell pellet resuspended in 2 ml NBCS and kept at 37 °C in the shaking water bath. Digestion of the ventricular fragments was repeated until all the fragments were dissociated. The resulting cell pellets were combined and centrifuged at 300 g for 5 minutes at room temperature. To obtain a non-fibroblast containing myocyte preparation, use was made of the isopycnic principle using Percoll with different gradients (Thandroyen et al 1989). The final cell pellet was resuspended in 1.082g/ml Percoll on top of which a 1.062g/ml and 1.050g/ml Percoll layer were loaded. The gradient was centrifuged at 1000g for 25 minutes at 21 °C. The cardiomyocytes were retrieved from between the 1.082g/ml and 1.062g/ml Percoll layers, washed twice with the buffer and then centrifuged at 300 g for 5 minutes at 21 °C. The pellet was diluted with Dulbecco's Modified Eagle's Medium (DMEM: with 10% Fetal Calf Serum and 1% penicillin) to a final density of ~800 000 cells/ml. Aliquots of 2ml were seeded into 35mm petri dishes coated with 5 mg/cm<sup>2</sup> fibronectin. After seeding, the cultures were incubated at 37 °C in an atmosphere of 95% air/5% CO<sub>2</sub>. After 3 days a confluent layer of beating cells was formed. The medium was changed every 24 hours.

### **2.1.2 Determination of cell viability in neonatal cardiomyocytes in culture**

For the assessment of cell viability a modification of the 3-[4,5-Dimethyl-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay described by Gomez et al (1997) was used. This assay is based upon the principle of reduction of MTT into blue formazan pigments in viable cells. This reduction occurs only in the presence of intact cell membrane and functional mitochondria, which makes it a marker of cell viability as described in Chapter 1. At the end of experiments, the medium was removed from the petri dishes and the cells washed twice with phosphate-buffered saline (PBS). One ml of HCl-isopropanol-Triton (1% HCl in isopropanol; 0.1% Triton X 100; 50:1) was added to each well and gently agitated for 5 minutes. This caused the cell membranes to lyse and liberate the formazan pigments. The suspension was then centrifuged at 14 000 rpm for 2 minutes. The optical density (OD) was determined spectrophotometrically (Spectronic<sup>®</sup> 20 Genesys<sup>™</sup> - Spectronic Instruments) using glass cuvettes, at a wavelength of 540 nm.

### **2.1.3 Preconditioning modalities: chemical hypoxia and $\beta$ -adrenergic receptor stimulation**

Two modalities were used to precondition neonatal cardiomyocytes:

- (1) chemical hypoxia (5 min) or
- (2)  $\beta$ -adrenergic receptor stimulation by exposure to isoproterenol ( $10^{-7}$  M) dissolved in Krebs Henseleit buffer (KHB) (5 min).

This chemical hypoxia solution was prepared by making a 1M KCN stock solution i.e., 0.326g/5ml H<sub>2</sub>O. From this stock solution, 1.15ml was mixed with 100  $\mu$ l of 10N HCl. From the resulting solution 250  $\mu$ l was added to 100ml KHB immediately before use. Two ml of this solution was added per well to induce chemical hypoxia.

### **2.1.4 Hypoxia modalities**

Two methods of chemical hypoxia were employed:

- (1) 5 mM KCN + 20 mM DOG in KHB for 5 min [Non-PC (DOG)] or
- (2) 5 mM KCN in KHB for 45 min.

The first modality for chemical hypoxia used both KCN and DOG for 5 minutes whereas the second modality only used KCN. This second modality was less severe and thus a longer period of exposure i.e., 45 min was employed.

### 2.1.5 Experimental protocols

The non-preconditioned cells were exposed to sustained chemical hypoxia only. All PC cell cultures were preconditioned as described below before exposure to simulated chemical hypoxia. The cells were washed twice with PBS between each step of the protocol. DMEM refers to removal of the simulated chemical hypoxia medium, washing with PBS and addition of DMEM with incubation in 95% CO<sub>2</sub>/5% O<sub>2</sub>.

Each preconditioning protocol was studied in triplicate and at least 6 different neonatal cell preparations were used. The following protocols were attempted:

- (I) Non-PC (DOG): oxygenated for 65 min + 5 min KCN + DOG + 10 min DMEM
- (II) 5 min KCN in KHB + 60 min DMEM + 5 min KCN + DOG in KHB + 10 min DMEM
- (III) Non-PC: oxygenated for 65 min + 45 min KCN + KHB + 10 min DMEM
- (IV) 5 min KCN in KHB + 60 min DMEM + 45 min KCN in KHB + 10 min DMEM
- (V) 5 min isoproterenol in KHB + 60 min DMEM + 45 min KCN in KHB + 10 min DMEM

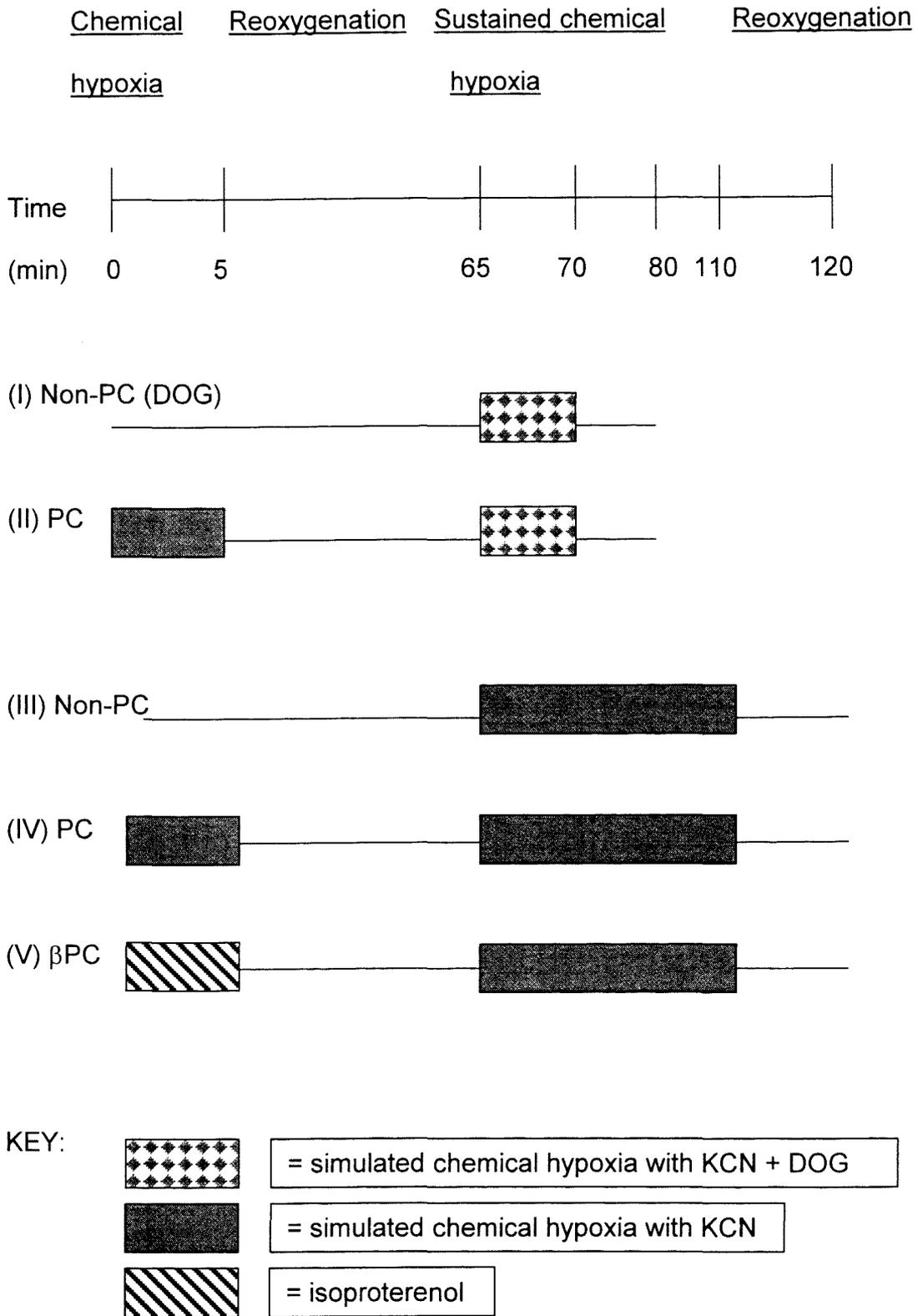


Fig 2.1 Experimental protocols for the neonatal cardiomyocytes in culture.

## **2.2 Isolated adult cardiomyocytes**

### **2.2.1 Isolation of adult cardiomyocytes**

For preparation of adult cardiomyocytes, a modification of the technique described by Fischer et al (1991) was used.

#### **2.2.1.1 Preparation of the buffers**

Solution A: Calcium-free buffer containing (in mM): KCl 6, Na<sub>2</sub>HPO<sub>4</sub> 1, NaH<sub>2</sub>PO<sub>4</sub> 0.2, MgSO<sub>4</sub> 1.4, NaCl 128, HEPES 10, glucose 5.5, pyruvate 2, pH 7.4

Solution B: Solution A containing 0.7% bovine serum albumin (BSA), fraction V, fatty acid free, 1.1 mg collagenase/ml, and 15 mM 2,3-butanedione monoxime (BDM).

Solution C: Equal volumes of solutions A and B were mixed and albumin (fraction V, fatty acid free) was added to obtain a final concentration of 1.65%. CaCl<sub>2</sub> was added to obtain a final concentration of 200 μM. The temperature of the solution was increased to 37°C in a shaking waterbath at 37°C, 180 strokes/min.

Solution D: Solution A containing 2% BSA (fraction V, fatty acid free), and 1.25 mM CaCl<sub>2</sub>

### 2.2.1.2 The perfusion technique

The perfusion system had two glass chambers containing solutions A and B respectively. The buffers were oxygenated by gassing with 95% O<sub>2</sub>/5% CO<sub>2</sub> and the temperature kept constant at 37 °C. The excised heart was arrested in ice-cold (4 °C) Solution A and mounted onto the aortic canula. It was then retrogradely perfused at a pressure of 100 cm H<sub>2</sub>O by the method of Langendorff (Langendorff 1895) for 5 minutes with Solution A to rinse blood from the coronary vessels. The perfusion was then switched to Solution B, and the first 8ml coronary effluent was discarded. The remaining Solution B was recycled in the system. After 20 minutes, 100 µM of CaCl<sub>2</sub> was added to Solution B and after 5 more minutes, another 100 µM of CaCl<sub>2</sub> was added. The completion of digestion by collagenase usually occurred after ± 30 min, as indicated by a marked increase in coronary flow rate. The heart was carefully detached and the atria removed with a pair of scissors. The ventricles were carefully dissected with a pair of tweezers and then transferred to oxygenated Solution C, agitated gently and incubated in a shaking water bath for 15 minutes (37 °C).

The cells were made calcium tolerant by increasing the calcium concentration of the buffer step-wise to 1.25 mM over a period of 5 minutes. The cells were filtered through a 200 x 200 µm mesh-filter and spun down at 100 rpm for 3 minutes at room temperature. The supernatant was removed and the cells resuspended in Solution D by gentle tilting.

The cells were allowed to settle for 5 minutes and the supernatant again removed. The cells were transferred into a smaller volume of Solution D and gently agitated for 1 hour at room temperature to stabilize. The cells were washed twice with Solution D before experimentation. All isolates prepared in this manner averaged 75-80% viability. A separate isolate was used for each experiment.

## **2.2.2 Viability of adult cardiomyocytes**

Cell viability was assessed by the trypan blue exclusion method and the MTT assay.

### **2.2.2.1 The trypan blue exclusion method**

This technique is based on the principle that an intact cell membrane excludes trypan blue. Thus non-viable cells take up the trypan blue dye and stain blue, whereas viable cells do not take up the dye, appear rod-shaped and have a yellowish appearance.

#### **2.2.2.1.1 Solutions**

KCN solution: KCN (0.035% w/v)-solution D (1:1) was used to stop the cells from beating.

Trypan blue solution: 1% trypan blue in the above KCN solution containing 0.5% glutaraldehyde was used as the counting medium.

#### 2.2.2.1.2 Viability determination

For the assessment of viability at the beginning of the experiments a 12.5  $\mu$ l aliquot was transferred from the initial pellet into 100  $\mu$ l KCN-solution D (1:1) and resuspended gently, but thoroughly. For the assessment of viability at the end of the experiments, 12.5  $\mu$ l was taken from each experimental cell pellet and added to 100  $\mu$ l KCN solution as described above. The cells were allowed to settle for 3-5 minutes and 25  $\mu$ l from this suspension was added to 25  $\mu$ l of the trypan blue solution and mixed gently. A 12.5  $\mu$ l aliquot from this homogenous solution was then transferred to a counting chamber. Microphotographs were taken of the non-beating myocytes (250-350 cells/sample), thereby allowing rapid recording of data and minimizing unnecessary time-dependent absorption of the dye by potentially viable cells. The number of viable cells was expressed as a percentage of the total number of cells in the sample.

#### 2.2.2.2 The 3-[4,5- Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay

For the assessment of cell viability a modification of the MTT technique described by Gomez et al (1997) was used. Upon completion of the experiment, a volume containing 50 000 cells was transferred from each experimental group into 35mm petri dishes. The volume per sample was made up to 500 µl with solution D. An equal volume of the MTT solution (1% MTT in Solution D) was added and incubated at 37 °C for 2 hours. The supernatant was removed and the cells washed twice with phosphate-buffered saline (PBS). One ml of HCl in isopropanol-Triton [1% HCl in isopropanol; 0.1% Triton X-100; (50:1)] was added and gently agitated for 5 minutes. The suspension was centrifuged at 14 000 rpm for 2 minutes. The optical density (OD) was determined spectrophotometrically (Spectronic<sup>®</sup> 20 Genesys<sup>™</sup> - Spectronic Instruments) at a wavelength of 540 nm. For each experimental group the protein content was determined using the Lowry method (Lowry et al 1951). Cell viability was expressed as OD/mg protein. Viability assays of a particular cell preparation were always done in triplicate.

### **2.2.3 Experimental protocols**

Three experimental groups were studied viz., oxygenated controls, Non-PC hypoxia, and preconditioned groups.

#### **2.2.3.1 Oxygenated controls**

Cells were incubated at 37 °C under oxygenated conditions for the entire duration of the experiment.

#### **2.2.3.2 Non-PC hypoxia groups**

For induction of hypoxia to adult cardiomyocytes, a modified technique of Armstrong et al (1995) was used. The cells were spun down at 250 rpm for 2 minutes (so-called ischaemic pelleting). The excess supernatant was removed until it equaled a third of the pellet size and a layer of mineral oil, just enough to cover the surface i.e., ~1 ml, was added to exclude air and induce hypoxia (care was taken to prevent mixing of the cells and mineral oil). The cells were then exposed to 2 hours of hypoxia at 37 °C.

### 2.2.3.3 Preconditioned groups

The cells were preconditioned by either a short episode of hypoxia or by  $\beta$ -adrenergic receptor stimulation. The cells were subsequently exposed to 2 hours of hypoxia as described above.

#### 2.2.3.3.1 Ischaemic preconditioning (IPC)

As with the hypoxia groups, cells were spun down at 250 rpm for 2 minutes (ischaemic pelleting), the supernatant removed until it was a third of the pellet size, and a layer of mineral oil added (hypoxia). These cells were then incubated at 37 °C for 10 minutes. The cells were removed from under the oil layer and resuspended in oxygenated solution D for 20 minutes, before being subjected to 2 hours of hypoxia as described in 2.2.3.2

#### 2.2.3.3.2 $\beta$ -adrenergic receptor stimulated preconditioning ( $\beta$ PC)

The cells were gently resuspended in Solution D containing  $10^{-7}$  M isoproterenol for 10 minutes at 37 °C. The supernatant was removed and the cells washed twice with oxygenated Solution D before allowing to settle for 20 minutes. After this, cells were exposed to 2 hours of hypoxia as described above.

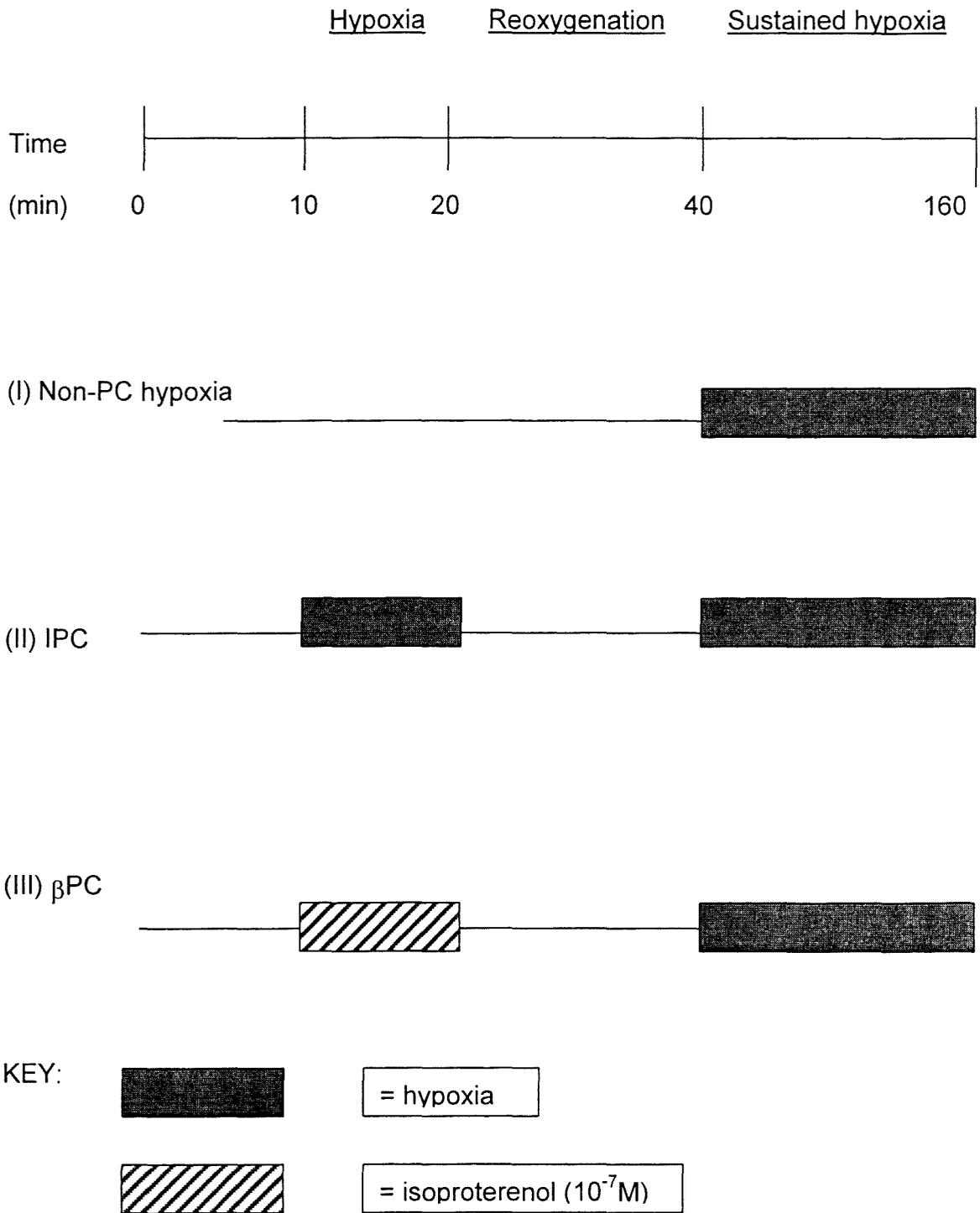


Fig 2.2 Experimental protocols for the isolated adult cardiomyocytes.

## **2.3 The isolated perfused heart**

### **2.3.1 Perfusion technique of hearts used for determination of infarct size**

For measurement of infarct size, hearts were perfused retrogradely at a constant pressure of 100 cm H<sub>2</sub>O. A water filled latex balloon was inserted into the left ventricle via the left atrium and the pressure recorded electronically and displayed on a computer monitor. Data was stored and analyzed using computer software developed by Mr. B. Genade. Left ventricular end-diastolic pressure was set between 4-8 mm Hg as baseline. Normothermic zero-flow global ischaemia was used to induce ischaemic preconditioning (3X5 min, alternated with 5 min reperfusion) but the sustained ischaemia was regional and induced by coronary artery ligation for 35 min followed by 30 min reperfusion. Reperfusion was initiated by loosening of the ligation. At the end of the experiment, the silk suture around the coronary artery was securely tied and ~1 ml of a 0.5% Evans Blue suspension slowly injected via the aorta cannula. The heart was then frozen overnight before being cut into 2 mm thick slices. After defrosting, the slices were stained with 1% w/v triphenyltetrazolium chloride in phosphate buffer (pH 7.4) at 37 °C for 15 minutes. Slices were then fixed in 10% v/v formaldehyde solution to enhance the contrast between stained viable tissue and unstained necrotic tissue.

The area of the left ventricle at risk and the area of infarcted tissue in the risk zone were determined using computerized planimetry (Summa Sketch II; Summa Graphics). The volume of infarcted tissue (I) and the risk zone (R) was then calculated by multiplying each area with the slice thickness and calculating the sum of the products. The infarct size was expressed as the percentage of the risk zone (I/R %).

### **2.3.2 Perfusion technique of hearts used for biochemical determinations**

The isolated working rat heart as described by Edoute et al (1988) was used as an experimental model. The hearts were rapidly excised and arrested in ice cold (4 °C) Krebs-Henseleit bicarbonate buffer containing (mM): NaCl 119, NaHCO<sub>3</sub> 25, KCl 4.75, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 0.6, NaSO<sub>4</sub> 0.6, CaCl<sub>2</sub> 1.25, Glucose 10, pH 7.4

After removal, the hearts were perfused by the Langendorff technique (Langendorff 1895) in a retrograde, non-recirculating manner with Krebs-Henseleit buffer oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37 °C. The left atrium was cannulated to allow atrial perfusion according to Neely et al (1984) as modified by Opie et al (1971).

Normothermic zero-flow global ischaemia was used to induce ischaemic preconditioning. The global ischaemia, for 25 min, was achieved by simultaneous clamping of both the aortic and left atrial cannulae. Temperature was carefully

controlled and maintained at 36.5 °C during sustained ischaemia by continuously monitoring using a temperature probe inserted into the pulmonary artery. Hearts were subsequently reperfused for 30 min (10 min retrogradely, 20 min working heart) by unclamping the tube to the aortic cannula. Hearts were freeze-clamped at the end of reperfusion for subsequent analyses.

### **2.3.3 Experimental protocols using infarct size as endpoint**

#### **2.3.3.1 Controls**

Control hearts were perfused for 30 or 60 min under oxygenated conditions before freeze-clamping of tissue.

#### **2.3.3.2 Non-preconditioned hearts (Non-PC)**

Hearts were stabilized for a period of 60 min (15 min retrograde, 15 min working heart, 30 min retrograde), followed by 35 min regional ischaemia and 30 min reperfusion (10 min retrograde, 20 min working heart).

#### **2.3.3.3 Ischaemic preconditioned hearts (IPC)**

Hearts were stabilized for a period of 30 min (15 min retrograde, 15 min working heart) and then preconditioned by 3 x 5 min global ischaemia, alternated with 5

min reperfusion. Hearts were subsequently subjected to 35 min regional ischaemia and 30 min reperfusion as described above.

#### 2.3.3.4 $\beta$ -adrenergic receptor stimulated preconditioned hearts ( $\beta$ PC)

Hearts were stabilized for 50 minutes (15 min retrograde, 15 min working, 20 min retrograde) after which  $10^{-7}$ M of isoproterenol was introduced for 5 minutes, and then subsequently washed out for 5 minutes, before induction of sustained regional ischaemia and reperfusion as described above.

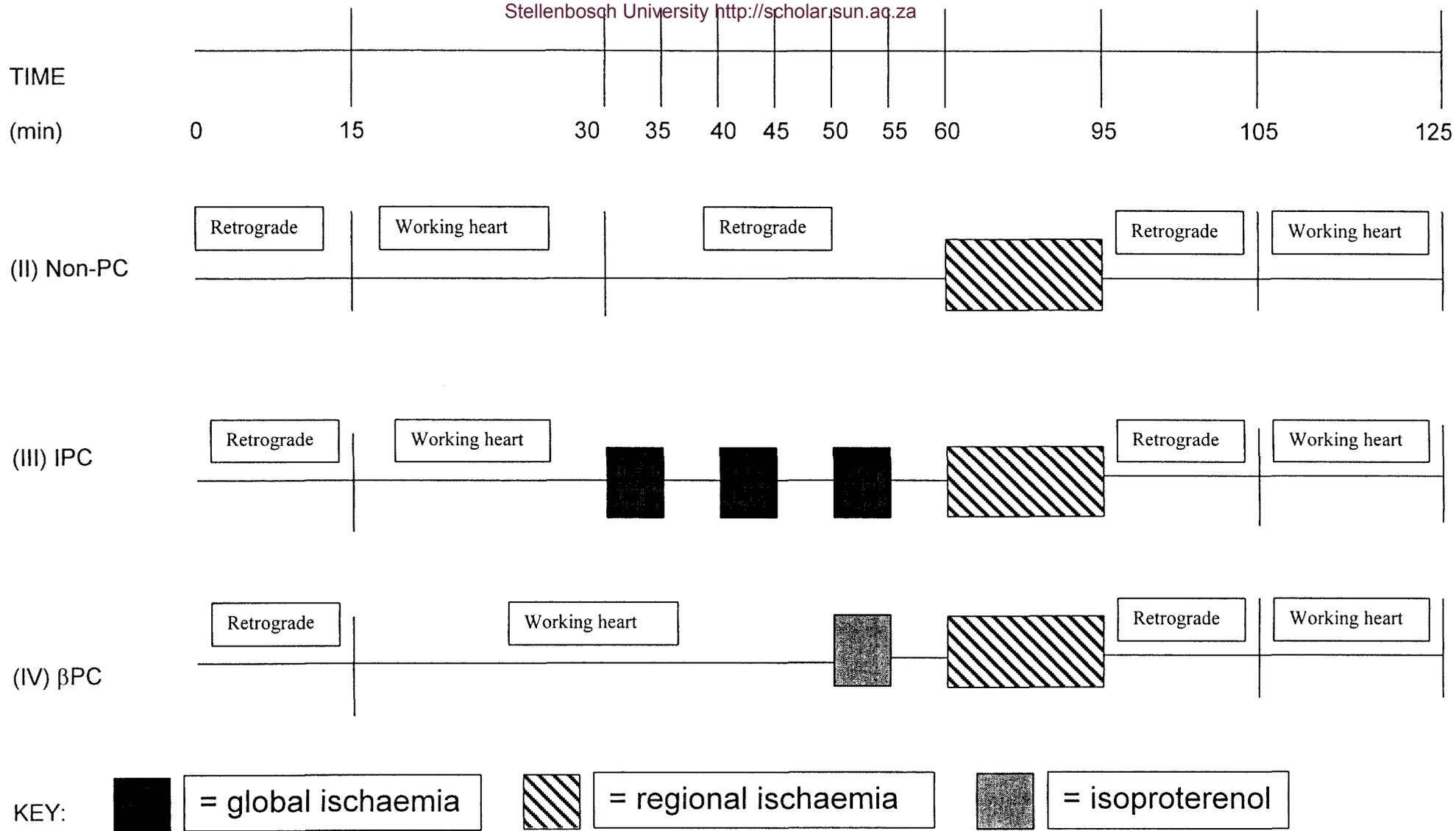


Fig 2.3 Experimental protocols for the effect of preconditioning on infarct size in isolated perfused hearts.

#### 2.3.3.5 SB203580 treated hearts

SB 203580, a blocker of p38 MAPK, was used at a concentration of 2  $\mu$ M in a number of studies. The following protocols preceded 35 min regional ischaemia and 30 min reperfusion:

- (1) hearts were stabilized for 50 min (15 min retrograde, 15 min working, 20 min retrograde), followed by 10 min administration of SB 203580,
- (2) hearts were perfused retrogradely for 15 min, followed by 10 min in the working mode. SB 203580 was administered for 5 min before and after the first PC episode and also after the second PC episode. After the third PC episode, hearts were perfused for 10 min to ensure washout of the drug.

#### 2.3.3.6 Anisomycin stimulated preconditioned hearts

Anisomycin at a concentration of 5  $\mu$ M was used. The following protocols preceded 35 min regional ischaemia and 30 min reperfusion (see above) of the isolated hearts:

- (1) 15 min retrograde, 15 min working, 20 min retrograde, followed by 10 min anisomycin
- (2) 15 min retrograde, 15 min working, 10 min retrograde, followed by 10 min anisomycin; 10 min reperfusion.

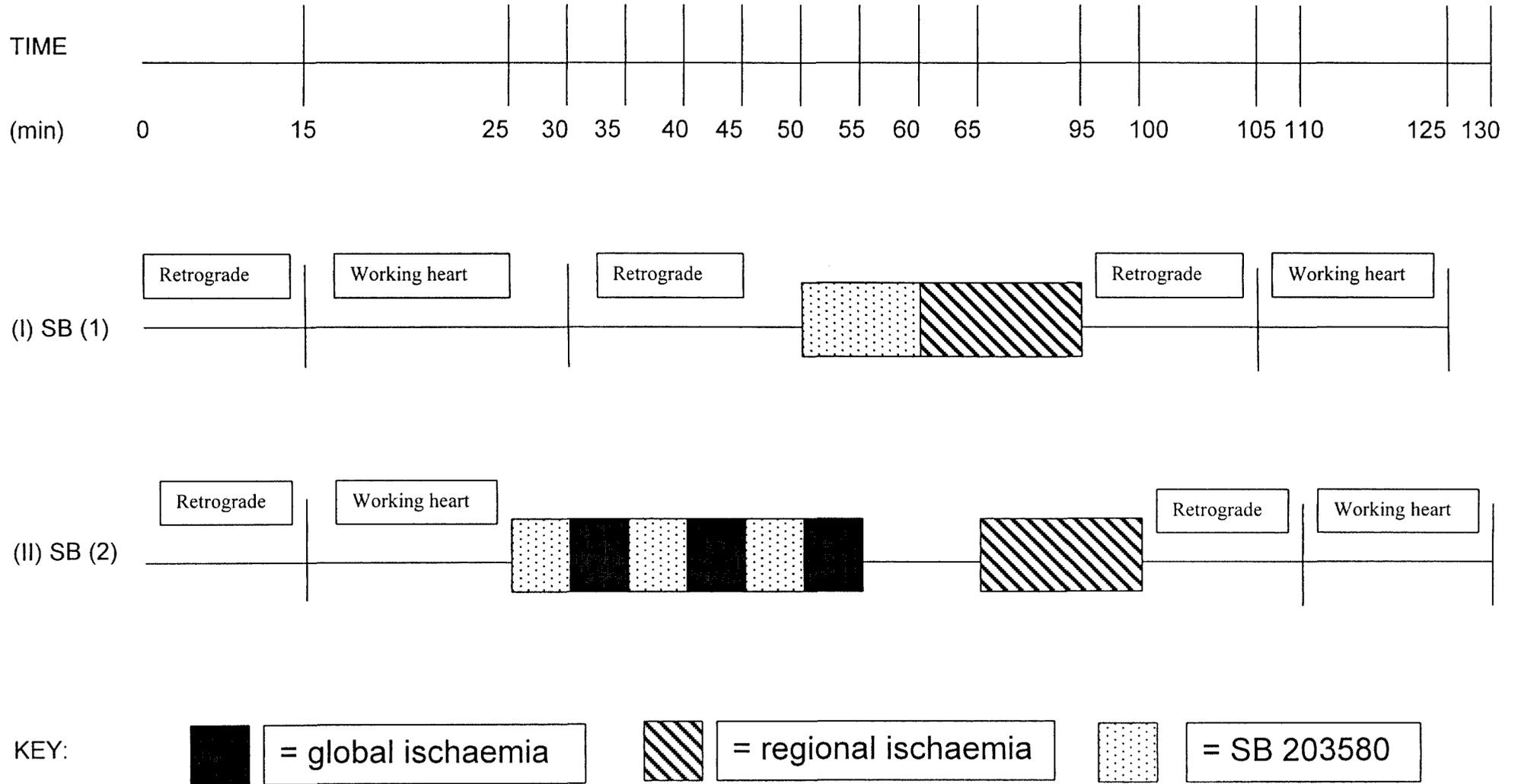


Fig 2.4 Experimental protocols for the effect of p38 MAPK inhibition with SB 203580 (1) before ischaemia/reperfusion and (2) during the IPC protocol on infarct size in isolated perfused hearts.

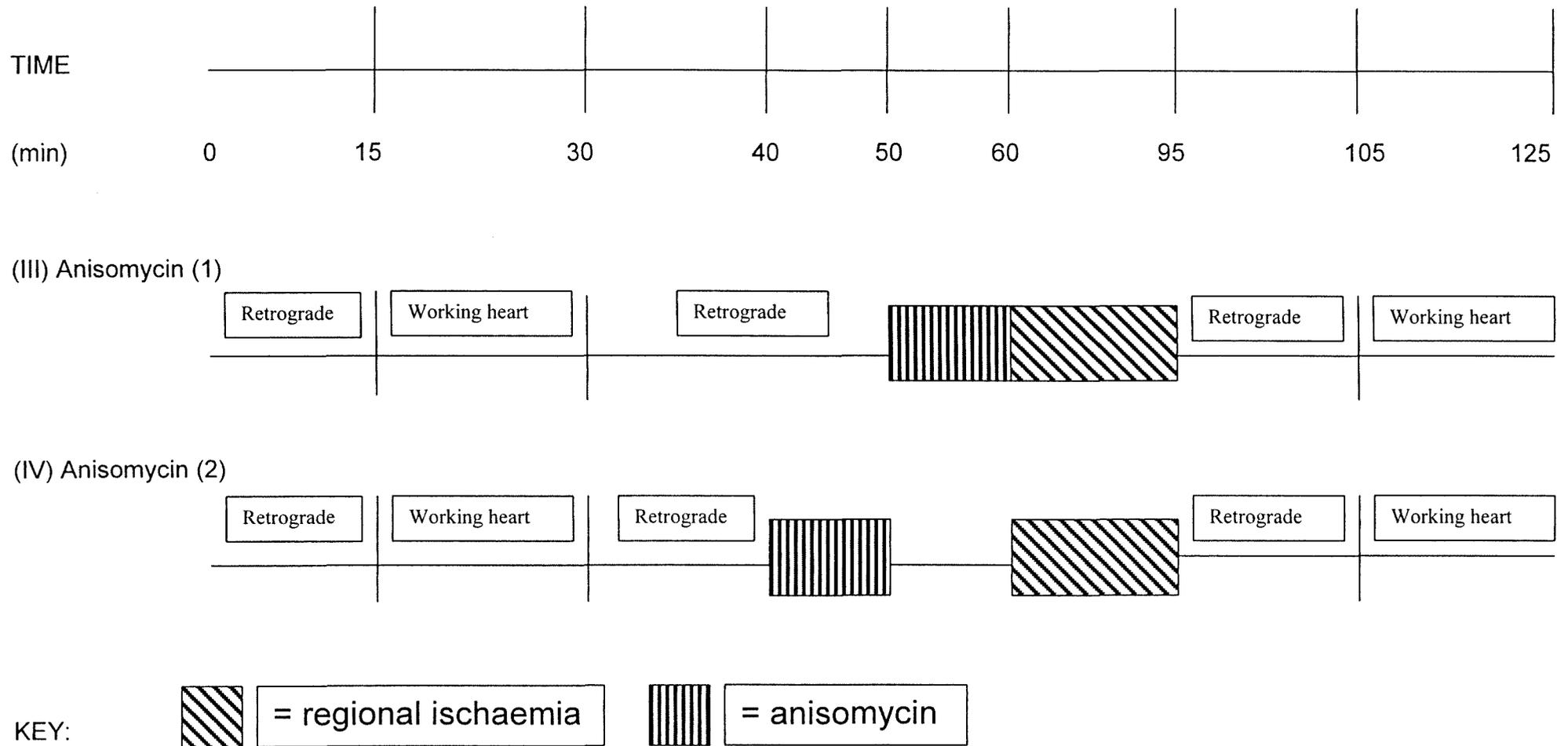


Fig 2.5 Experimental protocols for the effect of p38 MAPK activation with anisomycin (1) before ischaemia/reperfusion and (2) as a trigger on infarct size in isolated perfused hearts.

#### **2.3.4 Experimental protocols using markers of p38 MAPK activation and apoptosis as endpoint**

The same protocols as described in 2.3.3 were used. The only difference was that hearts were subjected to 25 min global ischaemia in contrast to 35 min regional ischaemia (Fig 2.6, Fig 2.7 and Fig 2.8).

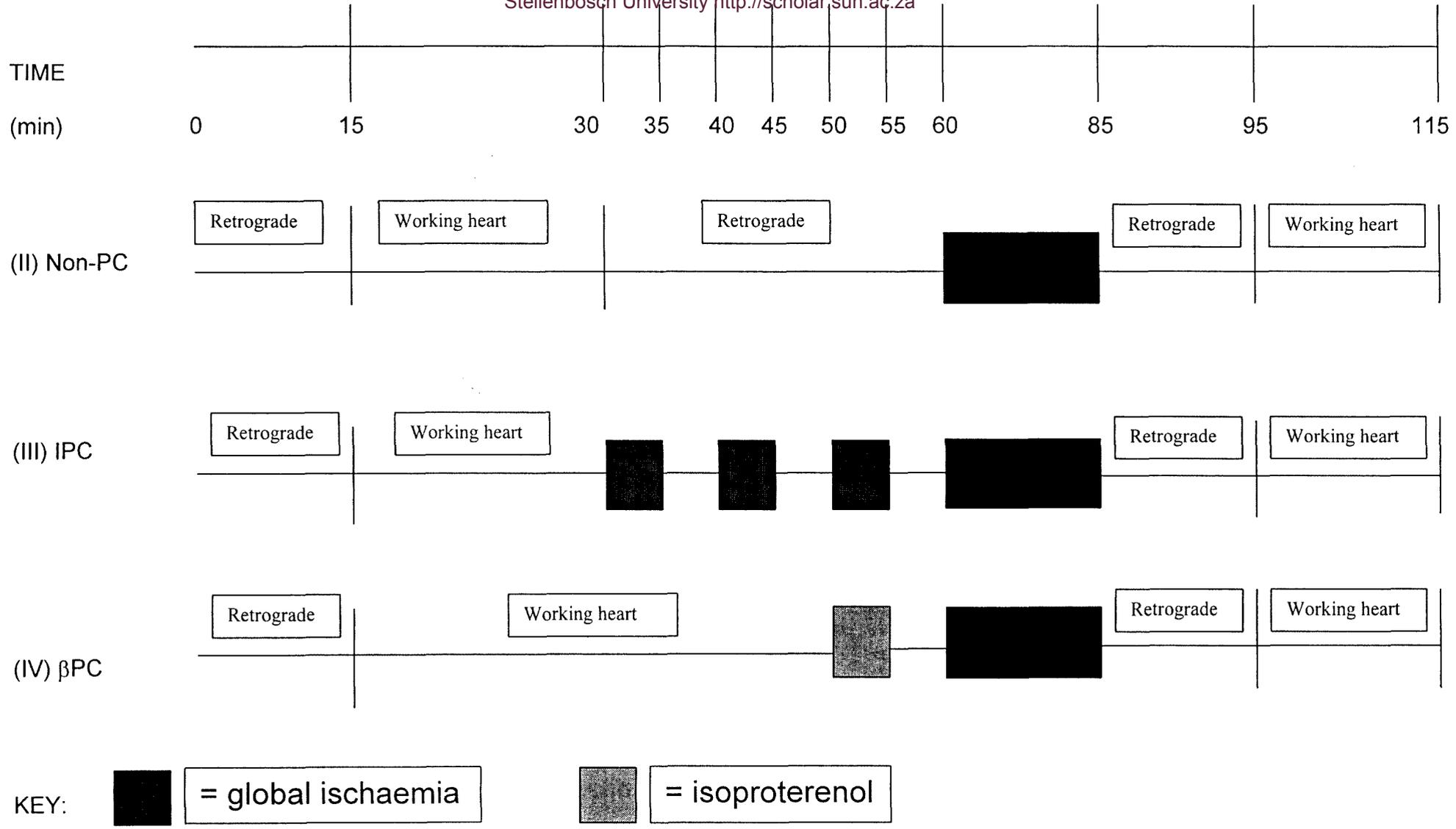


Fig 2.6 Experimental protocols for the effect of preconditioning on p38 MAPK activation, caspase-3 activation and PARP cleavage in isolated perfused hearts.

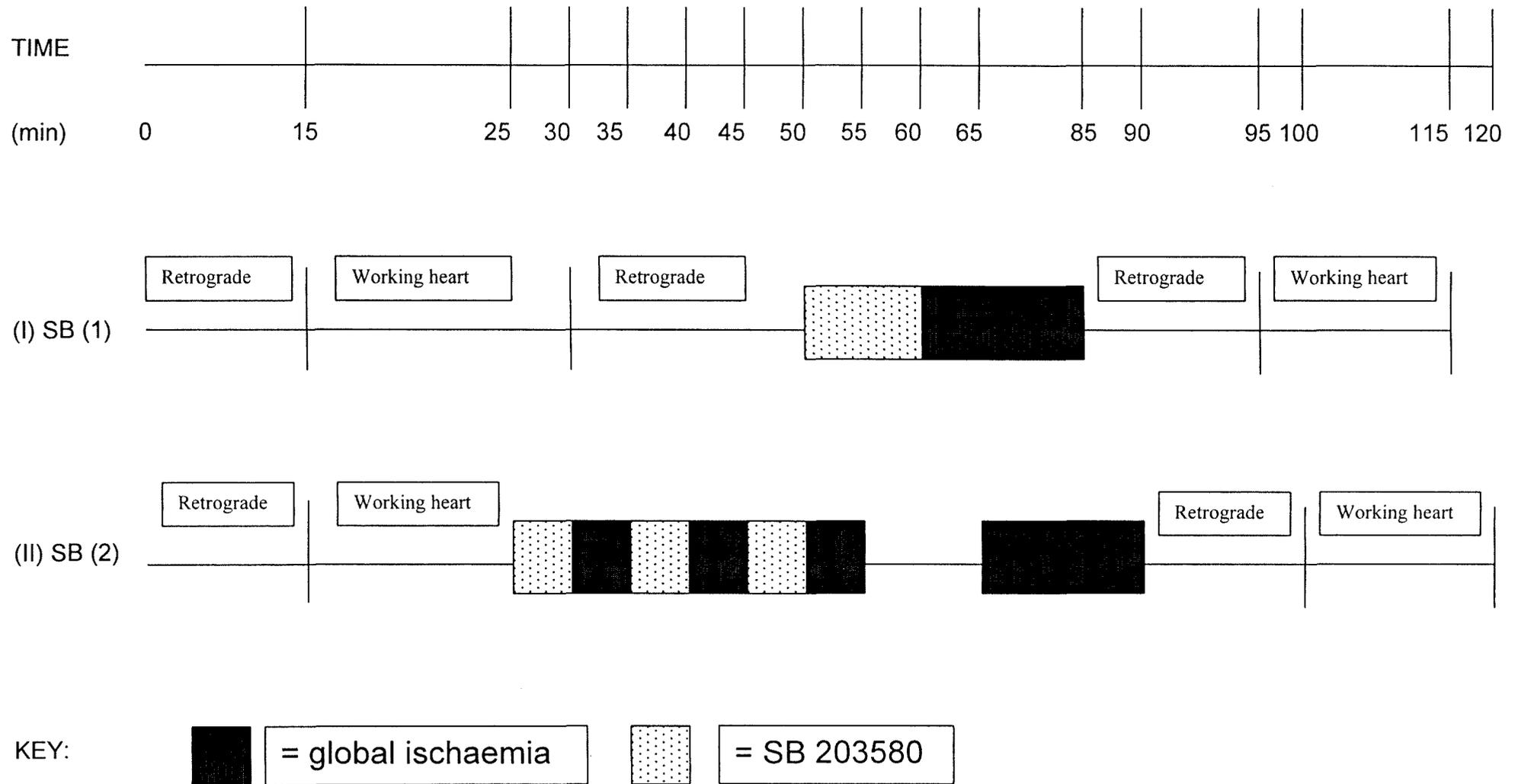


Fig 2.7 Experimental protocols for the effect of p38 MAPK inhibition with SB 203580 (1) before ischaemia/reperfusion and (2) during the IPC protocol on p38 MAPK activation, caspase-3 activation and PARP cleavage in isolated perfused hearts.

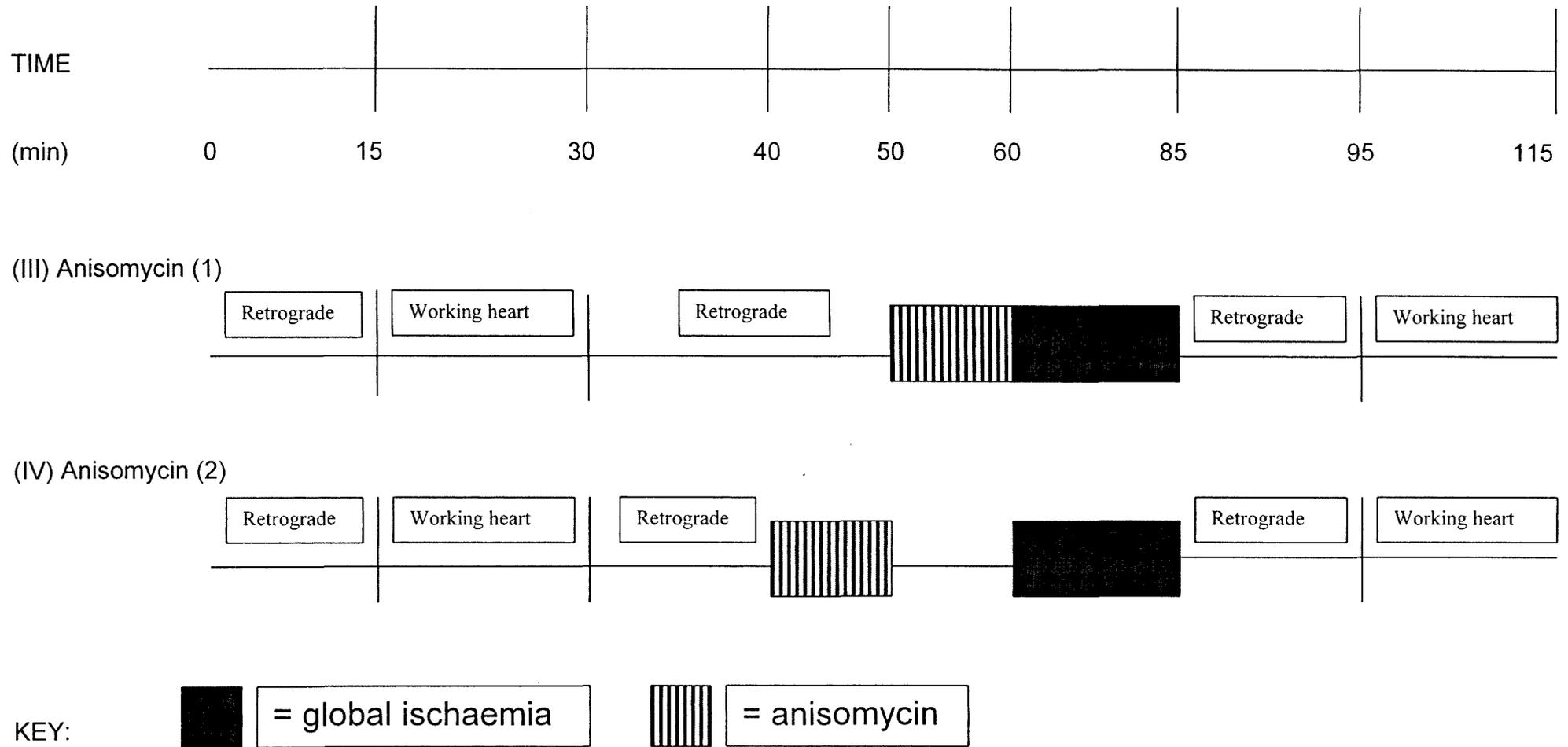


Fig 2.8 Experimental protocols for the effect of p38 MAPK activation with anisomycin (1) before ischaemia/reperfusion and (2) as a trigger on p38 MAPK activation, caspase-3 activation and PARP cleavage in isolated perfused hearts.

### 2.3.5 Experimental protocols for the collection of tissue samples

Hearts were freeze-clamped with precooled Wollenberger tongs at different times of the perfusion protocol and plunged into and stored in liquid nitrogen. The freeze-clamped tissue was homogenized and treated with a lysis buffer containing (in mM): Tris 20, P-nitrophenylphosphate 20, EGTA 1, NaF 50, Sodium orthovanadate 0.1, Phenylmethyl sulphonyl flouride (PMSF) 1, Dithiotreitol (DTT) 1, Aprotinin 10 µg/ml, Leupeptin 10 µg/ml. The tissue lysates were diluted in Laemmli sample buffer, boiled for 5 minutes and the appropriate amount of protein was separated by electrophoresis on the appropriate percentage polyacrylamide gel, using the Bio-RAD Mini-PROTEAN II System. The lysate protein content was determined using the Bradford technique.

The separated proteins were transferred to a PVDF membrane (Immobilon<sup>®</sup> P, Millipore). These membranes were stained with Ponceau Red for visualization of proteins. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline [Tris 4.84% w/v and NaCl 16% w/v in deionised water; pH 7.6]- 0.1% Tween 20 (TBST)]. The activated enzyme was visualized with the appropriate primary antibody. Membranes were subsequently washed with large volumes of TBST (3x1 min and then 2x5 min) and the immobilized antibody conjugated with a diluted horseradish peroxidase-labelled secondary antibody (Amersham LIFE SCIENCE). After thorough washing with TBST, membranes were covered with ECL detection reagents and exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through

a non-radioactive method (ECL Western blotting). Films were densitometrically analyzed (UN-SCAN-IT, Silkscience).

#### 2.3.5.1 p38 mitogen activated protein kinases (p38 MAPK)

10 µg of protein was separated by electrophoresis on a 12% polyacrylamide gel and the primary antibody used was dual phosphorylated p38 MAPK (Thr180/tyr182) (Cell Signaling – Massachusetts, USA).

#### 2.3.5.2 Caspase-3

The following procedure was done according to the technique of Schagger and Von Jagow (1987). 50 µg protein was separated by electrophoresis on a 4% T, 3% C stacking and a 16.5% T, 3% C separating gel. T denotes the total percentage concentration of both monomers (acrylamide and bisacrylamide). C denotes the percentage concentration of the crosslinker relative to the total concentration. The anode buffer used contained 0.2M Tris at a pH of 8.9 and the cathode buffer contained 0.1M Tris; 0.1M Tricine, and 0.1% sodium dodecyl sulphate (SDS) at a pH of 8.25. The primary antibody used was an anti-cleaved Caspase-3 (Asp 175) (Cell Signaling – Massachusetts, USA).

### 2.3.5.3 Poly ADP ribose polymerase (PARP)

50 µg of protein was separated by electrophoresis on a 12% polyacrylamide gel and the primary antibody used was an Anti-PARP p85 fragment (Promega Corporation – Wisconsin, USA).

## 2.4 Chemicals

Bovine serum albumin, bovine serum albumin fraction V, and collagenase for the neonatal cells in culture were obtained from Roche (Basel, Switzerland).

Collagenase for the preparation of isolated adult cardiomyocytes was obtained from Worthington Biochemical Corporation (New Jersey, USA). Isoproterenol was obtained from Adcock Ingram (Gauteng, RSA) and SB 203580 from Calbiochem (California, USA). All other analytical grade chemicals were purchased from Sigma (St. Louis, USA) or Merck (Darmstadt, Germany).

## 2.5 Statistical Analysis

Results are expressed as mean  $\pm$  SEM (standard error of the mean). P-values were determined by ANOVA and subjected to a TUKEY post-hoc analysis. Values of  $p < 0.05$  were regarded as statistically significant. In certain series of experiments, groups were compared using the unpaired Student's T-test (explained later).

## Chapter 3

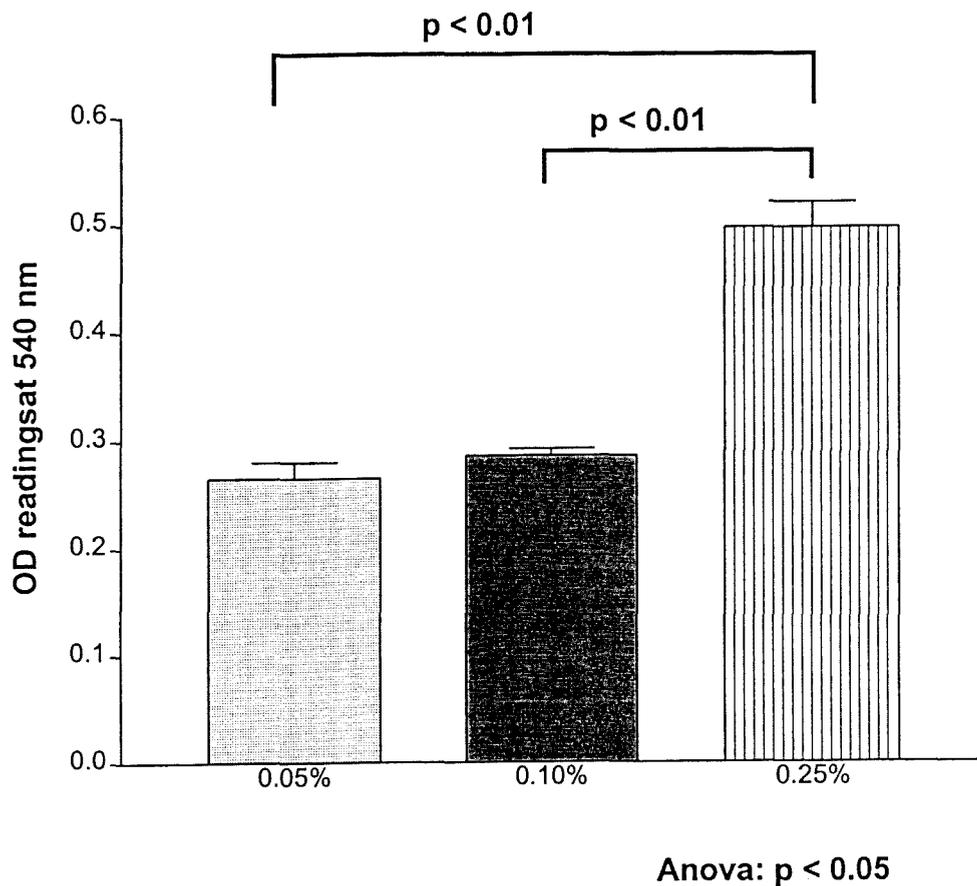
### Results

#### 3.1 Neonatal myocytes

##### 3.1.1 Optimization of MTT assay for cultured neonatal cardiomyocytes

The first aim was to optimize conditions for the MTT assay in order to evaluate viability of neonatal cardiomyocytes. The optimum concentration of MTT to use in cultured cardiomyocytes had to be established. Three final concentrations of MTT solution i.e., 0.05%, 0.1% and 0.25% were tested in the same cell population size (650 000 – 800 000 cells/ml). After 2 hours of incubation at 37 °C with the MTT solution, there was no significant difference in the optical density (OD) readings at 540 nm between the groups exposed to 0.05% and 0.1% of MTT solution respectively (Fig 3.1). However, 0.25 % of MTT solution produced a significant increase in the OD readings of the particular cell population when compared to both the 0.05% ( $p < 0.01$ ) and 0.1 % ( $p < 0.01$ ) of MTT solution respectively ( $p < 0.05$  for overall comparison). Further increases in MTT concentration did not increase the OD readings (results not shown). Therefore 0.25% of a MTT solution added to the wells was used throughout subsequent experiments on neonatal cardiomyocytes in which viability was assessed.

**Fig 3.1 Optimization of MTT concentration for neonatal cardiomyocytes in culture**



For each series, 2 different neonatal preparations were studied. Each condition was studied in duplicate.

### **3.1.2 Optimization of the preconditioning protocol for neonatal cardiomyocytes in culture**

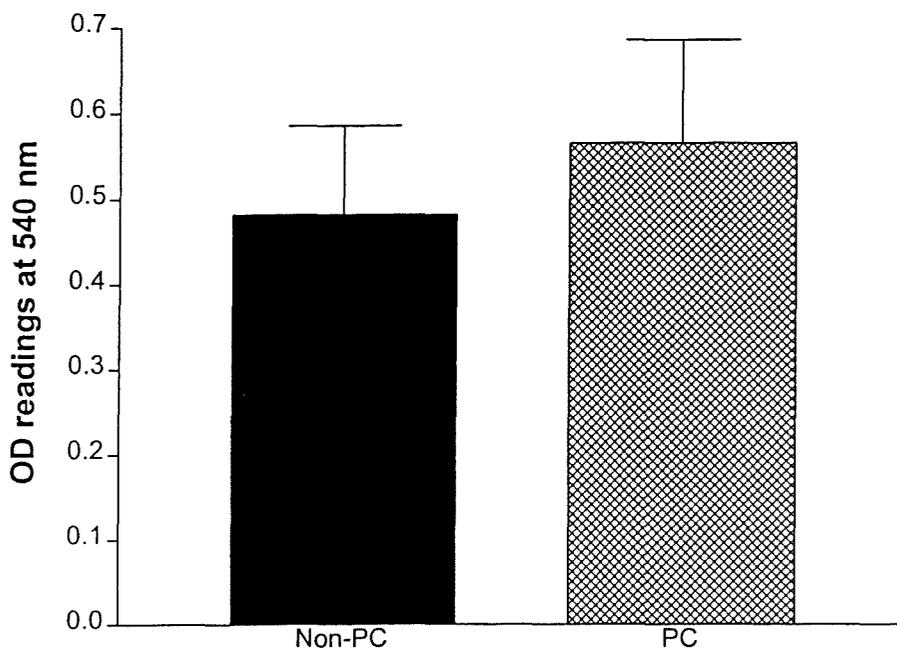
Three protocols were tested for their ability to elicit preconditioning in the cultured neonatal cardiomyocytes and were compared to the non-preconditioned (Non-PC) cells, which were exposed to chemical hypoxia only (see “Materials and Methods” 2.1.5). The MTT assay was used throughout the experiments as an indicator of cell viability. All experiments were performed at 37 °C.

The first protocol tested was:

- (i) 5 minutes exposure to 5 mM KCN as a preconditioning stimulus or “trigger” followed by 60 minutes of reoxygenation in DMEM. This was followed by 5 minutes exposure to a combination of KCN and deoxyglucose (DOG), used as the chemical simulated ischaemia (“test ischaemia”), followed by 10 minutes of reoxygenation in DMEM.

This protocol was not successful in preconditioning the cells, as the MTT assay showed no significant difference in viability at the end of 5 min chemical ischaemia (using KCN + DOG) and 10 min of reoxygenation between the PC and Non-PC groups (Fig 3.2).

**Fig 3.2 Effect of preconditioning on neonatal cell viability.**  
Preconditioning protocol: 5 min KCN, 60 min reox.,  
"Test ischaemia" 5 min KCN + DOG, 10 min reox.



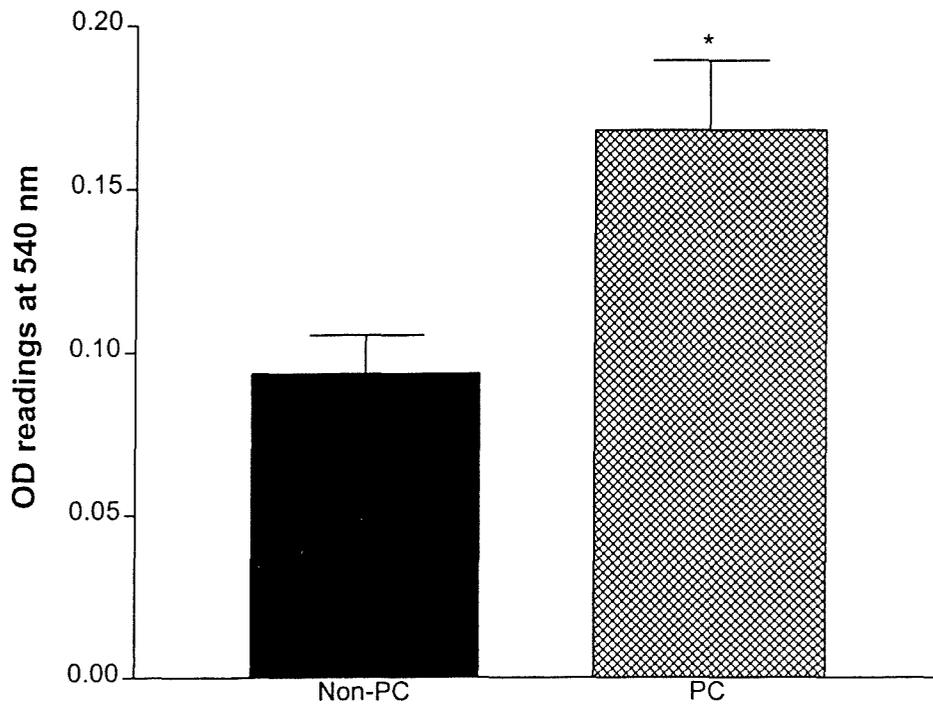
For each series, 4 different neonatal preparations were studied.  
Each condition was studied in triplicate or quadruplicate.

Exposure to simulated ischaemia of longer duration, but without DOG was decided upon:

- (ii) 5 minutes exposure to 5 mM KCN as a preconditioning stimulus followed by 60 minutes of reoxygenation in DMEM was used as before. This was followed by 45 minutes exposure to KCN only, as the chemical simulated ischaemia ("test ischaemia"), followed by 10 minutes of reoxygenation in DMEM.

In a limited number of experiments (n = 10 different neonatal preparations), cells that were preconditioned before 45 min of simulated ischaemia showed a significant increase in viability when compared to cells that were only exposed to 45 min of simulated ischaemia and 10 min reoxygenation i.e., Non-PC groups (Fig 3.3). This PC protocol however, failed to consistently precondition the neonatal cells from one cell preparation to the next. In an almost equal number of experiments (n = 11 different neonatal preparations), the viability of the PC groups was not statistically different from that of the Non-PC groups at the end of reoxygenation (Fig 3.4). The combined results of all these experiments (n=21) are shown in Fig 3.5A and as a scattergram in Fig 3.5B.

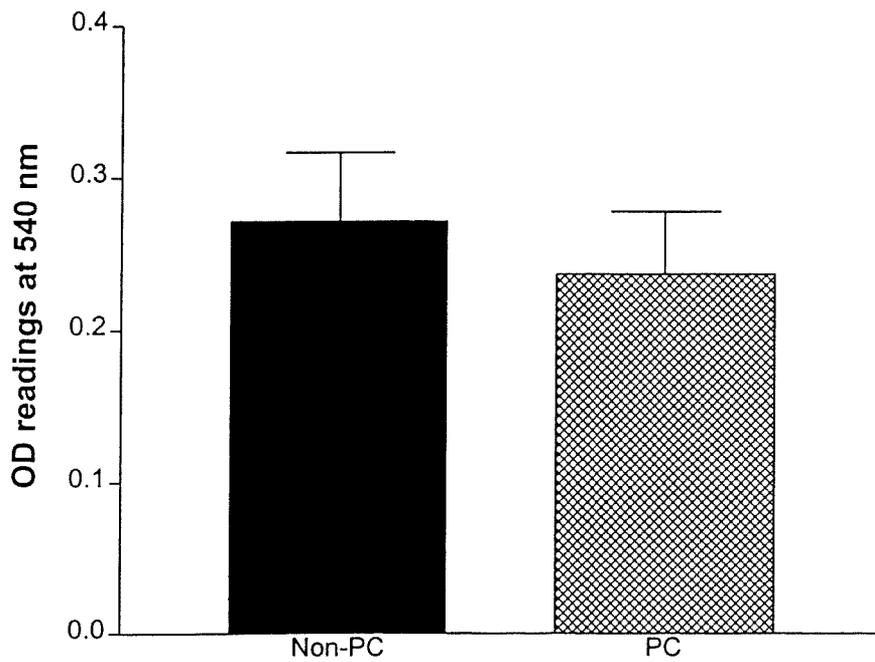
**Fig 3.3** Effect of preconditioning on neonatal cell viability. Preconditioning protocol: 5 min KCN, 60 min reox., "Test ischaemia" 45 min KCN, 10 min reox.



\*  $p < 0.005$  vs Non-PC

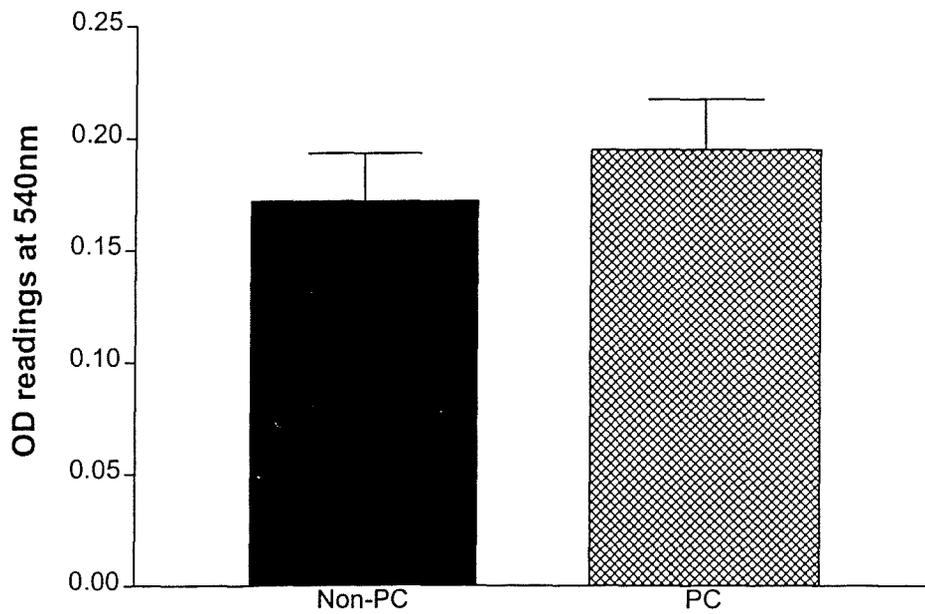
For each series, 10 different neonatal cell preparations were studied. Each condition was studied in triplicate.

**Fig 3.4** Effect of preconditioning on neonatal cell viability. Preconditioning protocol: 5 min KCN, 60 min reox., "Test ischaemia" 45 min KCN, 10 min reox.



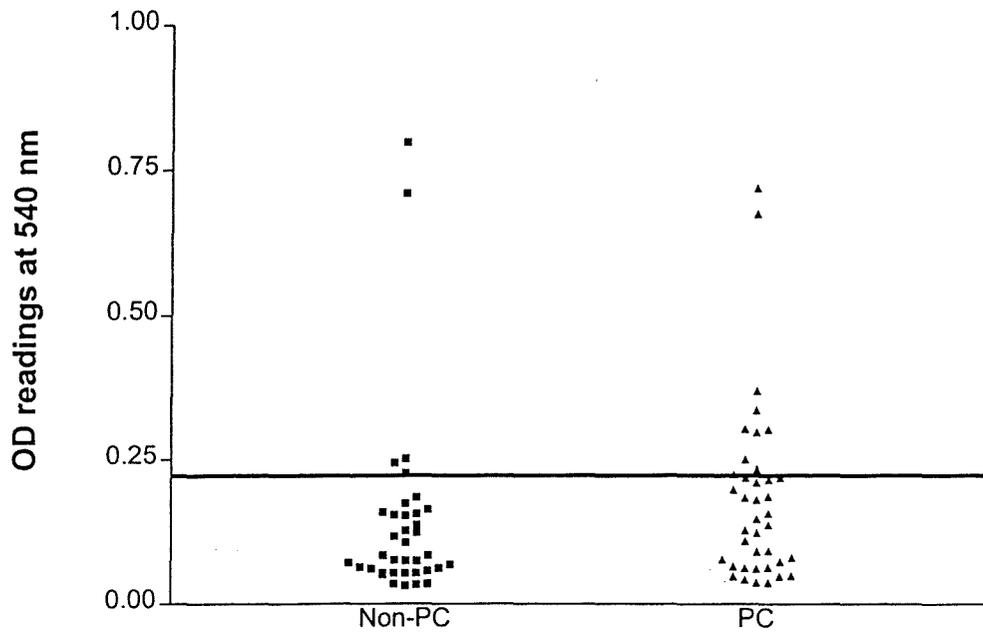
For each series, 11 different neonatal cell populations were studied. Each condition was studied in triplicate.

**Fig 3.5A** Effect of preconditioning on neonatal cell viability  
Preconditioning protocol: 5 min KCN, 60 min reox.,  
"Test ischaemia" 45 min KCN, 10 min reox.



For each series, 21 different neonatal cell populations were studied.  
Each condition was studied in triplicate.

**Fig 3.5B** Effect of preconditioning on neonatal cell viability.  
Preconditioning protocol: 5 min KCN, 60 min reox.,  
"Test ischaemia" 45 min KCN, 10 min reox.



All attempts to clarify this inability of the neonatal cardiomyocytes in culture to consistently precondition were unsuccessful. The following factors were considered:

- (i) Human error: more stringent attention to techniques employed in the preparation of the cultures such as aseptic technique; quantities, temperature and pH of buffers/solutions used in the preparation of the cultures was adhered to in order to prepare cultures that would be as identical as possible in terms of appearance and confluency on day 3. Day 3 was chosen as it represented the best degree of confluency in cultures that were previously successfully preconditioned.
- (ii) Age of the neonates: the age of neonates used to prepare the cultures were varied. Neonates ranging from 1 to 4 day were used. Cultures were prepared using only one particular age of neonate viz. 3 day old neonates.
- (iii) The growth medium was changed every 24 hours as opposed to every 48 hours to remove the cells that did not adhere to the fibronectin. These cells could deprive the adhering cells of nutrients and were seen as a possible factor that could affect confluency later on.
- (iv) Optimal confluency was reached from day 3 onwards, and experimentation was therefore done with these preparations. Neonatal cell cultures older than 3 days were also used to assess the influence of cell maturity on the results. However, no consistent preconditioning was observed with these older cultures either.

Since ischaemic preconditioning of the neonatal cells in culture was not producing the desired results it was decided to investigate the possibility that pharmacological preconditioning, for example  $\beta$ -adrenergic receptor stimulated preconditioning in particular, would provide us with the means to consistently precondition the neonatal cells in culture.

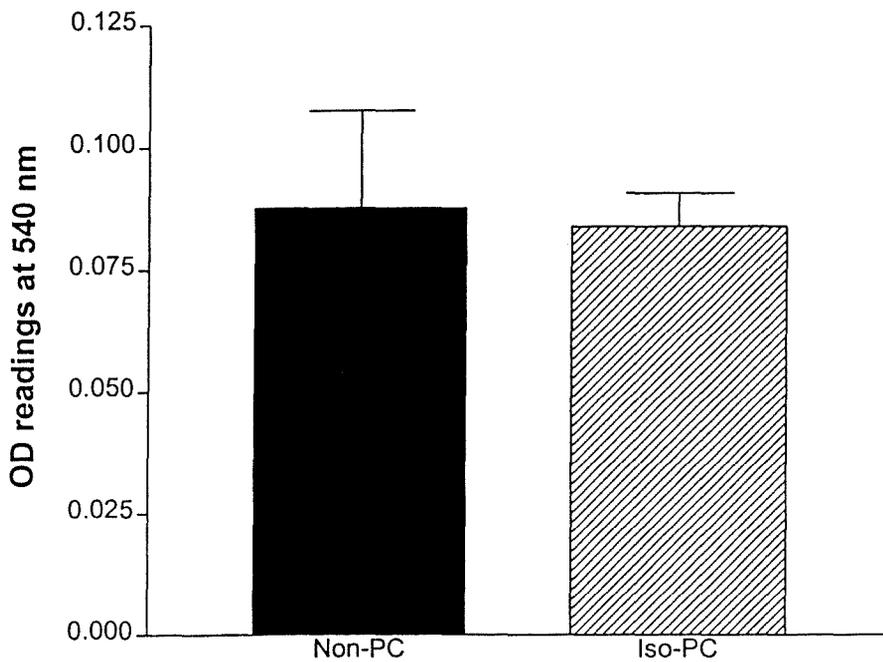
Thus the third protocol was:

- (iii) Exposure of the cells to 10 minutes of  $10^{-7}$  M isoproterenol, which was subsequently washed out with PBS. This was followed by 60 minutes reoxygenation in DMEM and 45 minutes in KCN (used as chemical simulated ischaemia) and finally 10 minutes reoxygenation in DMEM.

Unfortunately this PC protocol too caused no significant difference in cell viability after 45 min of simulated ischaemia and 10 min of reperfusion when compared to the Non-PC groups (Fig 3.6).

In order to assess the effects of ischaemia/hypoxia on myocardial necrosis and apoptosis, a reliable model of preconditioning was an absolute prerequisite. In view of the inexplicable variations in the outcome of the experiments performed, it was decided to switch to another model viz., the adult cardiomyocyte. It has been shown in our laboratory to precondition successfully (H. Strijdom, unpublished observations).

**Fig 3.6** Effect of preconditioning on neonatal cell viability.  
Preconditioning protocol: 5 min I, 60 min reox.,  
"Test ischaemia" 45 min KCN, 10 min reox.



Two different neonatal cell preparations were used.  
These were studied in triplicate.

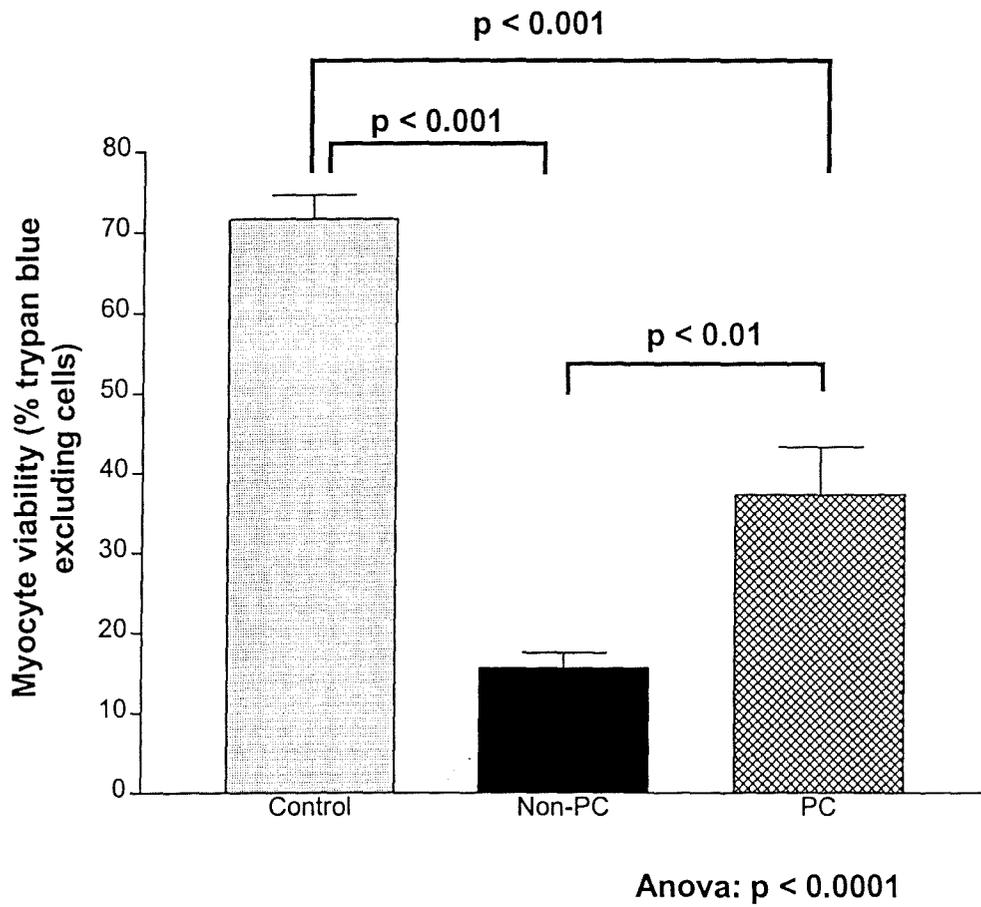
Iso = isoproterenol ( $10^{-7}$  M)

### 3.2 Preconditioning of isolated adult cardiomyocytes

Isolated adult cardiomyocytes used for the experiments were of optimal quality: Viability (expressed as the percentage trypan blue excluding cells) averaged between 70-80 % at time zero. Isolates with less than 70 % trypan blue exclusion were discarded. The difference between control cells, preconditioned cells (10 min hypoxia followed by 20 min reoxygenation) and Non-PC cells exposed to 2h of hypoxia was significant ( $p < 0.0001$ ). Cells exposed to 2 hours of hypoxia only i.e., Non-PC showed a significant reduction ( $15.6 \pm 1.9\%$ ) in viability when compared to cells incubated under oxygenated conditions ( $71.6 \pm 3.0\%$ ) ( $p < 0.001$ ). Preconditioned cells had a significantly higher viability ( $37.2 \pm 6.0\%$ ) after 2h of hypoxia compared to the Non-PC groups ( $15.6 \pm 1.9\%$ ) ( $p < 0.01$ ) (Fig 3.7). The MTT assay also showed similar significant increases in viability (OD/mg protein) when comparing the Non-PC groups to the controls and PC groups respectively (results not shown).

Although the isolated adult cardiomyocytes proved to precondition both successfully and consistently, the cell yield from one heart was insufficient to perform simultaneous evaluations of p38 MAPK, caspase-3 activation and PARP cleavage as well as cell viability. It was therefore decided to use the isolated perfused heart in all further experiments.

**Fig 3.7 Effects of preconditioning on viability of isolated adult cardiomyocytes (trypan blue exclusion method)**



Five isolated adult cardiomyocyte preparations were used.

### **3.3 The isolated perfused heart**

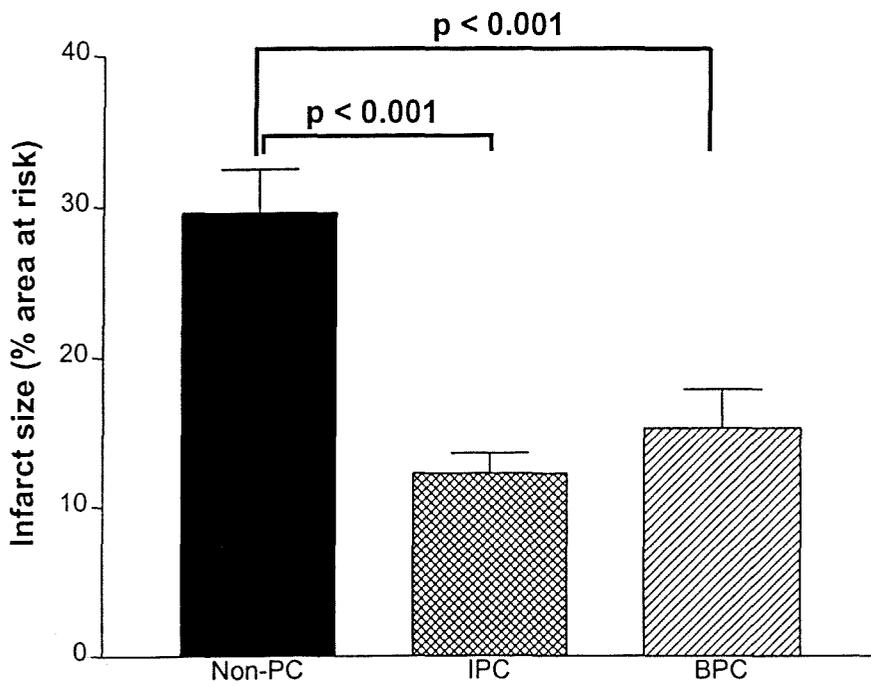
This model yields enough material for the simultaneous assessment of necrosis, apoptosis and p38 MAPK activation. This model also allows for pharmacological manipulation of p38 MAPK activation during both the ischaemic preconditioning protocol and during sustained ischaemia, and to evaluate the effects thereof on necrosis and apoptosis.

#### **3.3.1 Effects of ischaemic and pharmacological preconditioning on necrosis, p38 MAPK activation and apoptosis**

##### **3.3.1.1 Infarct size (% of area at risk) (Fig 3.8)**

The infarct size of the Non-PC and two PC groups differed significantly (Anova:  $p < 0.001$ ). Infarct sizes of hearts that were ischaemically preconditioned by 3 x 5 min global ischaemia ( $12.2 \pm 1.4\%$ ) and pharmacological preconditioning ( $\beta$ PC) with a 1 x 5 min cycle of isoproterenol ( $10^{-7}$ M) ( $15.2 \pm 2.6\%$ ) were significantly less than ischaemic hearts that were not preconditioned ( $29.6 \pm 2.9\%$ ) ( $p < 0.001$  for both comparisons).

**Fig 3.8 Effects of preconditioning on infarct size of perfused hearts (TTC staining)**



Anova:  $p < 0.0001$

Non-PC n = 8  
IPC n = 8  
BPC n = 9

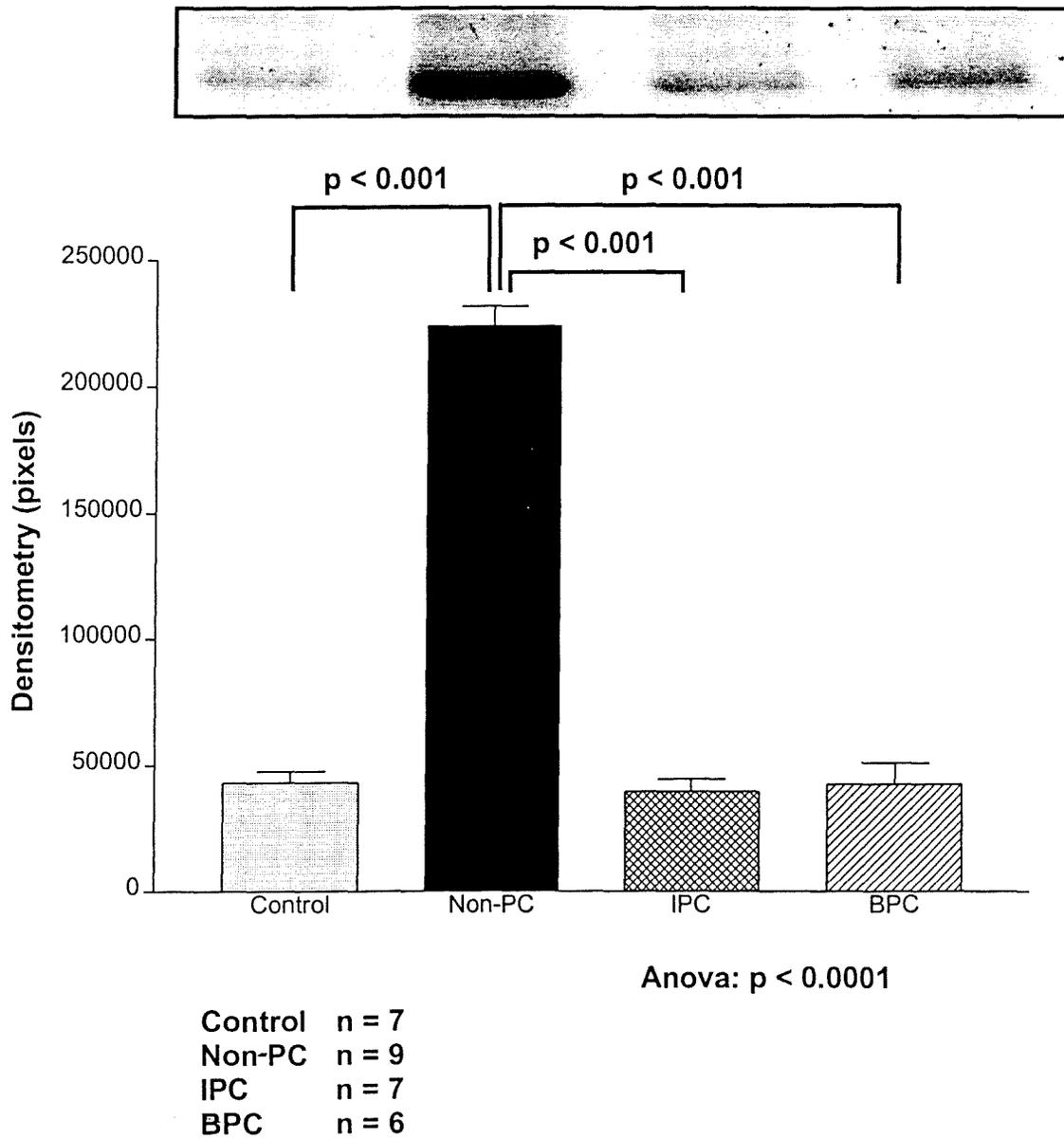
### 3.3.1.2 p38 MAPK (Fig 3.9)

p38 MAPK activation differed significantly between the groups (Anova:  $p < 0.0001$ ). Exposure of the heart to 25 min global ischaemia followed by 30 min reperfusion (Non-PC hearts) caused a significant increase in p38 MAPK activation ( $223\,800 \pm 7812$  pixels) when compared to the control hearts ( $43070 \pm 4535$  pixels) ( $p < 0.001$ ). p38 MAPK activation of IPC and  $\beta$ PC hearts were similar to controls and significantly lower than that of the Non-PC hearts ( $p < 0.001$  for both comparisons)

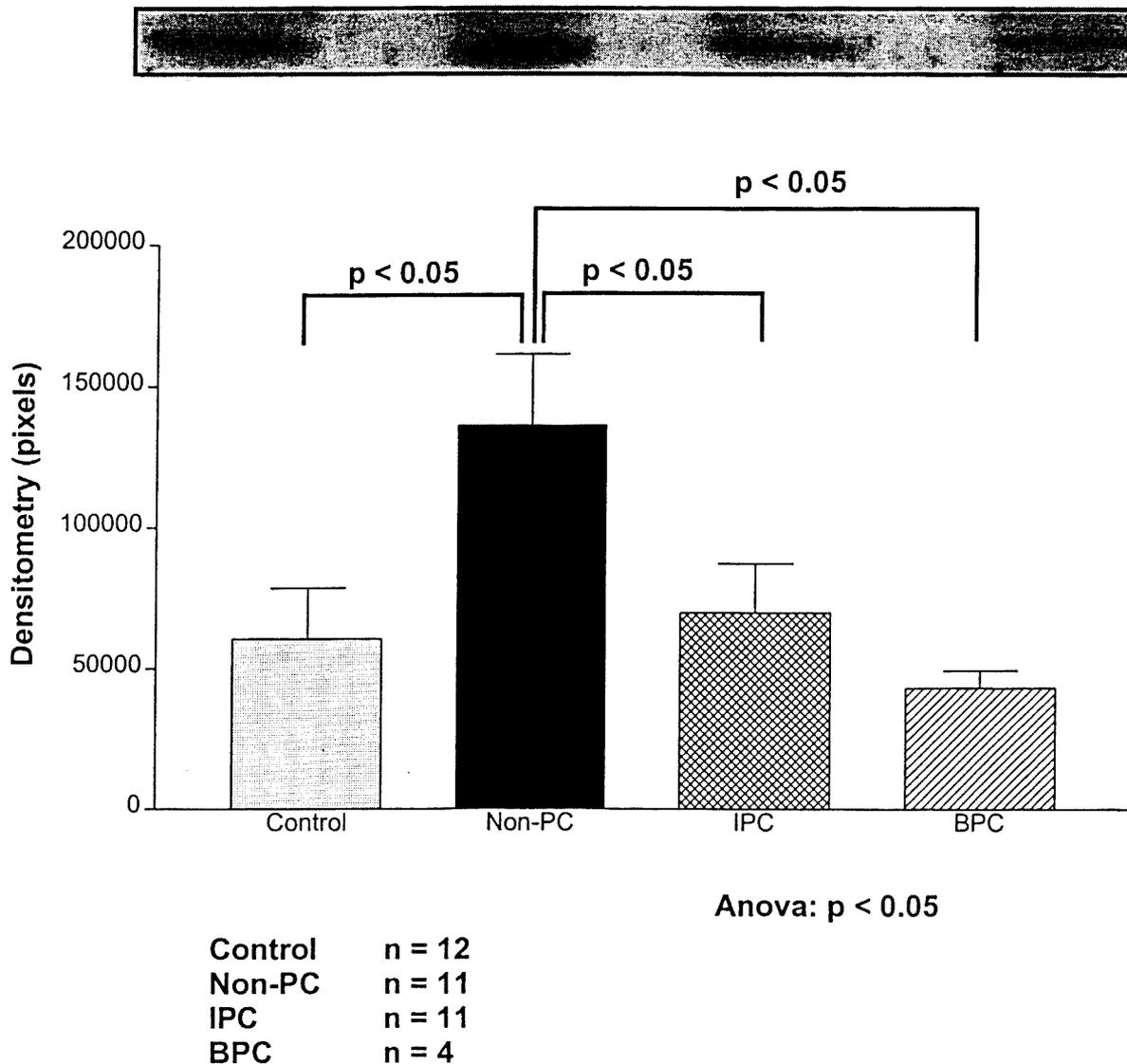
### 3.3.1.3 Caspase-3 (Fig 3.10)

Caspase-3 activation differed significantly between the different groups (Anova:  $p < 0.05$ ). Hearts that were not preconditioned showed a marked increase in caspase-3 activation ( $136200 \pm 25310$  pixels) when compared to the control hearts ( $60590 \pm 18090$  pixels) ( $p < 0.05$ ) (Fig 3.10). Preconditioned hearts (IPC and  $\beta$ PC) showed statistically significant lower caspase-3 activation ( $69750 \pm 17460$  pixels and  $43100 \pm 6168$  pixels respectively) compared to the Non PC hearts ( $p < 0.05$  for both comparisons).

**Fig 3.9 Effects of of preconditioning on p38 MAPK activation of perfused hearts at the end of reperfusion**



**Fig 3.10** Effects of preconditioning on caspase-3 activation of perfused hearts at the end of reperfusion

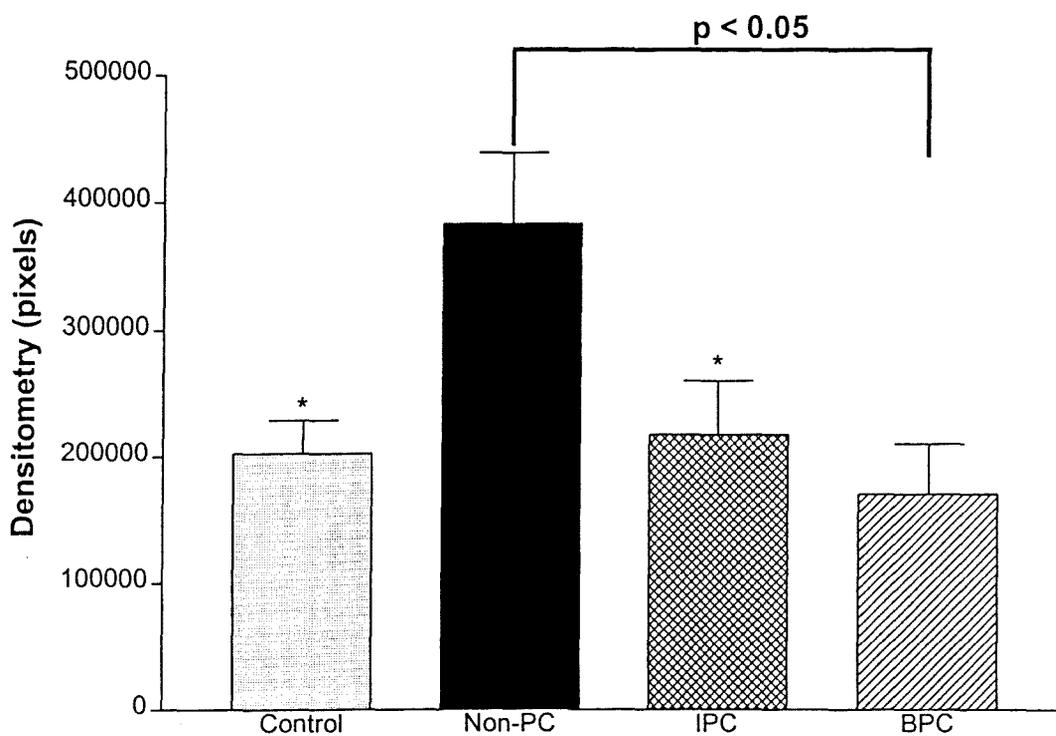


#### 3.3.1.4 PARP (Fig 3.11)

PARP cleavage as measured by Western blotting differed significantly between the different groups (Anova:  $p < 0.05$ ). Exposure of the heart to 25 min global ischaemia followed by 30 min reperfusion i.e., Non-PC hearts, caused a marked increase in PARP activation, and hence apoptosis, at the end of reperfusion ( $383100 \pm 55330$  pixels) when compared to the control hearts ( $262500 \pm 26540$  pixels) ( $p < 0.05$ ). Hearts preconditioned with both ischaemia and isoproterenol showed a significant decrease ( $p < 0.05$  for both comparisons) in PARP activation ( $217500 \pm 42550$  pixels and  $170300 \pm 39900$  pixels respectively) when compared to the Non-PC hearts (Fig 3.11).

These results indicate that apoptosis was decreased by IPC and  $\beta$ PC and was accompanied by reduced activation of p38 MAP kinase.

**Fig 3.11** Effects of preconditioning on PARP cleavage of perfused hearts at the end of reperfusion



Anova:  $p < 0.05$

\*  $p < 0.05$  vs Non-PC

Control n = 4  
Non PC n = 5  
IPC n = 5  
BPC n = 5

### **3.3.2 Effects of manipulation of p38 MAPK by SB203580 on necrosis, p38 MAPK activation and apoptosis**

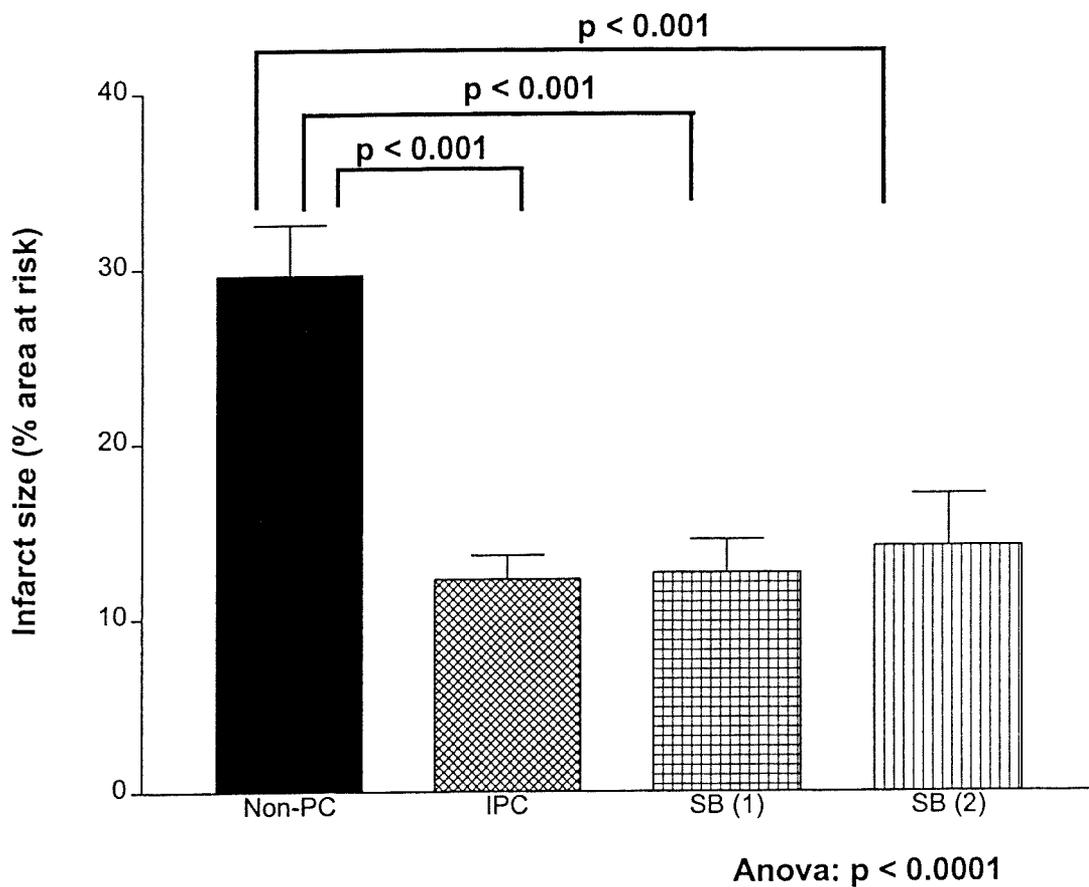
The next question that was addressed was the role of p38 MAP kinase in mediating the protective effect of preconditioning against ischaemia/reperfusion induced apoptosis.

Work performed in our laboratory (Marais et al 2001a) has shown that ischaemia of 5 min duration activated p38 MAPK. By appropriate use of the p38 MAPK blocker SB 203580, the role of p38 MAPK as a trigger and/or mediator of ischaemic preconditioning was evaluated. Administration of SB 203580 immediately before onset of sustained ischaemia and without washout allowed evaluation of its role as mediator of protection (SB 2035080 (1)). Administration of the drug, before and during the IPC protocol, but followed by washout before sustained ischaemia, allowed evaluation of its role as trigger (SB 203580 (2)).

#### **3.3.2.1 Infarct size (% area at risk) (Fig 3.12)**

Infarct size differed significantly between the different groups (Anova:  $p < 0.0001$ ). Hearts treated with SB 203580 10 min before ischaemia without washout ( $12.6 \pm 1.9\%$ ) and hearts treated with SB 203580 during the 3 x 5 min preconditioning protocol showed a significant reduction in infarct size at the end of reperfusion ( $14.1 \pm 2.9\%$ ) compared to the Non-PC hearts ( $29.6 \pm 2.9\%$ )

**Fig 3.12 Effects of SB 203580 on infarct size of perfused hearts (TTC staining)**



Non-PC	n = 8
IPC	n = 8
SB (1)	n = 5 (10 min before SI)
SB (2)	n = 8 (during the IPC protocol)

( $p < 0.001$ ). Infarct size of the two SB 203580 treated groups were similarly reduced.

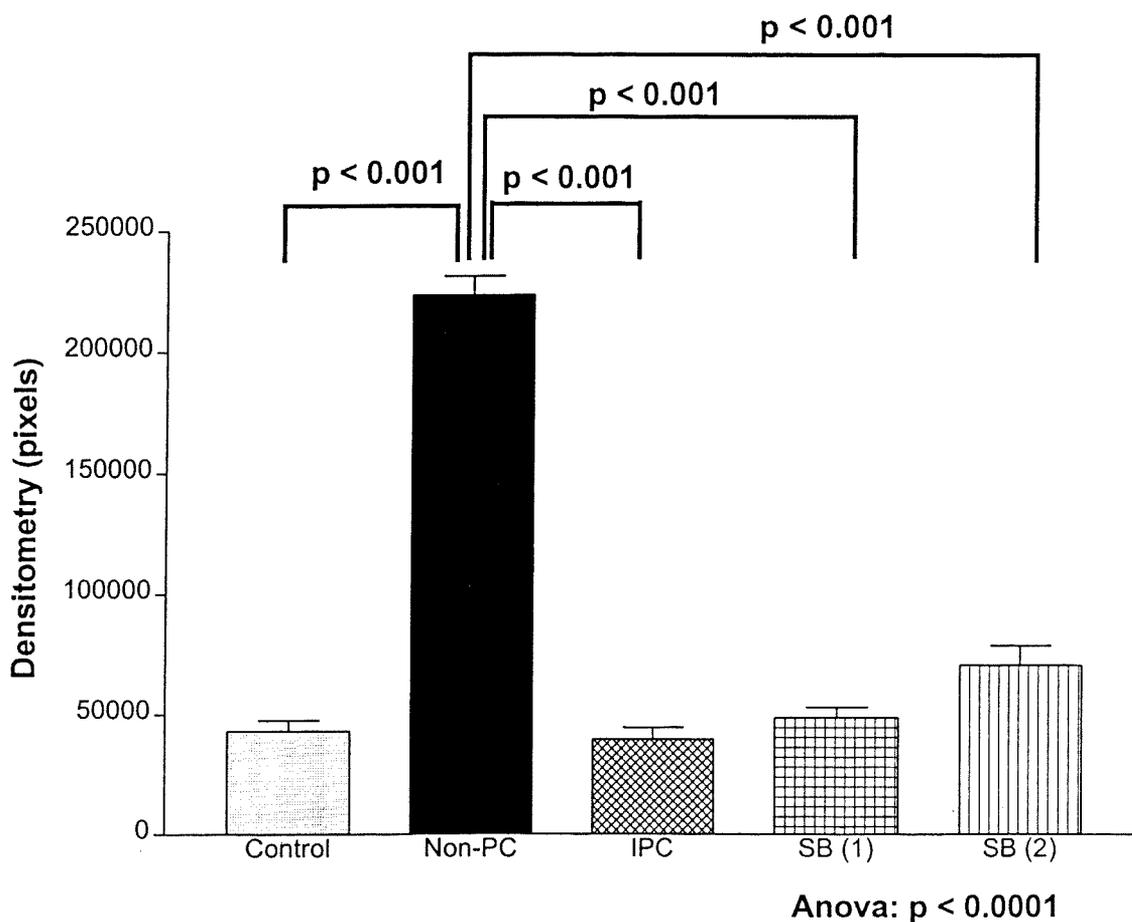
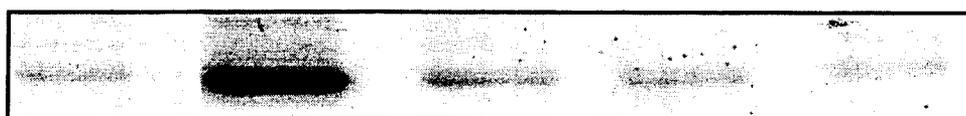
### 3.3.2.2 p38 MAPK (Fig 3.13)

p38 MAPK activation of all the different groups differed significantly (Anova:  $p < 0.0001$ ). p38 MAPK activation of hearts perfused with SB 203580 10 min (without washout) before ischaemia and bracketing of the IPC protocol (i.e., administered during preconditioning and washed out before sustained ischaemia) ( $48710 \pm 4192$  and  $70550 \pm 8011$  respectively) was significantly less than that of the Non-PC hearts ( $223800 \pm 7812$ ) ( $p < 0.001$  for both comparisons).

### 3.3.2.3 Caspase-3 (Fig 3.14)

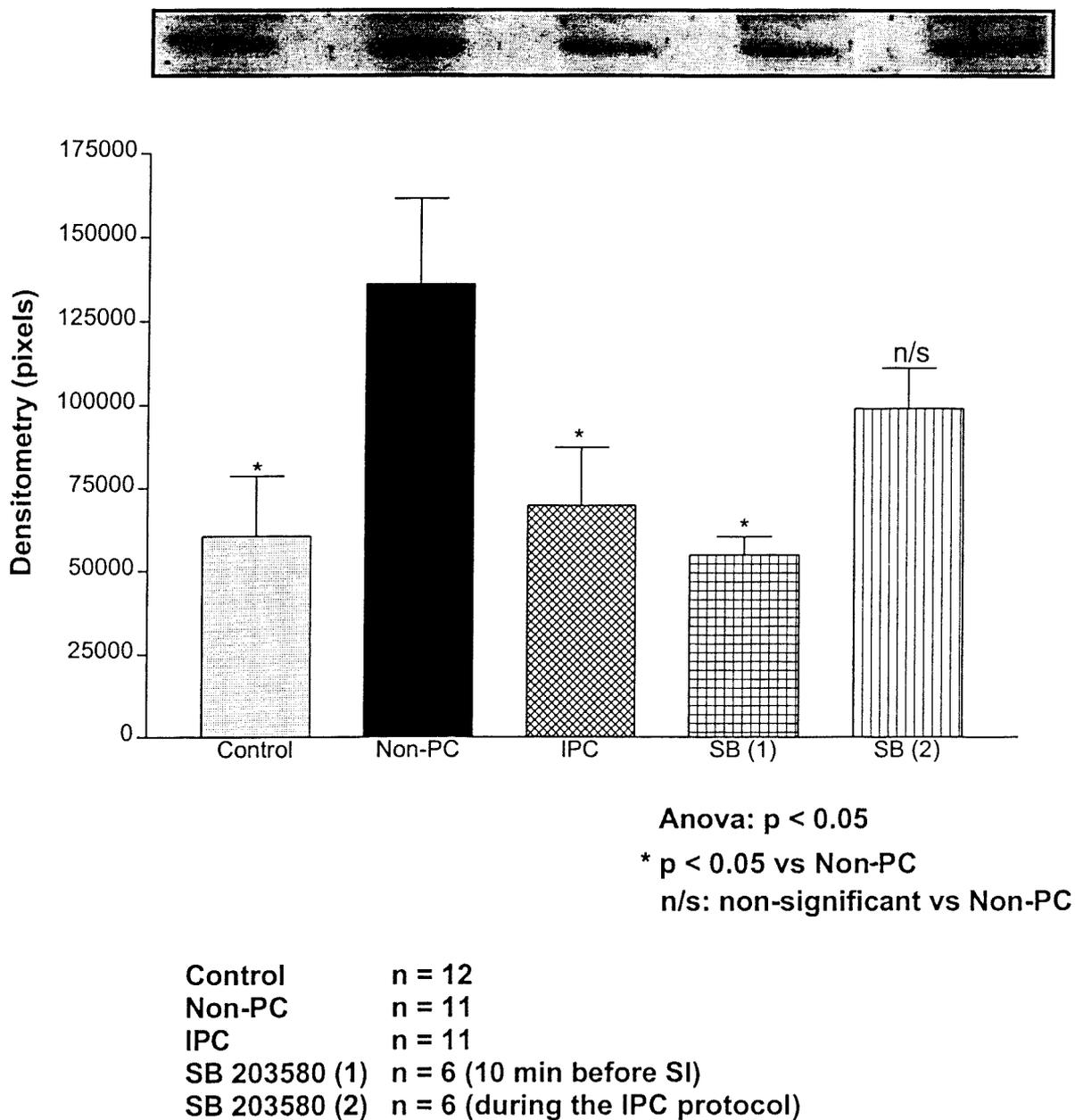
Caspase-3 activation of the different groups differed significantly (Anova:  $p < 0.05$ ). Inhibition of p38 MAPK with SB 203580 10 min before sustained ischaemia reduced caspase-3 activation ( $54860 \pm 5517$  pixels) when compared to the Non-PC groups ( $136200 \pm 25310$ ) ( $p < 0.05$ ). Hearts treated with SB 203580 during the 3 x 5 min IPC protocol, showed no significant difference in caspase-3 activation ( $98930 \pm 12060$  pixels) when compared to the Non-PC hearts.

**Fig 3.13** Effects of SB 203580 on p38 MAPK activation of perfused hearts at the end of reperfusion



Control	n = 7
Non-PC	n = 9
IPC	n = 7
SB 203580 (1)	n = 3 (10 min before SI)
SB 203580 (2)	n = 6 (During the IPC protocol)

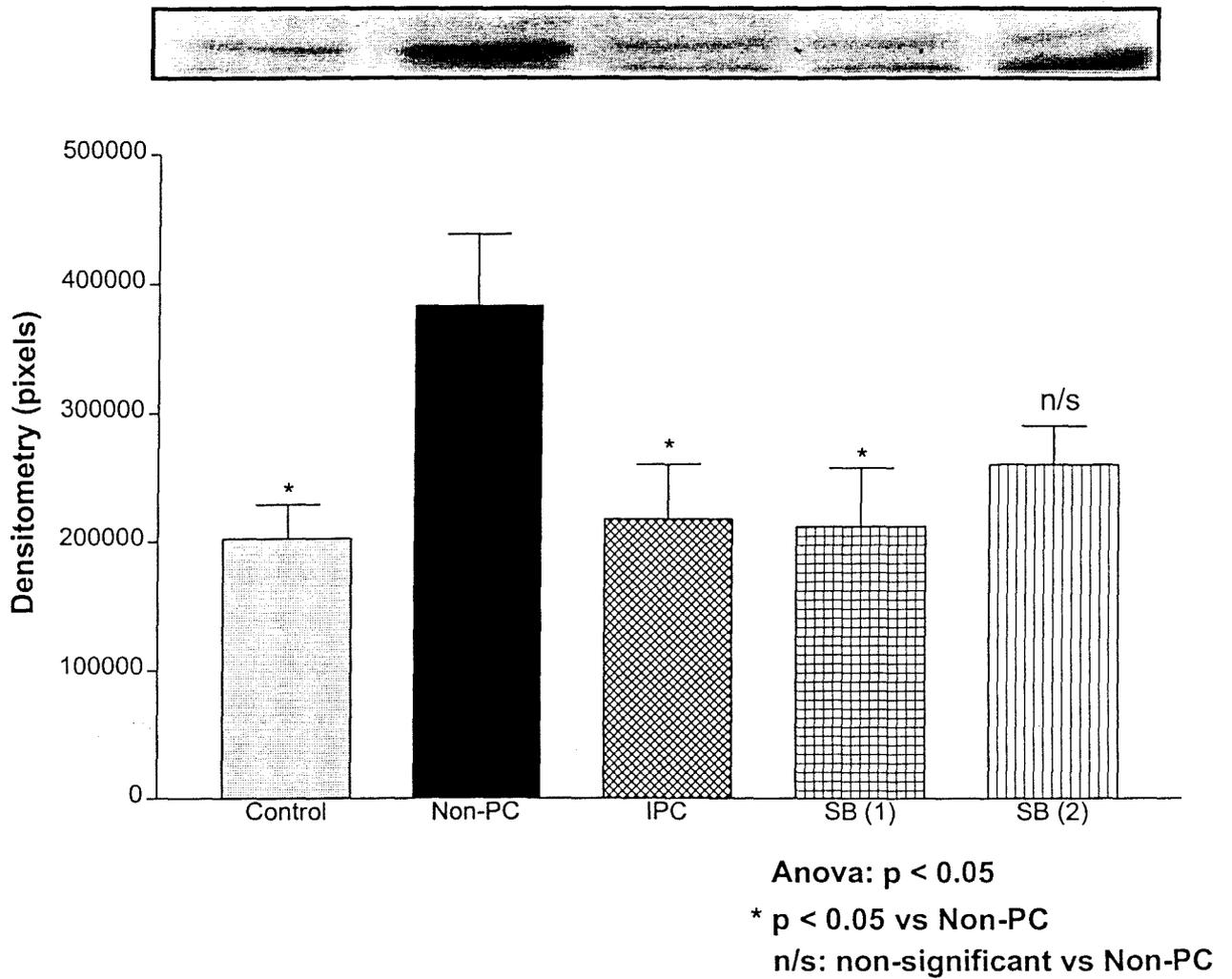
**Fig 3.14** Effects of SB 203580 on caspase-3 activation of perfused hearts at the end of reperfusion



#### 3.3.2.4 PARP (Fig 3.15)

PARP cleavage differed significantly between the different groups (Anova:  $p < 0.05$ ). Neither the Tukey nor the Newmans-Keuls post hoc tests could localise differences. This was probably due to large standard errors, which were attributed to inter-series differences. Intergroup differences were therefore investigated by an unpaired Students t-test. Hearts that were not preconditioned showed a marked increase in PARP cleavage ( $383100 \pm 55330$  pixels) at the end of reperfusion when compared to the control hearts ( $202500 \pm 26540$  pixels) ( $p < 0.05$ ). SB 203580, when administered 10 min before sustained ischaemia showed a statistically significant lowering of PARP cleavage ( $211780 \pm 45470$  pixels) when compared to the Non-PC groups ( $p < 0.05$ ) (Fig 3.15). When SB 203580 was administered during the 3 x 5 min IPC protocol, PARP cleavage ( $259900 \pm 29840$  pixels) was not significantly less than that of the Non-PC hearts ( $p = 0.07$ ) (Fig 3.15). A representative blot of one experiment is shown.

**Fig 3.15 Effects of SB 203580 on PARP cleavage of perfused hearts at the end of reperfusion**



Control	n = 4
Non-PC	n = 5
IPC	n = 5
SB 203580 (1)	n = 5 (10 min before SI)
SB 203580 (2)	n = 4 (during the IPC protocol)

### **3.3.3 Effects of manipulation of p38 MAPK by anisomycin on necrosis, p38 MAPK activation and apoptosis**

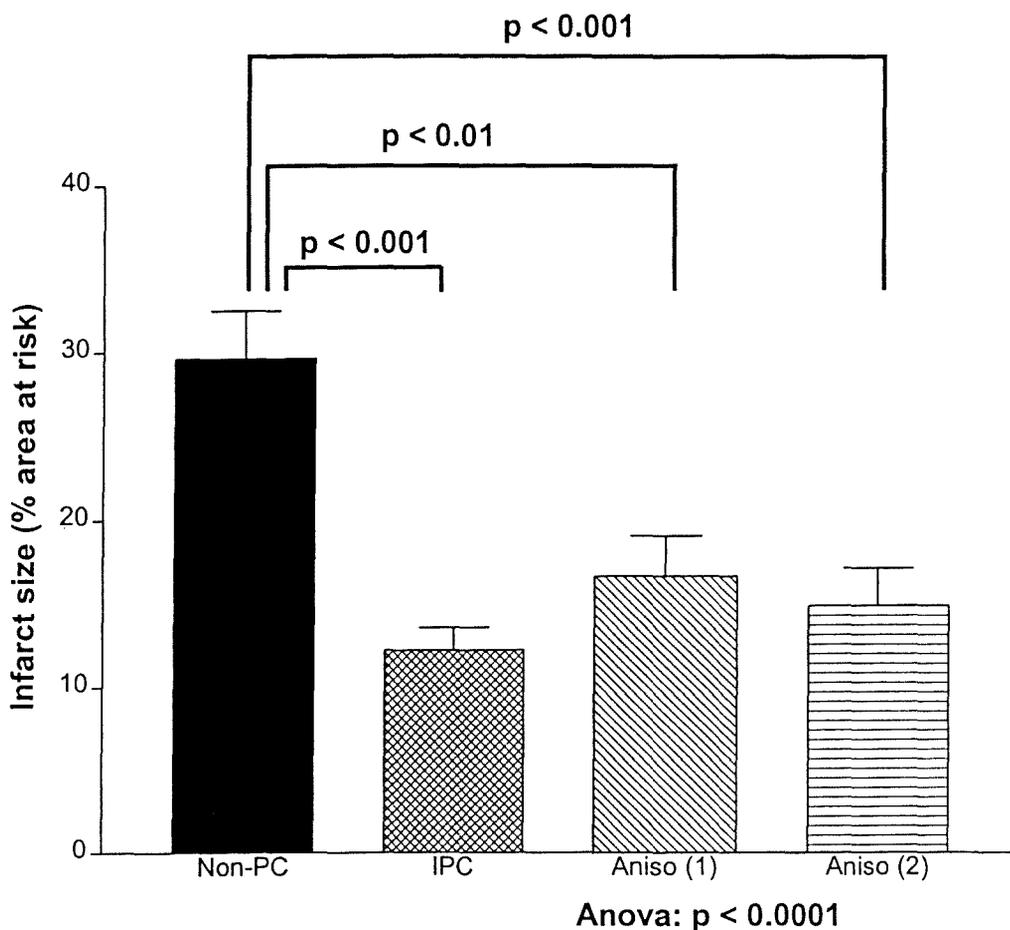
In the next series of experiments it was attempted to increase p38 MAPK activation in order to study its role in the protective effect of preconditioning on apoptosis and infarct size.

To determine whether activation of p38 MAPK by anisomycin can act as a mediator or trigger of preconditioning, the drug was administered either 10 min before sustained ischaemia (anisomycin (1)) or 10 min followed by washout (anisomycin (2)).

#### **3.3.3.1 Infarct size (% area at risk) (Fig 3.16)**

The infarct size of Non-PC and anisomycin-treated hearts differed significantly (Anova:  $p < 0.0001$ ). Administration of anisomycin for 10 min before sustained ischaemia resulted in an infarct size ( $16.6 \pm 2.4\%$ ) that was significantly lower than the non-preconditioned hearts ( $29.6 \pm 2.9\%$ ) ( $p < 0.01$ ). Administration of anisomycin for 10 min and washed out for 10 min before also caused a significant reduction in infarct size ( $14.9 \pm 2.2\%$ ) which was significantly lower than that of the Non-PC hearts ( $p < 0.001$ ) (Fig 3.16).

**Fig 3.16** Effects of anisomycin on infarct size of perfused hearts (TTC staining)



Non-PC n = 8

IPC n = 8

Anisomycin (1) n = 7 (10 min before SI)

Anisomycin (2) n = 7 (10 min, then washed out for 10 min before SI)

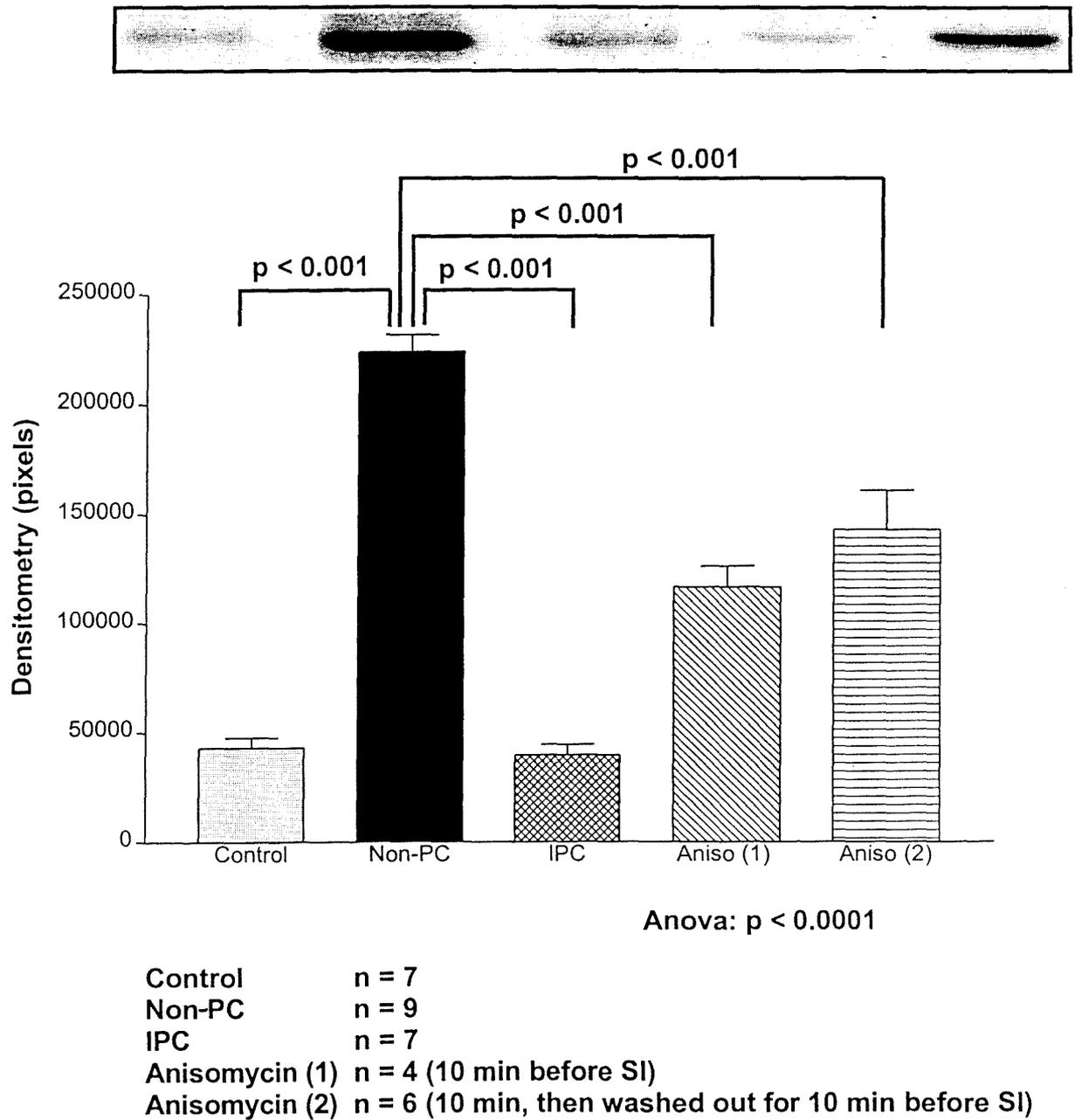
### 3.3.3.2 p38 MAPK (Fig 3.17)

p38 MAPK activation of all the different groups differed significantly (Anova:  $p < 0.0001$ ). Administration of anisomycin either 10 min before sustained ischaemia, or when anisomycin is administered for 10 min and then washed out for 10 min before sustained ischaemia both resulted in p38 MAPK activation at the end of reperfusion that was significantly higher than that of the control ( $p < 0.001$  for all comparisons). p38 MAPK activation of both anisomycin groups was however significantly less than that of Non-PC hearts ( $p < 0.001$  for all comparisons).

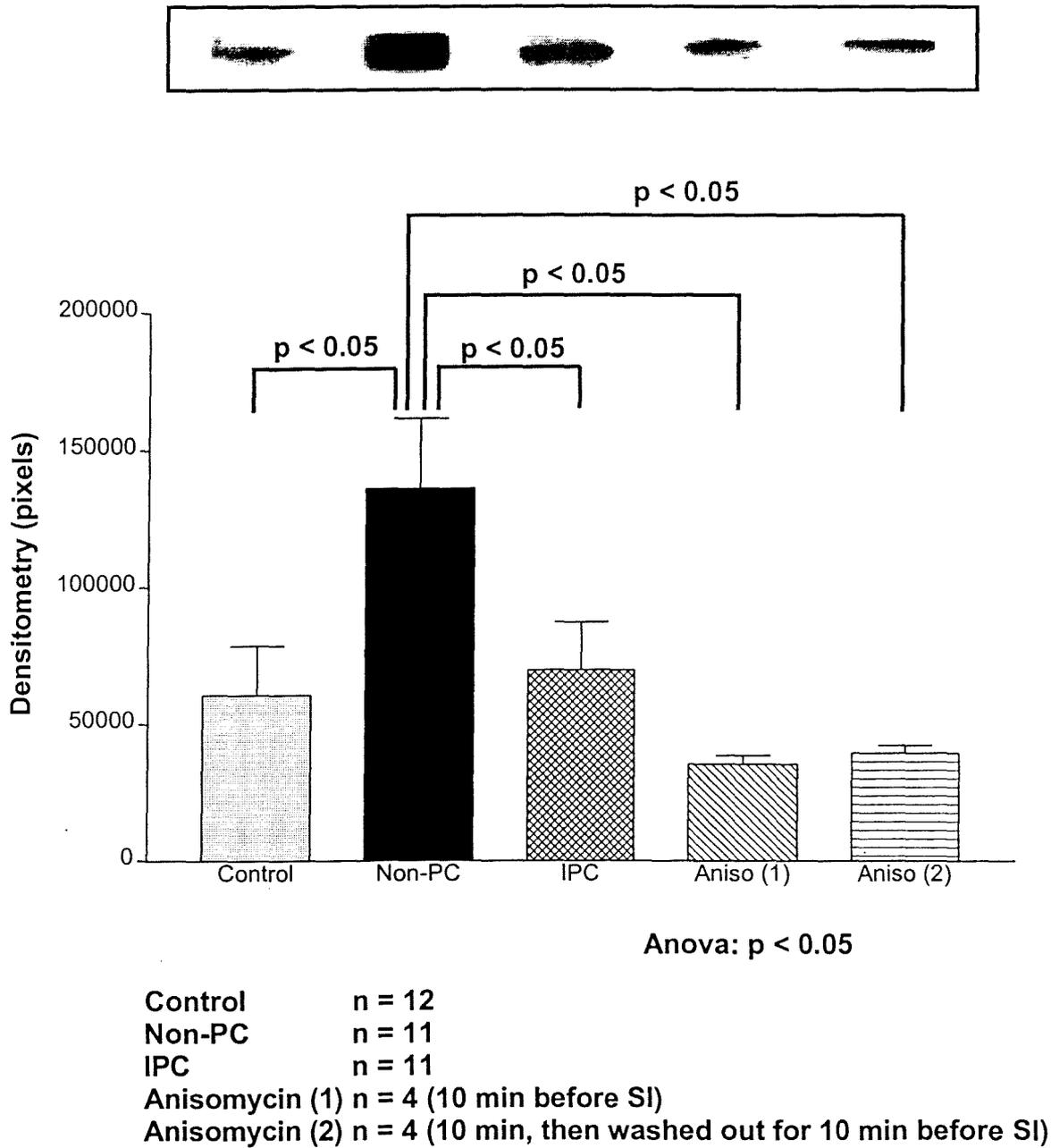
### 3.3.3.3 Caspase-3 (Fig 3.18)

Caspase-3 activation differed significantly between the different groups (Anova:  $p < 0.05$ ). Caspase-3 activation was significantly less when anisomycin was administered for 10 min before sustained ischaemia, and when administered for 10 min and then washed out before sustained ischaemia when compared to the values obtained in the Non-PC groups ( $136200 \pm 25310$  pixels) ( $p < 0.05$  for both comparisons).

**Fig 3.17** Effects of anisomycin on p38 MAPK activation of perfused hearts at the end of reperfusion



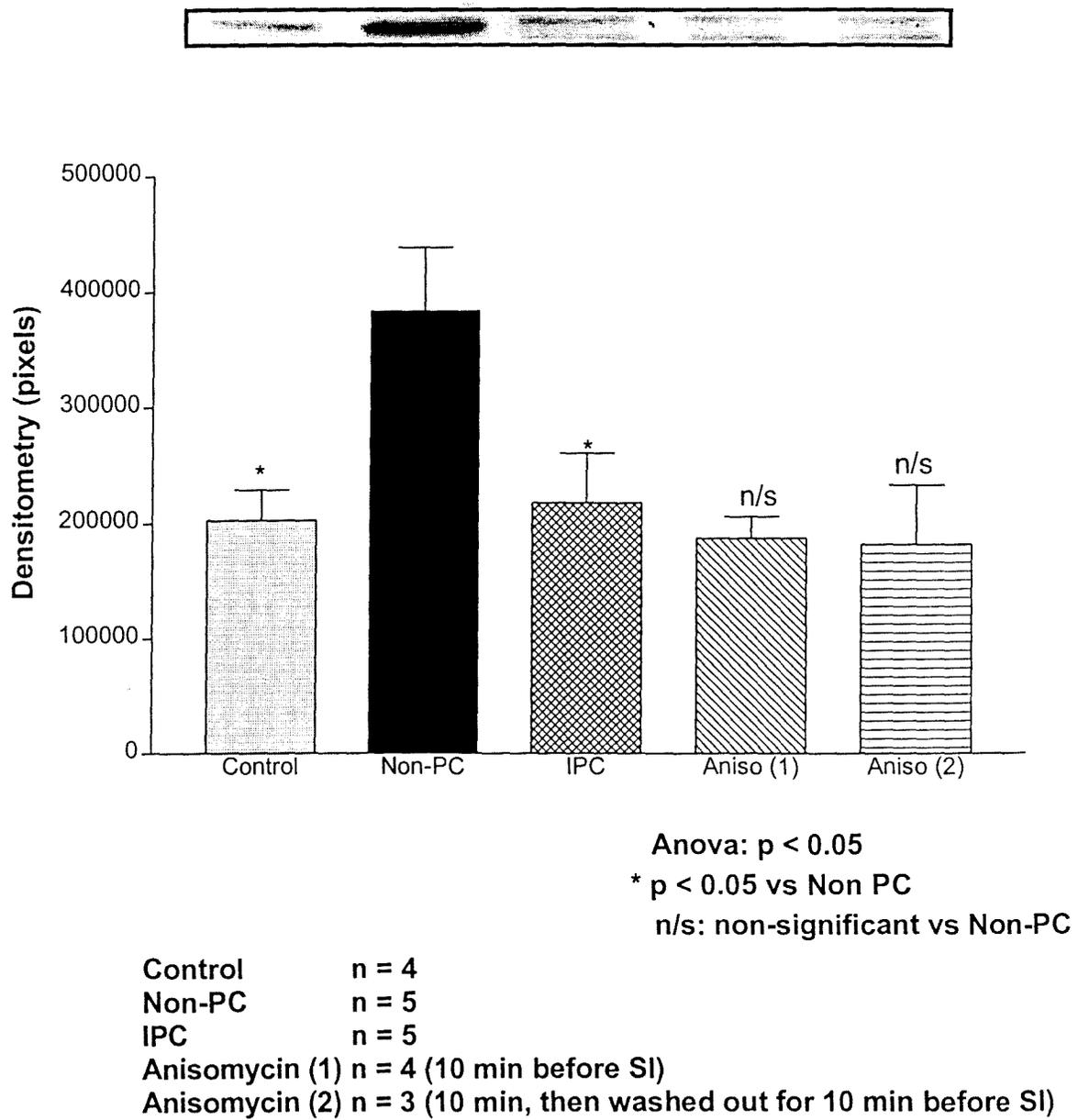
**Fig 3.18** Effects of anisomycin on caspase-3 activation of perfused hearts at the end of reperfusion



#### 3.3.3.4 PARP (Fig 3.19)

PARP activation differed significantly between the different groups (Anova:  $p < 0.05$ ). Anisomycin when administered for 10 min before sustained ischaemia or when administered for 10 min and then washed for 10 minutes before sustained ischaemia, caused no significant difference in PARP activation ( $181000 \pm 51540$  pixels and  $169900 \pm 8298$  pixels respectively) when compared to the control hearts.

**Fig 3.19 Effects of anisomycin on PARP cleavage of perfused hearts at the end of reperfusion**



## Chapter 4

### Discussion

#### Introduction

In this thesis we investigated the possible role of activation of the stress kinase, p38 MAPK, in the protective effect of preconditioning against ischaemia/reperfusion injury.

The main findings of the work are as follows:

1. A method for determination of cell viability was developed and established in the laboratory– the MTT staining method. The method is easy to use, and will be useful in future.
2. The attempt to develop a cell culture model of preconditioning using neonatal cardiomyocytes proved unsuccessful, and although a model of isolated adult cardiomyocytes was potentially useful, it was not utilized as the yield of material was too low to perform multiple simultaneous biochemical determinations.
3. The isolated perfused rat heart was subsequently used for experimental work, with infarct size as endpoint. This model demonstrated the phenomenon of preconditioning convincingly and reliably using two different triggers of preconditioning – ischaemia and pharmacological preconditioning with isoproterenol. Preconditioning with both ischaemia and isoproterenol protected against ischaemia/reperfusion damage as was demonstrated by both a reduction in infarct size and a reduction in apoptosis. This has not been shown before for isoproterenol mediated preconditioning, and is thus a unique finding.

4. Investigations into the role of activation of p38 MAPK in the protective effect of preconditioning against ischaemia/reperfusion damage showed that inhibition of p38 MAPK activation during sustained ischaemia mediated protection against both infarction and apoptosis. Furthermore, activation of p38 MAPK did not act as a trigger for protection against infarction. Inhibition of p38 MAPK during the triggering phase was however associated with a minor, but significant increase in apoptosis in the reperfusion phase, which suggest that activation of p38 MAPK prior to sustained ischaemia was important for protection against apoptosis.
5. The importance of activation of p38 MAPK was also demonstrated using an activator of p38 MAPK, anisomycin. Activation of p38 MAPK prior to ischaemia by administration of anisomycin immediately preceding ischaemia or as a trigger (i.e., administration followed by washout) both elicited protection against infarction and apoptosis, and was associated with reduced p38 MAPK activation upon reperfusion. The mechanism of these experimental observations was not investigated, but may relate to down-stream effectors of p38 MAPK, such as the small heat-shock protein, HSP27.

#### **4.1 The neonatal cardiomyocytes in culture**

The attempt to establish a cell culture model of preconditioning using neonatal cardiomyocytes had, as basis, the aim to enable one to elucidate the cardiac specific signal transduction pathways more accurately, as it would exclude the influence of non-cardiac cells. Furthermore, it potentially offered a way of doing multiple experiments (i.e. looking at different signal transduction components, and even different end-points) using a single cell culture preparation, and thus enable rapid progression of the

investigation. The use of isolated rat hearts limits one to a single experimental situation, and sets of at least 6 hearts per experimental point have to be performed.

#### **4.1.1 Establishing the MTT method for determination of cell viability**

Before it was attempted to set up a model of preconditioning in neonatal cardiomyocytes, a method of determining cell viability had to be established first. The MTT assay was chosen, as it has been a well-established method in assessing the effect of anti-cancer drugs in oncology research, and was also gaining popularity in cardiac research (Ghosh et al 2001, Gomez et al 1997, Ferrera et al 1993, Punn et al 2000).

Other methods employed to measure viability include osmotic fragility using trypan blue (Baines et al 1999) and morphological changes (Nojiri et al 1999). All these methods are labour intensive, and involve microscopic evaluation. Due to the inherent problem of investigator bias, these evaluations entail blinded evaluation of the samples by more than one person, which creates another problem – inter observer differences. The MTT assay is methodologically an easy method, involves one person who can even be the primary investigator, as the read-out is a spectrophotometer reading that cannot be manipulated.

The method relies on the conversion of the tetrazolium salt into spectrophotometrically measurable blue formazan pigments by viable cells. As the reaction involves availability of NADPH and NADH, they can only take place in cells with intact and functioning mitochondria. The optimal loading conditions thus had to be determined. The results show that at the cell density utilized ( $1,6 \times 10^6$  cells/35 mm Petri dish), a concentration

of 0.25% MTT had to be used. It can be seen that fully viable neonatal cardiomyocyte cell cultures not subjected to ischaemia cause the release of formazan pigments with an optical density higher than 0.5 (Fig. 3.1).

#### **4.1.2 Setting up a model of preconditioning in neonatal cardiomyocytes**

Numerous investigators have reported the use of neonatal myocyte cell cultures and simulated ischaemia to study preconditioning (see Table 4.1). In our hands, neonatal cardiomyocytes could however not be consistently preconditioned by the protocols that were employed, and using the MTT assay as an endpoint for viability.

Numerous attempts were made to modify the protocols in order to set up a successful model. In setting up the protocols, different aspects had to be kept in mind:

1. The nature of simulated ischaemia utilized – both for triggering preconditioning, and as “test ischaemia”, i.e. the sustained episode of oxygen deprivation.
2. The duration of both the triggering and the sustained ischaemic period.
3. The duration of reoxygenation following both triggering and sustained “ischaemia”.

It can be seen that the number of permutations possible could become extensive and time consuming.

In the first protocol, a combination of KCN + DOG of 5 min duration was used during the sustained ischaemia (SI) period. The OD of both groups did not differ, and were both in the range of 0.5 – i.e. in the range observed for cells not exposed to ischaemia. This suggested that the “test” simulated ischaemia was not severe enough to cause cell damage.

**Table 4.1 Literature review of experimental protocols for preconditioning cultured cardiomyocytes**

<b>Author</b>	<b>Species</b>	<b>Trigger + reoxygenation</b>	<b>Sustained ischaemia (SI) or hypoxia</b>	<b>Endpoint</b>
Gray et al (1997)	Neonatal rat cardiomyocytes	4 x 90 min hypoxia + 1h reoxygenation	9h hypoxia	Eukolight™ viability/ cytotoxicity assay
McPherson et al (2001)	Chick ventricular myocytes	10 min SI (ischaemia buffer) + 10 min reoxygenation + 10 min washout	1h SI (ischaemia buffer) + 3h reoxygenation	Propidium iodide
Rakhit et al (2000)	Neonatal rat cardiomyocytes	90 min SI (ischaemia buffer) + 30 min reoxygenation	6h SI (ischaemia buffer)	MTT assay LDH release
Saurin et al (2000)	Neonatal rat cardiomyocytes	90 min SI (ischaemia buffer) + 30 min reoxygenation	2.5h SI (ischaemia buffer)	MTT assay LDH release
Webster et al (1995)	Neonatal rat cardiomyocytes	25 min hypoxia + 30 min reoxygenation	6h hypoxia	Arachidonic acid release Contractile recovery
Zhao et al (1998)	Neonatal rat cardiomyocytes	90 min SI (ischaemia buffer) + 30 min reoxygenation	8h SI (ischaemia buffer)	TBE method LDH release

Thus, for the second protocol, the period of sustained simulated ischaemia was increased. It was thought that the combination of DOG and KCN would be too severe, and it was decided to only use KCN for 45 min. This proved effective to induce cell damage, as the OD readings of the non-preconditioned cells fell to below 0.3 (see Figures 3.3 – 3.5).

The preconditioning trigger was similar to that used in the first protocol – 5 min KCN. In some experiments this was adequate to elicit protection (Fig 3.3), but no consistent results could be obtained in 21 consecutive experiments. The scattergram in Fig 3.5B shows that the problem was unlikely to be related to an insufficiently long period of chemical hypoxia, as the OD readings of most of the experiments were below 0.25.

It is necessary to compare the methodology of authors who have been successful in preconditioning neonatal cell cultures with our unsuccessful attempts.

- (i) The triggers of preconditioning consisted of hypoxia with or without a so-called “simulated ischaemia buffer”. The ischaemia buffer contained (in mmol/l) sodium lactate 20; KCl 16 at a pH of 6.2. We employed a single-cycle 5 min administration of KCN without the addition of sodium lactate, high KCl or the reduction of the pH to 6.2. We do not think that the omission of lactate, a high potassium concentration or adjustment in the pH was the reason for our inability to elicit protection. Previous work in our laboratory have shown that 5 min KCN was adequate to cause rapid depletion of ATP, which would be accompanied by the same metabolic changes in potassium and lactate as was artificially induced by the other authors. It is known that it takes longer before hypoxia causes metabolic derangements in neonatal cell cultures – therefore the duration of

hypoxia used is 90 minutes in most cases as can be seen in Table 4.1. Therefore it is unlikely that our trigger was too short or not severe enough to elicit preconditioning.

- (ii) The time period for hypoxia with simulated ischaemia (SI) ranges from at least 1h to 8h. As discussed above, 45 min KCN was effective to induce cell damage, and does not explain the inability to set up the model.
- (iii) It should also be noted that reoxygenation after SI is not commonly used. Our model thus did not differ from established models in this respect.
- (iv) The endpoints employed in other studies included the MTT assay, the TBE method, propidium iodide, and LDH release measurement. The inability to demonstrate protection was therefore unlikely due to the inappropriate use of the MTT staining method.

It seems as if variations in the triggering and/or reoxygenation period following the triggering phase may have been useful in our efforts to elicit preconditioning, but in view of the time it could potentially consume, it was decided not to pursue that course.

Investigations into other reasons why the neonatal cardiomyocytes could not successfully and consistently precondition were discussed in the results section, but produced no definitive answers to the problem. One important aspect must be pointed out: sufficient attention was given to the effect of the age of the rat pups, and the confluence of the cultures, i.e. the maturity of the cells. This is important, as the sensitivity of neonatal cells to ischaemia is age dependent. Ostadalova et al in (1998) performed experiments with 1, 4, and 7-day old neonatal rat isolated perfused hearts.

They found that preconditioning with 3 x 3 min global ischaemia, separated by 5 min reperfusion, and followed by 40/60 min global ischaemia only protected the 7 day old neonatal hearts and not the 1, and 4 day old neonatal hearts. Awad et al (1998) performed similar experiments with 4, 7, 14, and 21-day old rat hearts, which were isolated and perfused in Langendorff mode. They found that preconditioning afforded cardioprotection to the 14, and 21-day old rats only and not the 4, and 7-day old neonatal rat hearts. This group concluded that protection by preconditioning develops after 7 days. The possibility that the 2-4 day old neonatal rat hearts may have been too young to elicit the protection afforded by preconditioning must therefore be considered. However, other researchers have been able to precondition neonatal cardiomyocytes. These results indicate that cell maturity can definitely influence outcomes, however even taking this into account, we could not identify a remediable factor bearing on this in our work.

Not only were we unable to consistently elicit ischaemic preconditioning in neonatal cells, but problems were also experienced with pharmacological preconditioning. It was known from previous work done in both our laboratory (Marais et al 2001b) and others (Miyawaki et al 1997b), that the isolated perfused rat heart could be preconditioned with beta-adrenergic stimulation. However, isoproterenol ( $10^{-7}$  M) was not able to elicit protection in neonatal cells, probably due to the fact that the OD reading was above 0.5 for both cell groups. It is thus possible that the neonatal cardiomyocytes were resistant to ischaemia, i.e. a maturity related effect, or that the episode of sustained simulated ischaemia was indeed insufficient in these particular sets of experiments.

**In conclusion:** It was not possible to consistently precondition neonatal cardiomyocytes and it seems likely that further modification of the triggering protocol, particularly the duration of ischaemia and reperfusion, could have been useful. Another possibility would be to use older neonates.

## **4.2 Isolated adult cardiomyocytes**

A reliable model of preconditioning was essential before proceeding to investigate the signal transduction pathways involved in its protection. It was decided to use the adult cardiomyocyte model, which has been shown in our laboratory to precondition successfully (H. Strijdom, unpublished observations).

Isolated adult cardiomyocyte models are well established in investigations of myocardial protection against ischaemia. Preconditioning initiated by either a short period of cell pelleting or by suspension of cells in glucose-free buffer shares many features with preconditioning in the intact heart, including the adenosine (Armstrong et al 1994), PKC (Liu et al 1999), and  $K_{ATP}$  (Liu et al 1998) dependence of protection. The trypan blue exclusion method is a well-established method for cell viability (Armstrong et al 1995, Armstrong et al 1999, Baines et al 1999, Liu et al 1999). However, the use of the MTT assay to measure viability in isolated cardiomyocytes is not common, and it was decided to utilize the method developed for the neonatal cardiomyocytes.

The preconditioning protocol employed in the isolated adult rat cardiomyocyte model successfully protected the cells against hypoxia. This was evident by a significant increase in viability, expressed as the percentage of trypan blue excluding cells, when comparing the Non-PC groups to the PC groups. The successful preconditioning was

further proven by the MTT assay expressed as OD/mg protein/ml. The MTT assay results were comparable with those obtained by the trypan blue exclusion method (results not shown) and this confirmed the effectiveness of this assay as a reliable method to test cell viability.

**In conclusion:** Isolated adult cardiomyocytes could be protected from hypoxia-induced damage by preconditioning, and the MTT assay could be used to determine cell viability.

However, this model, although successful in demonstrating preconditioning, could not provide us with sufficient material to perform subsequent simultaneous investigations on p38 MAPK, caspase-3 and PARP cleavage and hence it was decided to use the isolated perfused heart.

### **4.3 The Isolated perfused heart provided a reliable model of preconditioning**

#### **4.3.1 Infarct size reduction**

Preconditioning with both ischaemia and beta-adrenergic stimulation with isoproterenol (IPC and  $\beta$ PC respectively) significantly and convincingly reduced the infarct size after ischaemia/reperfusion compared to Non-PC hearts (Fig 3.8). TTC staining to quantify infarct size is considered the gold standard to determine the protective effect of ischaemic and pharmacological preconditioning and has been used by, amongst others, Baines et al (1997) in the isolated rabbit heart, Kitakaze et al (2000) in dog hearts, and Piot et al (1997) in isolated rat hearts.

Although the protective effect of  $\beta$ PC has been reported before (Asimakis et al 1994, Marais et al 2001b), no data has ever been published concerning its effect on infarct size. These results are thus unique. It may not appear to be an important point at first, but the issue is more complex. Recent data from our own laboratory (unpublished observations) suggested that the end-point used in preconditioning experiments is very important in determining the outcome. There is a distinct difference between infarct size and functional recovery, and protection as assessed by one end-point does not automatically imply protection as assessed by another. This seems to imply that different mechanisms of protection are active. Therefore, the finding that  $\beta$ PC and IPC both protect against infarction may indicate that the protection mediated by both triggers utilize the same mechanisms or signal transduction pathways.

The difference between the decrease in infarct size of IPC and  $\beta$ PC were not statistically different, which suggested that  $\beta$ PC was as effective as IPC in the reduction of infarct size. The importance of this observation are to be found in the potential clinical utilization of the intervention. Ischaemic preconditioning is regarded as the most potent form of protection yet described against ischaemia and infarction (Kloner et al 1998). The ultimate aim of all investigations in this field is to define the mechanism, in order to be able to prepare a pharmacological substance that can be administered to patients to protect them against foreseen or unforeseen ischaemic events. Ideally, pharmacological preconditioning should be as effective as possible. With this in mind the confirmation that  $\beta$ PC is at least as potent as IPC to protect against infarction assumes particular significance.

#### **4.3.2 Reduction in apoptosis**

The results presented in this study also show that both IPC and  $\beta$ PC protect against the apoptosis component of ischaemia/reperfusion injury. In this regard, the first question that must be addressed is the reliability of the method of apoptosis detection. Apoptosis was detected by using Western blotting to show caspase-3 activation and PARP cleavage.

These parameters are widely accepted and are reliable. Other methods that are commonly used to detect apoptosis include the TUNEL assay and DNA fragmentation using ELISA or gel electrophoresis. The TUNEL assay detects nick-end labeling of DNA of both single and double stranded DNA, which is present in both necrosis and apoptosis (Froelich-Ammon et al 1995). DNA fragmentation is not considered the most elegant way to detect apoptosis, and is not a quantitative method.

Measuring caspase-3 activation is the method used in more recent studies in this field (Table 1.2), and the PARP fragmentation is a more reliable parameter than DNA fragmentation (Gu et al 1995, Lazebnik et al 1994, Tewari et al 1995). We therefore regard the findings obtained in this study as valid proof of reduced apoptosis.

Ischaemic preconditioning has been shown to decrease apoptosis by, amongst others, Liu et al (2001) in isolated chick cultured cardiomyocytes and Piot et al (1997) in the isolated rat heart. Our results showing that IPC reduces apoptosis are thus in agreement with their findings. The finding that  $\beta$ PC reduces both caspase-3 activation and PARP cleavage and thus apoptosis are novel findings, as was the case with protection against infarction. At the same time the question can be posed whether this is such an unexpected finding – i.e. whether the protection against apoptosis is divorced from the protection against infarction, or whether the reduced apoptosis is only the consequence of reduced damage induced by sustained ischaemia. It is not possible to answer this question with the information available from these experiments. One possible way to obtain an answer would be to determine LDH or CK release into the effluent during reperfusion. On the other hand, it is known that some cells undergoing apoptosis may switch to necrosis, and would then release cardiac enzymes, rendering this parameter useless. Indeed, Gottlieb et al (1999) has noted that for this reason the distinction between apoptosis and necrosis in the myocardium may be an elusive goal to pursue, and that it may be wiser to include both concepts in a wider theme, namely “reperfusion damage”. Another way to investigate whether separate anti-apoptotic mechanisms were activated would be to test whether IPC or  $\beta$ PC could protect against other mediators of apoptosis. If this were the case, it would support the concept of the involvement of separate pathways in the activation of apoptosis and mediators of necrosis.

In the absence of definite scientific evidence, our results could indicate that the two triggers of preconditioning (IPC and  $\beta$ PC) have a common pathway which protect against apoptosis and necrosis.

#### **4.3.3 Mechanism of protection: Can the protection against necrosis and apoptosis be attributed to p38 MAPK?**

Our data clearly show that protection against necrosis and apoptosis was accompanied by decreased activation of p38 MAPK upon reperfusion. The question was therefore whether reduced p38 MAPK activation was the cause or consequence of protection. The role of p38 MAPK activation as a mechanism of protection remains controversial. Weinbrenner et al (1997) and Barancik et al (1999), showed that activation of p38 MAPK during sustained ischaemia was beneficial to the ischaemic heart and have attributed the protection to the activation of heat shock protein 27 (HSP27). This protein causes stabilization of the actin and fodrin proteins, which are involved in maintaining plasma membrane integrity. Others, including Marais et al (2001a) and Sato et al (2000), have shown that decreased activation of p38 MAPK during sustained ischaemia, and inhibition of p38 MAPK activation with the specific p38 MAPK inhibitor, SB 203580, protected against ischaemia. Also, there is sufficient evidence from the literature that p38 MAPK is involved in apoptosis under many harmful situations, including oxidative stress, which is a component of ischaemia/reperfusion (Han et al 2001).

**In conclusion:** IPC and  $\beta$ PC caused a significant reduction in infarct size and apoptosis as determined by caspase-3 activation and PARP cleavage at the end of ischaemia/reperfusion, which was associated with a reduction in p38 MAPK activation

upon reperfusion. The question that remains to be addressed concerns the role of p38 MAPK in preconditioning.

#### **4.4 Defining the role of p38 MAPK in protection against ischaemia/reperfusion**

Apart from the possibility that attenuated activation of p38 MAPK during sustained ischaemia was involved in the protective effect of preconditioning, another aspect had to be kept in mind. Ischaemia/reperfusion of 5 minutes duration, such as used in our preconditioning protocol, has been shown to cause an increase in p38 MAPK activation (Armstrong et al 1999, Bogoyevitch et al 1996, Marais et al 2001a, Maulik et al 1996). The possibility that p38 MAPK was involved in preconditioning thus had to be approached from two viewpoints – attenuated activation during sustained ischaemia as a mediator of the protective effect, and activation of p38 MAPK as a trigger of protection. To investigate these aspects, two approaches were used:

1. inhibition of p38 MAPK with SB 203580 during the triggering and mediating phase, and
2. activation of p38 MAPK with anisomycin during the triggering and mediating phase.

##### **4.4.1 The inhibition of p38 MAPK activation**

###### **4.4.1.1 Attenuation of p38 MAPK activation as mediator of protection**

Administration of SB 203580 before sustained ischaemia/reperfusion resulted in significant protection against ischaemia/reperfusion damage as evidenced by a reduction in infarct size and markers of apoptosis. The protection was of the same magnitude as that of IPC. These results suggested that the attenuation of p38 MAPK activation during ischaemia was not just an associated finding, and provided evidence

that attenuated p38 MAPK activation could indeed be a mediator of the protective effect of ischaemic preconditioning (Fig 3.12, 3.13 and 3.14).

These results contradict the findings of Weinbrenner et al (1997) who found that p38 MAPK was activated in preconditioned isolated rabbit cardiac cells during sustained ischaemia, and that SB 203580 added prior to simulated ischaemia abolished the reduction in fragility. Their results therefore suggested that p38 MAPK *activation* acted as a mediator of protection. Nakano et al (2000b) found no role for p38 MAPK in reducing infarct size *in vivo* in rabbits when SB 203580 was administered before sustained ischaemia. However, our results are in agreement with data by Ping et al (1999), who have shown that p38 MAPK activation was downregulated by six cycles of ischaemia/reperfusion and Sato et al (2000), who found that SB 203580 administered before sustained ischaemia decreased infarct size in isolated rat hearts. Similarly, Marais et al (2001a) found that, in isolated rat cardiomyocytes, SB 203580 when administered before hypoxia caused a significant improvement in both cell morphology and viability. Saurin et al (1999) also provided compelling evidence that the protective effect of preconditioning was accompanied by attenuated activation of p38 MAPK- $\alpha$  during sustained ischaemia.

Although the model used by Han et al (2001) was a fibroblast cell line and not cardiac cells, the results of this excellent study clearly showed that a decrease in p38 MAPK activation was associated with a decrease in apoptosis. This is in agreement with the current notion that activation of the p38 MAPK is pro-apoptotic in many situations (Chen et al 1996, Johnson et al 1996, Obata et al 2000) and although not conclusive, is certainly supportive of our findings.

The exact reason for the above disparate results are not clear. It should be noted that species differences may be important – rabbits and rats are not necessarily comparable. Whereas the literature supports a role for adenosine as a trigger of preconditioning in the rabbit, there is no consensus on its role as a trigger in the rat (Ganote 2000). The role of activation of PKC in the rat has also not been as equivocal as was the case in the rabbit (Simkhovich et al 1998). As noted before, the end-point used was also important – measuring osmotic fragility may not be relevant in comparison to evaluating protection against necrosis or apoptosis.

#### 4.4.1.2 p38 MAPK as a trigger of protection

As mentioned before, an IPC protocol of 5 min ischaemia causes activation of p38 MAPK (Sato et al 2000, Marais et al 2001a). The role of p38 MAPK activation could therefore be assessed by inhibiting it during the triggering phase. The inhibition of p38 MAPK activation by bracketing the ischaemic preconditioning protocol did not abolish the protective effect of IPC on infarct size (Fig 3.12), but caused a slight but significant increase in markers of apoptosis (Fig 3.14 and Fig 3.15).

Our results are in disagreement with data by Sato et al (2000) who found that SB 203580, when given before the preconditioning protocol, abolished the infarct-reducing effect of IPC. Also, in the study on the fibroblast cell line by Han et al (2001) referred to above, inhibition of p38 MAPK during the triggering phase abolished the protection against radical oxygen species.

Our results agree with those of Marais et al (2001a), who found that in isolated rat cardiomyocytes, administration of SB 203580 before and during the IPC protocol had no effect on morphology and viability after 2 hours of hypoxia compared to untreated preconditioned isolated rat cardiomyocytes.

**In conclusion:** The inhibition of p38 MAPK activation with SB 203580 during the IPC protocol did not abolish the protective effect of IPC on infarct size reduction, but caused a slight increase in apoptosis. This suggests that separate pathways are operating in mediating protection against necrosis and apoptosis.

#### **4.4.2 The activation of p38 MAPK**

In order to confirm the validity of the conclusions made in the previous section by using inhibitors of p38 MAPK, the opposite approach was used, namely to observe the effect of activators of p38 MAPK. In order to elucidate the role of p38 MAPK activation as either a mediator or as a trigger of the protective effect of preconditioning, anisomycin, an activator of p38 MAPK, was administered (i) immediately preceding sustained ischaemia and (ii) for 10 min followed by a washout period before sustained ischaemia.

##### **4.4.2.1 Activation of p38 MAPK with anisomycin either as a mediator or as a trigger elicits protection**

p38 MAPK activation with (i) anisomycin immediately before sustained ischaemia (i.e. as mediator) or (ii) for 10 min followed by washout (i.e. as trigger) resulted in a significant decrease in infarct size, (Fig 3.16), and less apoptosis as evidenced by reduced caspase-3 activation. The effect on PARP cleavage was not as pronounced as

that of caspase-3 activation due to large standard errors. A representative blot is shown in Fig 3.19, which demonstrates the extent of PARP cleavage under these circumstances. p38 MAPK activity was significantly lower upon reperfusion in both groups treated with anisomycin.

The question that needs to be addressed is how the data with p38 MAPK activation fits in with the data obtained in experiments using the inhibitor, SB 203580. The observation was made that anisomycin administered before sustained ischaemia elicited *protection* against both necrosis and apoptosis, both when administered as trigger and as mediator, whereas the inhibition of p38 MAPK mediated protection during sustained ischaemia, and only seemed to play a role as a trigger in the protection against apoptosis.

If attenuation of p38 MAPK activation was indeed a mediator of protection, one would expect anisomycin mediated activation of p38 MAPK during sustained ischaemia to *decrease* protection against infarction and apoptosis. One explanation is that the administration of anisomycin immediately prior to sustained ischaemia *did not result in sustained p38 MAPK activation during ischaemia* – the finding that p38 MAPK activity was decreased at the end of reperfusion supports this notion. It is therefore possible that the unexpected beneficial effect of anisomycin can be attributed to the activation of protective mechanisms prior to sustained ischaemia. The only approach to activate p38 MAPK during ischaemia alone would be to infuse the agent directly into the myocardium – this was done by Barancik et al (2000) in similar work in a regional infarct model in pigs.

The role of p38 MAPK as a trigger is also questioned by these results – if it was true that p38 MAPK was indeed not important as a trigger of protection against necrosis, anisomycin would have been expected to have no effect on it. On the other hand, as inhibition of p38 MAPK during the triggering phase caused an increase in apoptosis, it would be expected that anisomycin mediated activation of p38 MAPK should decrease apoptosis, which did occur.

One approach would be to regard the two sets of anisomycin experiments as variants of the same phenomenon – namely that the activation of p38 MAPK prior to sustained ischaemia activates protective downstream mechanisms which protect against both necrosis and apoptosis. This would take into account the fact that p38 MAPK was not activated during sustained ischaemia in this model. Indeed, this data is in agreement with Weinbrenner et al (1997) who found that, in isolated rabbit cardiomyocytes, anisomycin added prior to simulated ischaemia was as protective as IPC. Similarly Barancik et al (1999) showed that, in the pig heart, anisomycin reduced infarct size when administered before I/R. Also, in the isolated rat heart, Sato et al (2000) documented that the activation of p38 MAPK with anisomycin mimicked the infarct size-lowering effects of IPC.

What could this downstream effector be? The strongest candidate is HSP27, a small heat shock protein that is activated by p38 MAPK activation. HSP27 has been documented to have strong anti-apoptotic effects, as well as an actin stabilizing action that could contribute to protection against ischaemia induced cell disruption (Guay et al 1997). It can be postulated that the activation of HSP27 by anisomycin (acting via activation of p38 MAPK) elicited the protection that was observed.

This explanation does not account for the fact that SB 203580 did not abolish the protective effect of a 3 X 5 min cycle of ischaemic preconditioning. On the other hand, it may indicate that p38 MAPK activation only contributed to part of the protective effect of such a multi-cycle ischaemic preconditioning protocol, and that other factors independent of p38 MAPK were also involved. This would explain why a small increase in apoptosis was observed when SB 203580 bracketed the ischaemic preconditioning protocol, yet no increase in infarct size was observed. It has been shown that the activation of p38 MAPK which is seen with repetitive intermittent ischaemia as used in a multi-cycle preconditioning protocol decreases with every cycle (Marais et al 2001a), which may mean that only the initial activation of p38 MAPK is of any importance. Furthermore, there is evidence that multi-cycle ischaemic preconditioning protocols activate more than one signal transduction pathway. Sandhu et al (1997) have found that inhibitors of adenylyl cyclase could attenuate the protective effect of a single episode of ischaemic preconditioning, but not of a multi-cycle protocol. Unpublished data from our own laboratory also suggest a more marked role for p38 MAPK as trigger in a single episode ischaemic preconditioning protocol.

Other factors have to be considered too. Anisomycin also activates the JNK stress kinases. In order to evaluate the contribution of JNK to the observed results, the experiments would have to be repeated using an inhibitor of JNK, such as curcumin or SP 600125. However, JNK activation is unlikely to be involved in the activation of protective mechanisms as it is usually involved in activating apoptosis. Although anisomycin activates p38 MAPK, it is not known which isoform of p38 MAPK is activated. It is possible that administration of 5  $\mu$ M anisomycin activated p38 $\beta$  in the isolated adult rat perfused heart, an isoform known to be anti-apoptotic and thus

protective. Unfortunately, no antibodies are yet available to distinguish between the isoforms.

**In conclusion:** p38 MAPK activation with anisomycin administered either as a trigger or as a mediator decreased infarct size and apoptosis, which suggests a role for p38 MAPK activated downstream effectors as protectors against ischaemia/reperfusion damage. The logistical problem of being unable to activate p38 MAPK during ischaemia only (and not prior to it) makes it impossible to make valid deductions about the role of p38 MAPK activation during ischaemia. The data using the p38 MAPK inhibitor, SB 203580, therefore must carry more weight in the assessment of the role of p38 MAPK.

Furthermore, activation of p38 MAPK as a trigger caused a reduction in infarct size and suggests a role for p38 MAPK as a trigger of preconditioning. Due to the possibility that a multi-cycle protocol of ischaemic preconditioning may activate multiple mechanisms, the data using anisomycin should be considered to be more important to define the role of p38 MAPK activation than the data in which the inhibitor of p38 MAPK was used. These concepts are summarized in Fig 4.1 and Fig 4.2

**Final conclusion:**

These results provide evidence for the involvement of the p38 MAPK pathway in the protective effect of preconditioning against ischaemia/reperfusion damage. Manipulation of this pathway may constitute a new therapeutic approach to counter the detrimental consequences of ischaemia/reperfusion.

Fig 4.1 Role of p38 MAPK activation as a trigger of protection as a trigger of protection

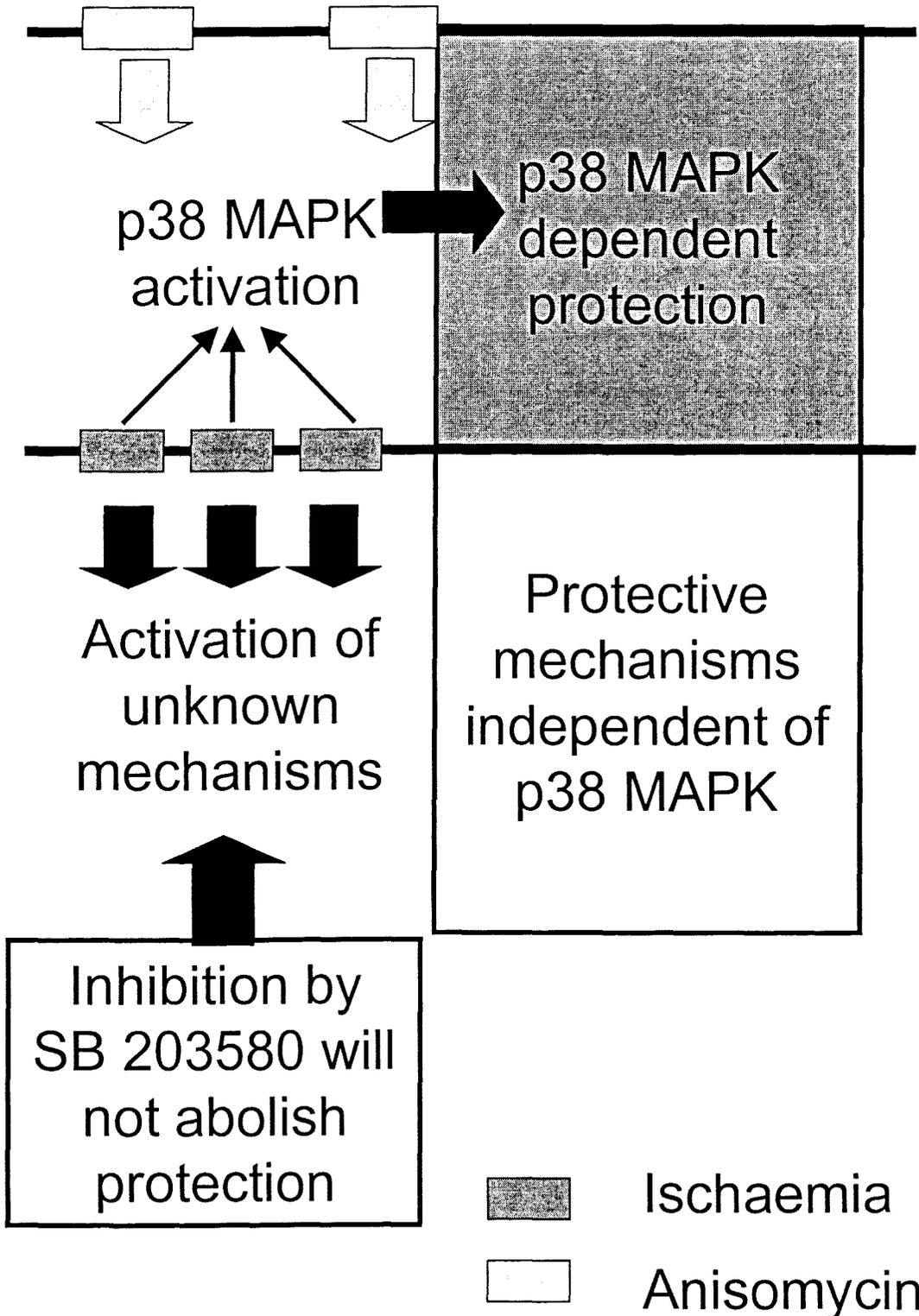
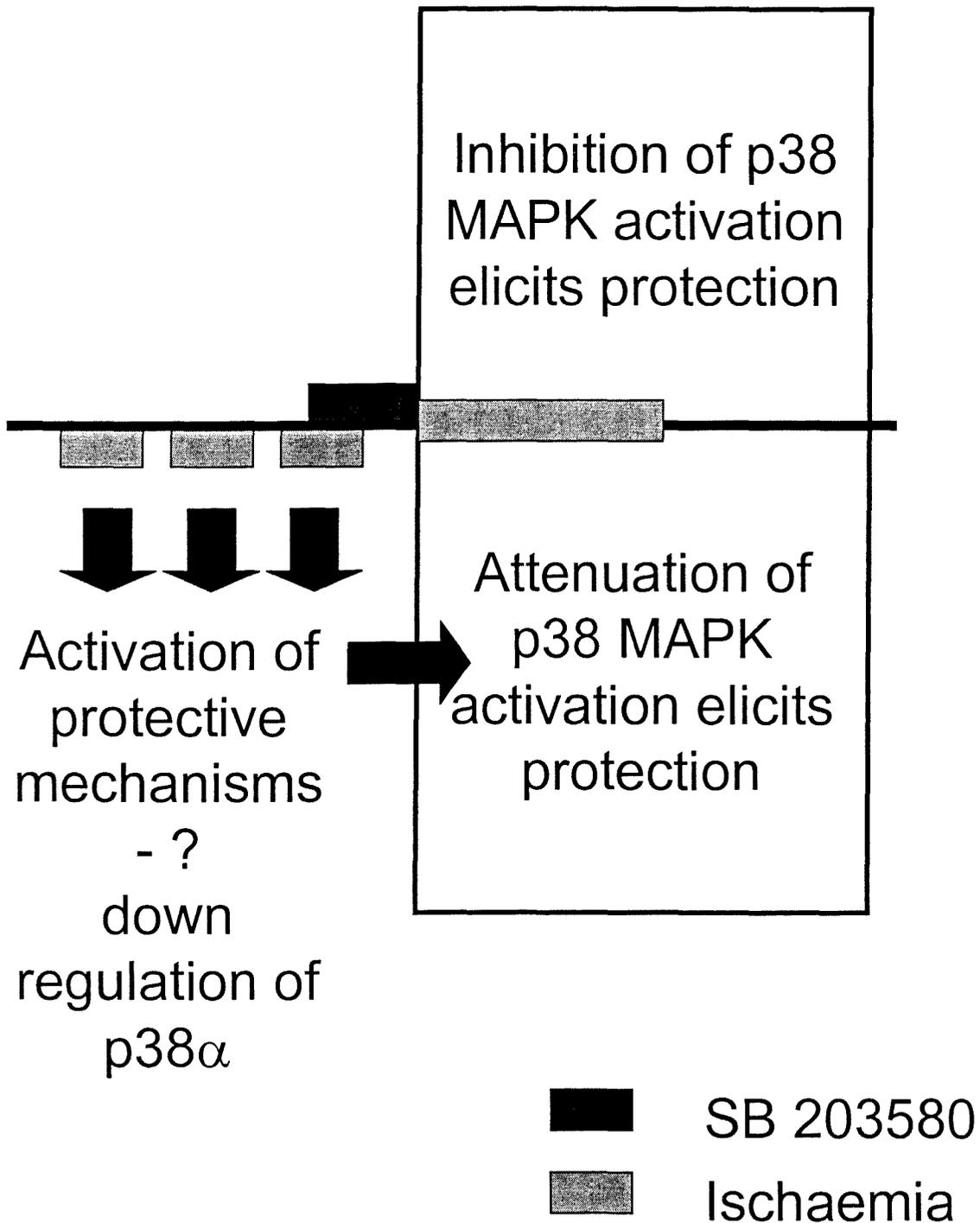


Fig 4.2 Role of p38 MAPK attenuation as a mediator of protection



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