THE NUCLEAR FACTOR –κB IN THE SURVIVAL SIGNALS OF THE ENDOGENOUS ISCHAEMIC CARDIOPROTECTION

PhD Thesis

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CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
AP-1	activation protein-1
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
COX	cyclooxygenase
DAG	diacilglicerol
DNA	dezoxyribonucleic acide
EMSA	electrophoretic mobility shift assay
ECG	elecrocardiograph
FV	ventricular fibrillation
GSH	reduced glutathione
HSP	heat shock protein
iNOS	inducible nitric oxide synthase
Ik-B	inhibitor kappa-B
IKK	inhibitor kappa kinase
IP3	inositol triphosphate
LAD	left anterior descendens coronary artery
LV	left ventricle (of the heart)
MAP kinase	mitogén aktivated protein kinase
MDA	malondialdehyde
MnSOD	manganese superoxide-dismutase
MPG	N-2-mercaptoproprionylglycine
NFkB	nuclear factor-kappaB

NO	nitric oxide
NSAID	non-steroid anti-inflammatory drugs
OFR	oxygen free radicals
ONOO	peroxinitrit
PC	preconditioning
PIP2	phosphatitil/inositol/ diphosphate
PKC	protein kinase C
PLC and PLD	phospholipase C and D
ROI	reactive oxygen intermediers
SOD	superoxide dismutase
SWOP	second window of protection
TyrK	tyrozin kinase
TTC	triphenyltetrazolium chloride

1. INTRODUCTION

It is now 11 years since the phenomenon termed "ischaemic preconditioning" was formally recognised [1]. There can be little doubt that our understanding of the mechanisms underlying the pathogenesis of ischaemia-reperfusion injury has been enhanced significantly by the extensive research stimulated by interest in endogenous myocardial protection. In the basic experimental setting, the triggers, mediators and effectors of the preconditioning phenomenon are being extensively investigated. The results of recent clinical experiments suggest that preconditioning can protect against ischaemic injury, although at this stage they must be interpreted with caution.

1.1 HISTORY OF ISCHAEMIC PRECONDITIONING

In 1986 Reimer et al.[2] reported a series of experiments in the dog heart designed to dissect the contributions of ATP depletion from catabolite accumulation in the genesis of lethal ischaemic injury. Their experimental model involved repetitive brief ischaemic episodes, working on the premise that each ischaemic episode would cause cumulative ATP depletion while the intermittent reperfusion would wash out ischaemic catabolites. To their surprise they found that, following the initial ischaemic period, ATP levels were not depleted further by subsequent similar ischaemic challenges. They also noted that no infarction occurred in six of the seven dogs studied. This result was contrary to the previously accepted view that repetitive ischaemia would cumulatively lead to infarction. The observation led the same group [3] to test the hypothesis that the preservation of high energy phosphates was due to a

slowing of consumption during ischaemia associated with a rapid and protective adaptation of the myocyte. They tested this hypothesis by subjecting the myocardium to four 5 minute coronary occlusions, separated by 5 minutes' reperfusion, before a sustained 40 minute ischaemic insult. They found that the preceding brief periods of ischaemia and reperfusion were protective, reducing infarct size to 25% of that seen in the control group. This phenomenon was termed "preconditioning with ischaemia". Following these initial studies, the protection obtained has been further characterised both in terms of time course and various end-points of cellular injury.

1.2. CLASSIC ISCHAEMIC PRECONDITIONING

Brief episodes of ischaemia-reperfusion protect the myocardium from the damage induced by subsequent more prolonged ischaemia. When first described by Murry et al.[4] such ischaemic preconditioning was elicited by brief coronary occlusion, and the endpoint was reduced infarct size. This protection, termed *classic preconditioning*, appears to be an acute and immediate response lasting not more than a few hours. Soon, however, a variety of preconditioning stimuli were uncovered including hypoxia[5], rapid cardiac pacing[6, 7] , thermal stress[8], stretch[9], and various pharmacological agents. Also various endpoints of ischaemic preconditioning have been used: ischaemic preconditioning protects against infarction in all species tested so far, and there is also evidence that it might be operative in human myocardium[10]. Ischaemic preconditioning also reduces the extent of apoptosis[11, 12]. Other studies have used recovery of contractile functions as an end point of ischaemic preconditioning. Although it appears logical that less infarcted myocardium in preconditioned hearts should result in improved regional and subsequently global function, ischaemic preconditioning does not improve regional myocardial function within the first hours of

reperfusion (thus it does not attenuate stunning) in animal models[13, 14, 15]. However, with longer reperfusion ischaemic preconditioning diminishes adverse left ventricular remodelling following infarction and improves long-termfunctional recovery in chronically instrumented rabbits[16]. Iscaemic preconditioning protects against arrhythmias in mice[17], rats[18], rabbits[19], and dogs[20]. In pigs, however, ischaemic preconditioning not only fails to reduce the incidence of ventricular fibrillation during ischaemia-reperfusion, but even accelerates the onset of ventricular fibrillation during sustained ischaemia and decreases the ventricular fibrillation threshold[21].

This manuscript does not want to go into details of the cellular pathways leading to classic preconditioning. It is believed that PKC phosphorlates other kinase, including p38 mitogen activator protein kinase (p38 MAP kinase), and eventually leads to the opening of the mitochondria ATP-sensitive potassium (K_{ATP}) channels. In support of this theory, studies using K_{ATP} channel inhibitors such as glibenclamide or 5-hydroxydecanoate have blocked protection (10,11). K_{ATP} channels open when intracellular ATP concentration falls. An influx of potassium ions follows, shortening action potential duration and reducing the influx of calcium ions into the cell. K_{ATP} channel opening is believed to be the key effector in conferring early protection to the myocardium. There are emerging views as to the mechanism behind this form of protection.

1.3. DELAYED PRECONDITIONING

In addition to the initial phase of protection in 1993 two separate studies by Kuzuya et al. and Marber et al. both observed that, a second wave of protection appears 24 hours following the

preconditioning protocol[22 23]. This second wave of protection is now referred to as the second window of protection (SWOP), late preconditioning or delayed preconditioning. SWOP has certain characteristics, distinct from classic preconditioning. It appears gradually, yet lasts as long as 72 hours or more.

Early studies about the late preconditioning studies took infarct size reduction as the end point of cardioprotection, and there was little data regarding any delayed antiarrhythmic effect in the second window. Végh et al. in 1994published a study that positively confirmed delayed protection against reperfusion arrhythmias in the canine heart, using ventricular rapid pacing to globally precondition the heart[24].

1.3.1. TRIGGERS OF DELAYED PRECONDITIONING

As with investigations of classic preconditioning, the mechanisms of delayed preconditioning may be conceptually divided into `upstream' and `downstream' components. In the rabbit, adenosine A_1 receptor activation during preconditioning is an important trigger of delayed protection against infarction. Adenosine receptor blockade during preconditioning abolishes the protective response 24 hours later[25] and conversely, stimulation of A_1 receptors with a selective agonist results in marked protection against infarction 24–72 hours later[26 27 28]. In the pig, delayed preconditioning against stunning does not appear to involve adenosine, but free radicals and nitric oxide are important triggers [29 30]. At present it is not possible to say if this divergence is due to differences in experimental endpoint (infarction versus stunning) or species (rabbit versus pig).

Since it is clear that delayed myocardial protection can be induced by means other than transient ischaemia, investigation of these stimuli may ultimately be relevant, not only for our understanding of the mechanisms of delayed preconditioning but to the development of *practical therapeutic approaches*. For example, bacterial endotoxin treatment is known to

induce delayed myocardial protection, probably by upregulating various cytoprotective proteins, including antioxidants and inducible nitric oxide synthase. The endotoxin derivative monophosphoryl lipid-A induces myocardial protection 24 hours after administration and the opening of the K_{ATP} channel may be integral to this late protective response[31]. There is considerable evidence that bradykinin (synthesised by endothelial cells) is involved as a trigger in preconditioning, and may contribute to the cardioprotective effects of ACE inhibitors[32 33]. ACE inhibition results reduced breakdown of bradykinin, thus could start up the signaling cascade leading to delayed protection[34].

1.3.2. THRESHOLD HYPOTHESIS OF TRIGGERING THE ISCHAEMIC PRECONDITIONING

Not all combinations and durations of ischaemia and reperfusion will trigger the preconditioning phenomenon and protect ischaemic myocardium. There appears to be a critical threshold. Very short preconditioning ischaemia with subsequent reperfusion prior to the index ischaemia has no protective effect in pigs[35] and humans[36]. Above this threshold the protection conferred by ischaemic preconditioning is independent from the intensity of the preconditioning stimulus. There is a definite and fairly rigid time frame for ischemic preconditioning. Somehow the myocardium `remembers' that it has been preconditioned by a brief ischemic period, which has occurred up to several hours before the index ischemia. The exact nature and location of this memory is one of the great, unsolved mysteries of ischemic preconditioning.

1.2.3. SIGNALLING ASPECTS OF DELAYED PRECONDITIONING

Activation of PKC appears to be a crucial intermediate step since pharmacological inhibition of PKC during the preconditioning stimulus abolishes protection 24 hours later in the rabbit

infarct model[37]. Direct measurements of PKC activity and translocation have not been widely studied so far but Parratt's group have recently provided evidence that sustained PKC-ε translocation to the membrane fraction occurs in the hearts of dogs subjected to rapid cardiac pacing[38], a stimulus that induces delayed protection against ischaemia-reperfusion arrhythmias. It has also been reported that brief repeated periods of coronary artery occlusion in the conscious rabbit cause the translocation of PKC-ε[39], and that this can be blocked with chelerythrine[40].

The involvement of other parallel and downstream kinases is under investigation. Considerable interaction exists between PKC and other kinase systems including tyrosine kinase and MAP kinase cascades. Tyrosine kinase activation may be an obligatory component of the signalling cascade since administration of genistein during preconditioning in rabbits abrogates the delayed anti-infarct effect[41]. Interestingly, delayed protection induced by adenosine A_1 agonist in rabbits is dependent on both PKC and tyrosine kinase activation since protection can be abolished by pretreatment with either chelerythrine (a PKC inhibitor) or lavendustin-A (a tyrosine kinase inhibitor)[42]. The interactions of these complex signalling systems and their involvement or interaction with membrane channels or new protein synthesis still needs to be evaluated.

The transcription factor nuclear factor-kappaB (NFkB) appears to be a critical regulator for gene expression induced by diverse stress signals including mutagenic, oxidative and hypoxic stresses. NFkB is a ubiquitous transcription factor, which is translocated in response to oxidative stress from its inactive cytoplasmic form by releasing the inhibitory subunit Inhibitor kappaB (IkB) from NFkB[43 44]. Activation of NFkB is likely to be involved in the induction of gene expression associated with the ischaemic adaptation, since this transcription

factor has recently been found to play a crucial role in the regulation of ischaemia-reperfusion-mediated gene expression and consecutive protein synthesis[45].

1.2.4. PROTEIN EFFECTORS OF DELAYED PROTECTION

A fundamental difference between classic and delayed preconditioning may be in the means by which cardioprotection is conveyed. In the former, K_{ATP} channels are suspected to be the end-effectors, in the delayed preconditioning newly synthesised cardio-protective proteins are claimed to convey protection. The time course of delayed preconditioning is suggestive of a mechanism involving new protein synthesis.

Two early reports raised the possibility that the delayed phase of protection involves either increased activity of manganese-superoxide dismutase (SOD)[46] or elevation of the myocardial content of the major inducible heat shock protein, HSP72[47]. Both proteins are stress-induced proteins that have cytoprotective properties. Manganese-SOD is a mitochondrial antioxidant which detoxifies superoxide anions. HSP72 is a chaperone protein involved in regulation of protein folding, transport and denaturation during the cellular response to injury. Hoshida et al.[48] described the temporal dynamics of manganese-SOD activity following preconditioning in canine myocardium and reported a biphasic pattern of enzyme activity over a 24 hour period similar to the biphasic timecourse of the anti-infarct effect[49]. Similarly, myocardial content of HSP72 was elevated in rabbits 24 hours after preconditioning, a time when increased tolerance to infarction was observed[50]. Relationships between enhanced ischaemic tolerance and stress-inducible cytoprotective protein activity have been pursued most convincingly in gene transfection studies[51 52], antisense oligonucleotide studies[53] and studies with transgenic mice constitutively over-expressing human HSP72[54]. These studies tend to suggest, but do not confirm, that the appearance of the delayed protection could be related to changes in activities of stress-inducible cytoprotective proteins in preconditioned

myocardium. However, it is important to note that since the regulation of a large number of proteins is altered by sublethal ischaemia, it is likely that delayed preconditioning involves other proteins in addition to anti-oxidants and heat shock proteins. A number of new gene products not yet identified may be involved and techniques such as differential gene display and other advanced molecular techniques will be very relevant to future research directions.

2. AIMS AND HYPOTHESIS

In the last decade the extensive research in the field of ischaemic preconditioning has greatly extended our understanding of the signalling mechanism of cardiac adaptation. Although many questions are yet to be elucidated, especially with regard to the role of transcription factors in the second window of protection, it remains one of the most powerful experimental tools in cytoprotection that may one day translate into a clinical reality.

In the first part of our investigations we aimed to monitor the time fluctuaion of the activation of two transcription factors, nuclear factor (NF)-kB and activation protein (AP)-1, which are conferred to play essential role in the gene-expression induced by the ischaemic preconditioning. We aimed to measure the DNA binding activity of these two transcription factors in the nuclear fraction of cardiomyocytes in different, definitive times of the reperfusion following the stimulus of ischaemic preconditioning.

In the second part of our investigations, we aimed to define the activation level of NFkB after repeated number of preconditioning stimuli. The protective effect of delayed preconditioning is an “all or nothing” response: the strength of the evoked cardioprotection is independent from the strength (duration and number of the ischaemic-reperfusion cycles) of the preconditioning (PC) stimuli. We hypothetized that the activation of the transcription factors in the delayed adaptation is also independent from the PC stimulus, thus the all or nothing response evolves in the level of the triggers.

We also aimed to investigate the role of oxygen free radicals (OFR) in the signaling cascade of this adaptive process leading to the induction of NFkB. The Department of Experimental Surgery in Pécs has many years of experience investigating free radicals in relation to ischaemic-reperfusion, especially in heart tissue. This provided us with the ideal opportunity to set upon the task of examining the role of OFR in delayed IPC. Numerous studies suggest that OFRs act as trigger and as mediators of delayed ischaemic preconditioning in an additive interaction with the other triggers. Accordingly, we aim to measure the NFkB activation after repeated cycles of preconditioning ischaemia-reperfusio after blocking the OFRs. To monitor the haemodynamic parameters of the heart during the preconditioning and in the reperfusion period we measure the heart rate, systolic and diastolic heart pressure and calculate the rate pressure product of the animals.

It has been demonstrated that the nonsteroid anti-inflammatory drog, aspirin - which is widely used for patient with ischaemic heart disease - *in vitro* blocks the activation of the transcription factor NFkB, which is necessary for the delayed cardiac adaptation. **In the third part of our investigations we aimed to demonstrate that aspirin (acetylsalicylic acid, ASA), used in low and medium concentration do not inhibit the activation of NFkB *in vivo* in the preconditioned myocardium.** Furthermore we investigate the effect of ASA on the delayed cardioprotection against myocardial infarction.

3. DYNAMISM OF ACTIVATION OF NUCLEAR FACTOR-κB AND ACTIVATION PROTEIN-1 IN THE SIGNALLING OF DELAYED MYOCARDIAL PRECONDITIONING

3.1. INTRODUCTION

The sublethal ischemic stress initiate a complex signal transduction cascade that modulates the activation of the severe transcription factors, which lead up to expression of cardioprotective genes. Recent studies assume, that the activation and translocation of Nuclear Factor (NF) -κB and Activation Protein-1 (AP-1) is a key component in the signal transduction mechanism of ischaemic preconditioning[55,56].

3.1.1. NFkB IN THE SIGNALLING OF ISCHAEMIC PRECONDITIONING

NF-kB is a redox sensitive transcription factor involved in transcription of proteins in response to mutagenic, oxidative and hypoxic stress[57 58]. Under normal physiologic conditions NF-kB is found in the cytoplasm of cells in an inactive form in association with the inhibitor IkappaB-alpha. Upon cellular stress stimulation, IkB-alpha is phosphorylated on two serine residues (S32 and S36), ubiquitinated, and degraded by a proteosome-dependent pathway, allowing active NF-kB to translocate to the nucleus[59], [60]. NF-kB translocation leads to expression of a large number of NF-kB related genes, encoding various signaling and defense proteins, which are supposed to bring the cellular protection in the late preconditioning[61].

In contrast to the role NF-kB activation has in the destructive events of inflammation, NF-kB also mediates the expression of cytoprotective proteins (Heat Shock Proteins) that block

apoptosis or inhibit inflammation in response to several types of cellular stress[62]. In a negative feedback manner, these cytoprotective proteins inhibit NF-kB[63].

The NF-κB pathway displays the capacity to activate, in a cell- and stimulus –specific manner, only a subset of the total repertoire of NF-κB –responsive genes. The seemingly promiscuous nature of NF-κB activation poses a regulatory quagmire as to how specificity is achieved at the level of gene expression. Rapid growth in our understanding of signal transduction in general, and NF-κB in particular, provide intriguing insight as to how this may occur.

3.1.2. AP-1 IN THE SIGNALLING OF ISCHAEMIC PRECONDITIONING

Ap-1 is another well-characterized eukaryotic transcription factor that is highly regulated by the redox status of the cell. It is composed of various subunits (jun, fos, and Fra) as dimers, which recognize with different affinities the AP-1 DNA-binding site. Through the mechanism of reactive oxigen intermediates (ROI) – generated by ischaemia-reperfusion – induction of AP-1 is not clear, alteration of cell thiol redox status has been shown to induce c-fos and c-jun expression, and phosphorylation of jun proteins. Many factors including ionizing radiation, cytokines, oxidative stress, and growth factors lead to AP-1 activation. Similar to NF-κB, activation of AP-1 induces expression of a variety of genes whose protein products may either protect the cells from, or make the cells more susceptible to oxidative stress. Oxidative stress caused by the brief ischaemia-reperfusion of preconditioning induce NF-κB and AP-1 activation, both of them activate pro- and anti-apoptotic signals in vivo in the myocardium.

For understanding the role, and the detailed regulation of these transcription factors in the phenomenon of ischaemic PC, the purpose of this study was to examine the time fluctuation of NF-κB and AP-1 levels in the preconditioned myocardium. We measured the DNA binding

activity of NF-κB and AP-1 at various times after brief ischaemic episode in reperfused myocardium.

3.1.3. AIMS

Because the exact mechanism of the activation of these factors is still not clear in the signaltransduction of the ischaemic preconditioning of the myocardium, we aimed to monitor the time fluctuation of the NFkB and AP1 induction in an in vivo animal model. We aimed to follow the changes in the activation rate of NFkB and AP1 in the reperfusion period after ischaemic preconditioning stimuli.

3.2. MATERIALS AND METHODS

The present study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23,revised 1996) and was approved by the local institutional Committee on Animal Research of Pécs University (BA02/2000-29/2001).

3.2.1. SURGICAL PREPARATION

A marginal ear vein was cannulated in 42 New Zealand White rabbits weighing 2,6-3,3 kg (mean 2,8kg), after local anaesthesia was induced using lidocaine cream. The animals were anaesthetised with intravenous (iv.) xylazine (6mg/kg), ketamine (6 mg/kg) and propofol (10mg/kg). The trachea was intubated (tube 3 mm internal diameter) and the lungs were ventilated (Sulla 808, Drager , Lübeck, Germany) at a frequency of 30-35 breaths/min and a tidal volume of 15-20 ml. Anaesthesia was maintained by inhalation of isoflurane (2-4 Vol.%) and nitrous oxide (50 Vol.%).

The chest was opened by midline sternotomy. A 5-0 prolene (Ethicon 5/0, 1-metric, TF) ligature was passed around the left anterior descending (LAD) coronary artery and through a snare. In general the site of vessel encirclement was on the long axis of the left ventricle towards the apex approximately one-fourth of the distance from the atrioventricular groove to the left ventricular apex. Fifteen minutes after completion of surgical preparation animals were heparinized with 500 U of heparin sodium. Temperature was measured inside the pericardial cradle (Siemens Sirem, Digital Thermometer, Düsseldorf, Germany) and maintained between 38,3˚C and 38,7˚C by adjusting a heating pad and an infrared lamp.

In the ischaemic preconditioned (PC) groups the snare was tightened for 5 min, thereby inducing occlusion of the coronary artery. Myocardial ischaemia was readily discernible by

the development of a dusky, bulging region of myocardium (careful note was made of anatomic landmarks of this region). The effectiveness of this manoeuvre was verified by the appearance of epicardial cyanosis and by the immediate occurrence of ST-segment elevations in the electrocardiogram (ECG) (Siemens Sirecust 1260, Düsseldorf, Germany). At the end of the 5-min period of coronary artery occlusion, the suture was released and removed to ensure proper reperfusion, which was verified by the disappearance of the ECG changes within 5 min in every animal.

3.2.2. EXPERIMENTAL PROTOCOL

In our experiments the animals (42 rabbits) were randomly listed in 7 groups. In control animals (group 1, 6 animals) the heart was excised right after thoracotomy and tissue sample was taken from the untreated heart. In the preconditioned groups, after 5 min ischaemic period animals were assigned to 10 min (group 2, 6 animals), 30 min (group 3, 6 animals), 1hour (group 4, 6 animals), 2 hours (group 5, 6 animals), 3 hours (group 6, 6 animals), or 4 hours (group 7, 6 animals) reperfusion period (R) before taking tissue sample from the ischaemic zone of the heart. (figure 1.)

After the experimental period the heart was rapidly excised and rinsed in ice-cold physiological saline. The ischaemic zone was excised on the basis of the previously defined landmarks. The tissue was snap frozen in liquid N2, and stored for not more than 3 days at -82C before EMSA analysis.

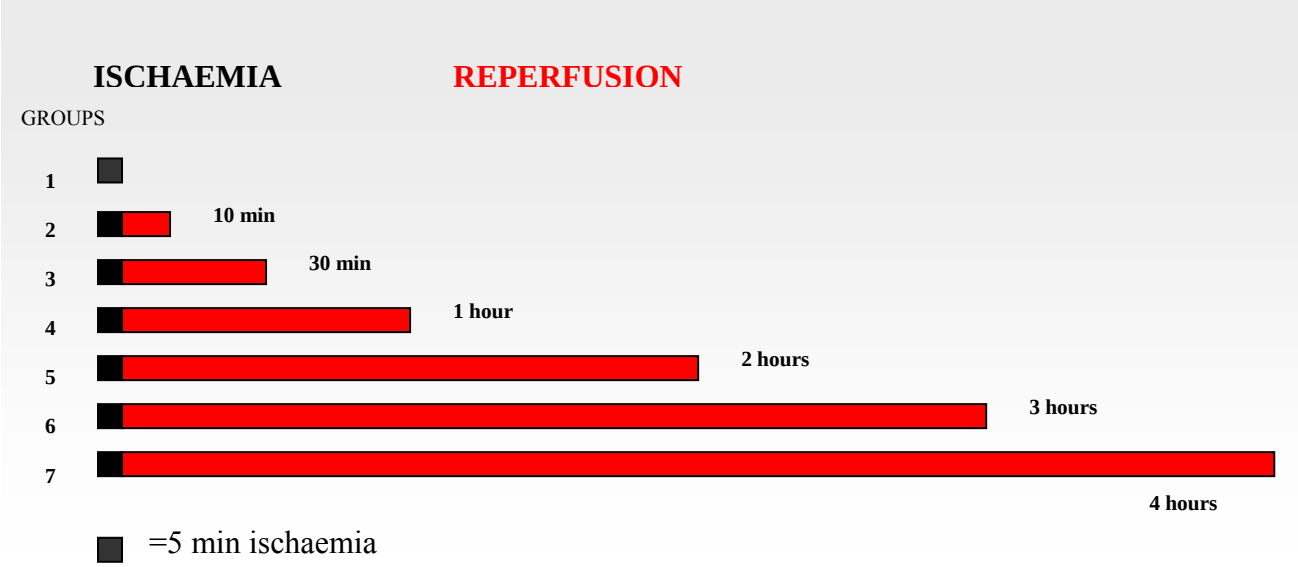


Figure 1. Experimental groups with different time of reperfusion.

3.2.3. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

100 mg tissues were homogenized in 1 ml TE buffer (1.5 mM EDTA, 0.01 M Tris Base, pH 7.4) containing 10 μM PMSF. Nuclei were separated from cytosol by centrifugation at 1400 xg for 20 min at 4°C, and this separation procedure was repeated for 3 times. The last pellet was resuspended in 2 volumes of buffer containing 20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitors (Complete Mini, Boehringer Mannheim, Germany) and placed on ice for 20 min. After 10s centrifugation the supernatants were saved, alliquoted and stored.

Protein concentration was determined with the Bio-Rad Protein Assay kit. 5'-end labelling of oligonucleotides was performed using [γ-^{32}P]-ATP and T4 polynucleotide kinase (Amersham Pharmacia Biotech Inc., UK) according to the manufacturer's protocol.

20 μg nuclear proteins were mixed with 1 μg poly(dl-dC), 100 ng non-specific single-stranded oligonucleotide and 4 μl buffer containing 10 mM HEPES pH 7.5, 10% glycerol, 1 mM EDTA, 100 mM NaCl. After 15 min incubation at room temperature the mixtures was

completed with 2 μl, approximately 100 000 cpm of ^{32}P oligonucleotide and then incubated for another 30 min. DNA-protein complexes were electrophoresed in a 5% non-denaturating polyacrylamide the gel using a Tris Base, Borate, EDTA buffer system (pH 8.3) for 2.5 h at 200 V. Gel was dried and were quantitated using an image scanning densitometer (Cyclone Phosphorlmager System, Packard I. C. USA).

3.2.4. STATISTICAL ANALYSIS

For statistical analysis EMSA pictures were appreciate with Colim 2000 Color Image Measuring (Pictron Kft, Hungary) densitometry software. The data (mean ± SEM) for levels of expression of individual protein (EMSA) were subjected to analysis of variance with post hoc Dunnett's 1-tailed t-tests (NF-κB, and AP-1 EMSA), and two-sample Student's t-test for significance. $P < 0,05$ was considered significant.

3.3. RESULTS

3.3.1. TIME COURSE OF NF-KB ACTIVATION

Fig. 2. shows changes in NF-kB levels in preconditioned myocardium after various reperfusion (R) times. Specificity of the signal was verified in a competition assay wherein the signal detected by labeled NF-kB was abolished, when the protein homogenate was preincubated with excess unlabeled NF-kB oligo before the addition of labeled NF-kB. The signal was not abolished, when the competition assay contained excess unlabeled non NF-kB binding oligonucleotide. Low and consistent levels of NF-kB were detected in normal myocardium (untreated: group 1) at steady state. Significantly higher levels were detected at 30 min R (group 3) in all 6 animals (densitometry: 2,35-fold; $p<0.0001$ vs. controls), and then fell to lower state at 1 h R. Again at 3 h R (group 6), the levels rose significantly higher (2,59-fold; $p<0.0001$). At 4 h R the levels decreased to basic rate, indicating a biphasic regulation (with an emphatic up- and downregulation) of NF-kB in preconditioned myocardium.

3.3.2. TIME COURSE OF AP-1 ACTIVATION

Fig. 3. shows changes in AP-1 levels in control (untreated) and in preconditioned myocardium. After a weak signal elevation at 30 min R, significant increase of AP-1 levels were detected at 1 h R (group 4) ($p<0.001$). Though the levels declined gradually, they were still signal at 2, 3, and 4 h R. Preincubation of protein homogenate with excess unlabeled AP-1 consensus oligo abolished specific signals obtained by labeled AP-1, demonstrating the specificity of signals. Signal specificity was established the above mentioned way in all cases.

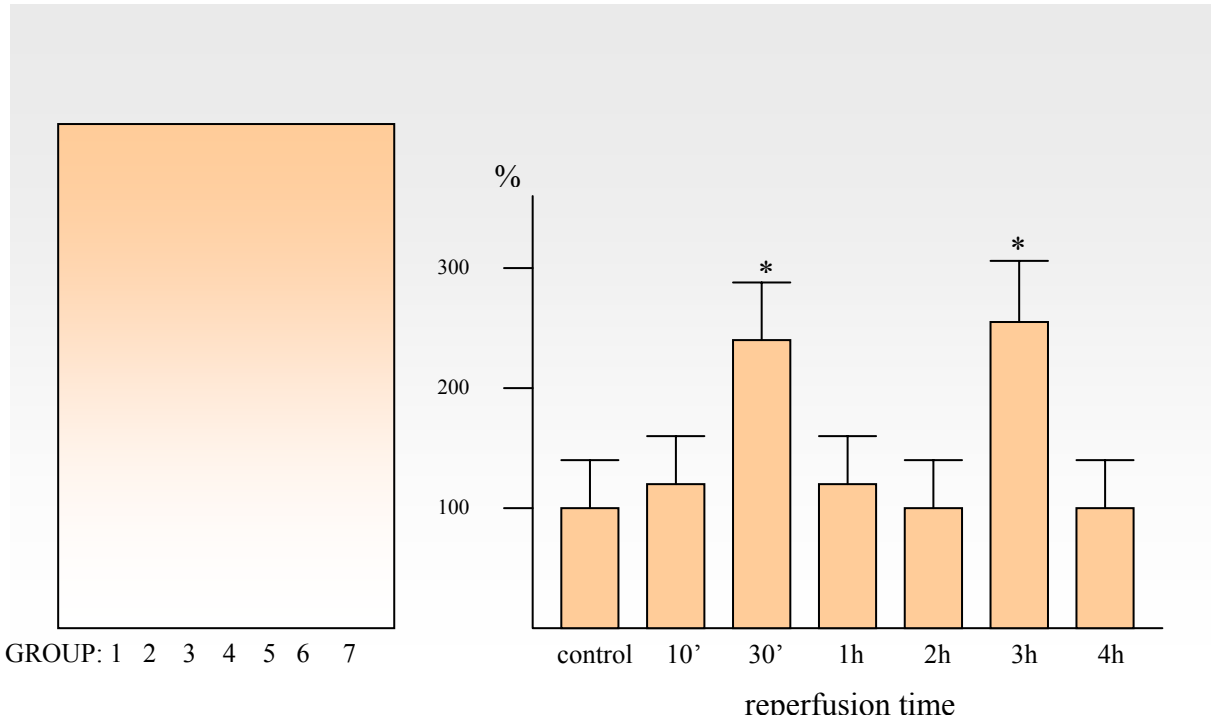


Figure 2. Nuclear levels of NFkB after different reperfusion period in the preconditioned myocardium. (time course). Activated NF-kB bands (a p50/p65 heterodimer and a p50 homodimer) are shown. EMSA analysis.(left) Densitometry measurements expressed as percent of control. * p<0,0001 v control.(right)

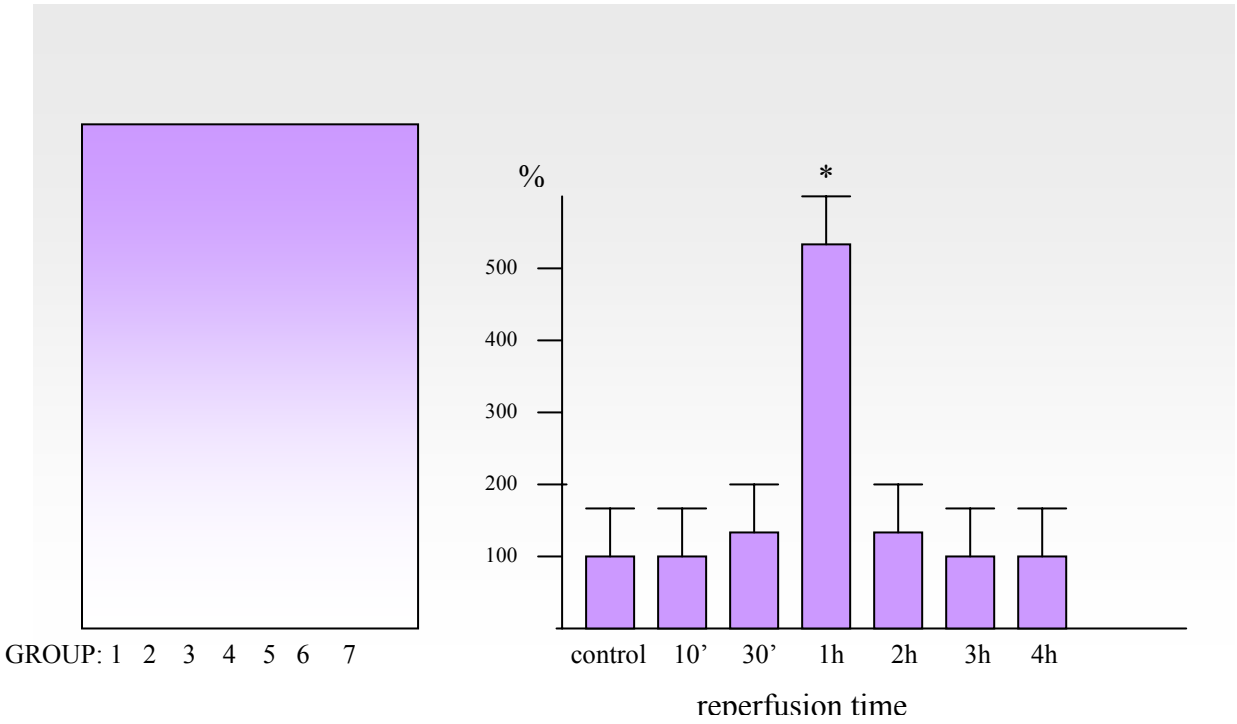


Figure 3. Nuclear levels of AP-1 transcription factor after different reperfusion period in the preconditioned myocardium. (time course) EMSA analysis. (left) Densitometry measurements expressed as percent of control. * p<0,001. (right)

3.4. DISCUSSION

Recurrent episodes of myocardial ischaemia are commonly observed in patients with coronary artery disease who suffer from frequent angina pectoris or angioplasty of the left anterior descending coronary artery. Reversibly injured myocardium (by short episode of ischaemia followed by another short period of reperfusion) renders the heart more resistant to a longer ischaemic-reperfusion period. Such adaptation – ischaemic precondition (PC) - is mediated through the upregulation of the heart's own cellular defense via the accumulation of intracellular mediators and reprogramming of gene expression. Recent studies suggest that NF-kB and AP1 transcription factors have a possible role in the signaltransduction pathways of this cytoprotection are resulted from ischaemic adaptation.

3.4.1. DYNAMISM OF NFkB ACTIVATION

The results of our experiments show that there is a biphasic activation of NF-kB in the preconditioned myocardium, with increased levels at an early time point (30 min), and again at 3 hour R. There is presumed to be two different ways leading to the early NF-kB activation after ischaemic PC. Through receptor-dependent triggers (AdenosineA_1 agonists[64], opioid δ_1 agonists[65], bradykinin, prostaglandins, norepinephrine, angiotensin, endothelin): the receptor is coupled through G proteins to, among others, phospholipase C (PLC) and D (PLD). PLC catalyzes the hydrolysis of membrane inositol-containing phospholipids into inositol trisphosphate and diacylglycerol (DAG)[66]. DAG stimulates the translocation and activation of protein kinase Cε (PKCε). The onset of the PLC reaction is typically very rapid, and DAG production is short-lived, peaking at 30 s[67]. PKCε activation then triggers a complex signaling cascade that involves Src and-or Lck tyrosine kinases and probably other kinases, leading to

phosphorylation of Inhibitor-κBα (IκBα) and to mobilization (nuclear translocation) and activation of the transcription factor NF-kB[68].

Another possible way of NF-kB activation in ischaemic PC is came off through the increased production of nitric oxide (most likely via eNOS) and $\cdot O_2^-$ (leading to formation of secondary reactive oxygen species (ROS)) after a brief episode of myocardial ischaemia/reperfuson[69]. Both NO and $\cdot O_2^-$ derived ROS could directly activate the ε isoform of PKC via nitrosylation and oxidative modification, respectively; alternatively, NO and $\cdot O_2^-$ are known to react to form ONOO- which, in turn, could activate PKCε. Thus PKC is thought to be a critical component in both pathways[70,71].

Ischaemic PC has recently been found to activate Janus Activated Kinase 1 and 2 (JAK1, JAK2) with a subsequent tyrosine phosphorylation and activation of STAT1 and STAT3, which is essential for iNOS upregulation. Binding of NF-kB and STAT1/3 to the inducible Nitric Oxide Synthase (iNOS) promoter results in transcriptional activation of the iNOS gene and lead to synthesis of new iNOS protein (eNOS-dependent iNOS induction). iNos –derived NO is supposed to make a second wave of PKCε activation, leading to the late phase of NF-kB activation and translocation[72].

The other possible explanation of the late increase in NF-kB activation might be the feed-back mechanism of NF-kB induced pro-inflammatory cytokines (TNFα, IL-1β). After ischaemia-reperfusion, even in case of ischaemic preconditioning, NF-kB can induce TNFα and IL-1β gene expression[73,74]. Trough a certainly unknown mechanism these cytokines generate a mass of ROI in the myocardium, which can - via the above mentioned pathway – newly activate NF-kB in the cytoplasm and lead to a delayed wave of nuclear translocation of this transcription factor. TNFα and IL-1β can also start up a signaling pathway leading to IkB

phosphorylation, - and thus NF-kB activation - through cell membrane receptors[75]. A number of signal transduction proteins have been identified as associated with these receptors, including TNF-receptor associated factors 2 and 6 (TRAF2 and 6) death domain-containing proteins (TRADD and FADD), kinases associated with IL-1 receptor (IRAK1 and 2, and MYD88). These kinases phosphorylate members of the IkB family at specific serines within their N-termini, leading to site-specific ubiquitination and degradation of NF-kB by the 26S proteosome. This circle cascade (NF-kB→TNFα, IL-1β→NF-kB feedback) might also be an explanation of the biphase activation of NF-kB after ischaemic PC[76]. (figure 4.)

3.4.2. DYNAMISM OF AP-1 ACTIVATION

The second observation of our study is the detection of increased AP1 levels in the preconditioned myocardium. In contrast to NF-kB, where after an initial increase at 30 min a second peak was observed at 3 hR, AP1 levels increased in a monophasic manner at 1 h R. Though we did not measure the levels of AP1 during the ischaemic period, in various in vitro systems, substantial increase in AP1 levels was demonstrated during hypoxic conditions. In a cancerous cell line (HeLa), Rupec and Baeuerle have shown increased NF-kB activity within 15 min after initiation of reperfusion, while increased AP1 was detected during hypoxia itself[77]. They argued that during reoxygenation, increased intracellular ROI activate existing NF-kB by dissotiation from its inhibitor IkB, while low levels of free radicals during hypoxia, a condition similar to that observed during antioxidant treatment, induced AP1.

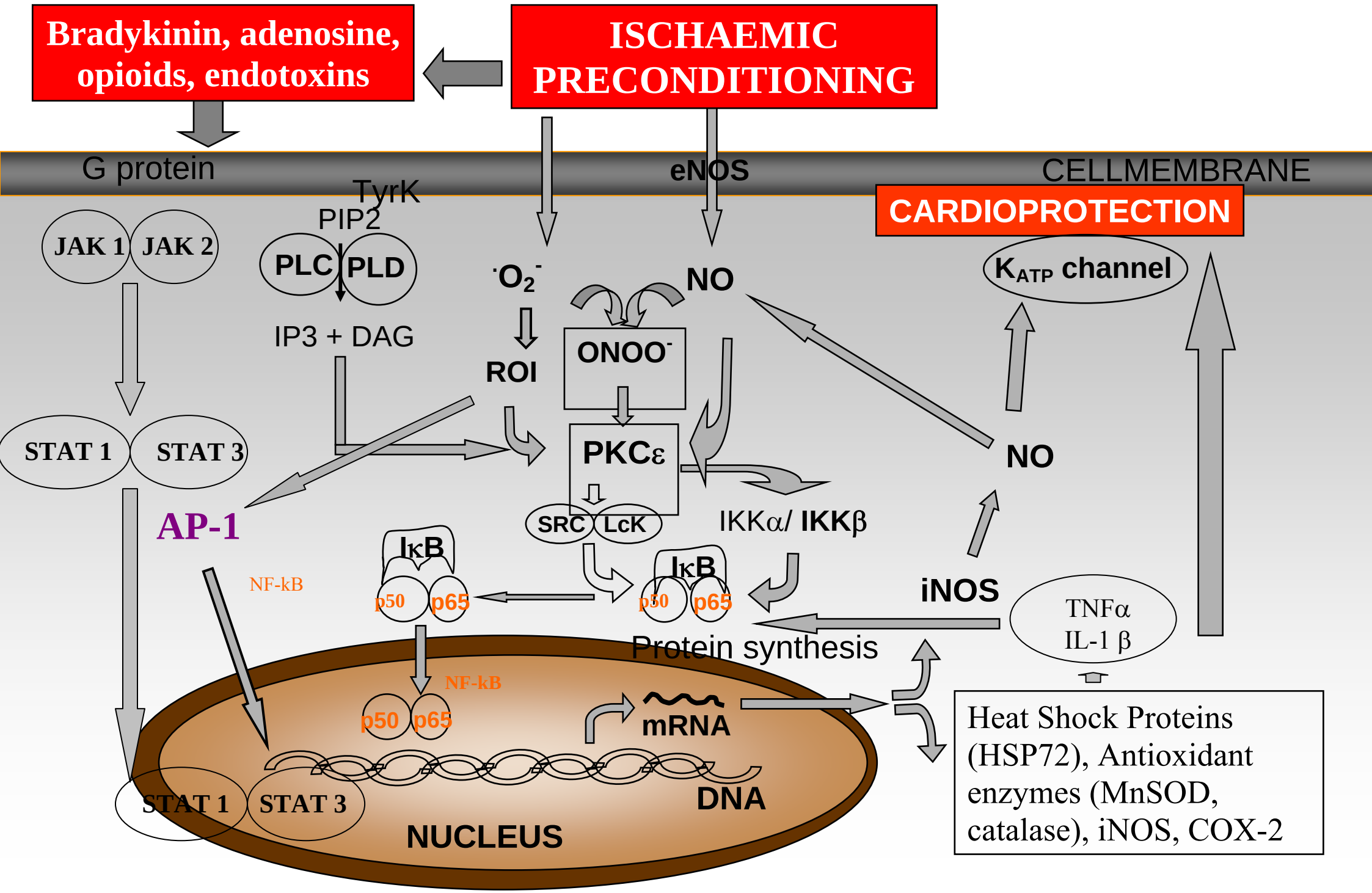
Because both NF-kB and AP1 are activated by cytokines such as IL-1 and TNFalpha, the positive synergy between NF-kB and the subunits of AP1 might have important implications for both immune and inflammatory responses. Stein et al. have shown functional cross-coupling of NF-kB p65 and AP1 families of transcription factors, resulted in increased DNA

binding activity of NF-kB. Both s-fos and c-jun synergised with NF-kB by physically interacting with p65 subunit[78]. Whether such interaction exists in preconditioned myocardium is not known.

3.4.3. CONCLUSION

From our study we conclude, that after the ischaemic preconditioning stimuli the activation of NFkB is biphasic with peak levels at 30min and at 3 hour of reperfusion in the preconditioned myocardium. The activation rate of AP1 increased monophasically, with peak level at 1 hour of reperfusion. These data show that the activation of NFkB and AP1 have a specific time curve in the signaling of endogenous cardioprotection.

Figure 4.(next page) Shematic representationof the intracellular signaltransduction of ischaemic preconditioning. (NO-nitric oxide; ROI-reactive oxygen intermediers; ONOO-peroxinitrit; PLC and PLD-phospholipase C and D; PIP2-phosphatitil/inositol/ diphosphate; Ip3-inositol triphosphate; DAG-diacilglicerol; PKC-protein kinase C; IKK-inhibitor kappa kinase; MAP kinase-mitogén aktivated protein kinase; TyrK-tyrozin kinase; NFkB-nuclear factor-kappaB; iNOS-inducible nitric oxide synthase; MnSOD- manganese superoxide-dismutase)


Bradykinin, adenosine, opioids, endotoxins
ISCHAEMIC PRECONDITIONING
G protein
eNOS
CELLMEMBRANE
CARDIOPROTECTION
TyrK
PIP2
JAK 1
JAK 2
PLC
PLD
IP3 + DAG
·O2-
ROI
NO
ONOO-
KATP channel
PKCε
NO
STAT 1
STAT 3
AP-1
SRC
LcK
IKKα/ IKKβ
IκB
p50
p65
NF-kB
iNOS
TNFα
IL-1 β
Protein synthesis
mRNA
DNA
NUCLEUS
Heat Shock Proteins (HSP72), Antioxidant enzymes (MnSOD, catalase), iNOS, COX-2

4. THE ROLE OF OXYGEN FREE RADICALS IN THE ACTIVATION OF NF-KB IN THE PRECONDITIONED MYOCARDIUM

4.1. INTRODUCTION

4.1.1.GENERAL BACKGROUND

Oxygen free radicals are highly reactive molecules with an unpaired electron, associated widely with ischaemic-reperfusion injury[79 80 81]. Although better known for their toxicity, when in large quantities they overwhelm the endogenous antioxidant systems, recently it has been suggested that at low concentrations they can modulate survival functions within the cell. As previously mentioned Murry *et al* was the first to investigate their potential role as triggers of classic IPC[82]. Since then various species and models have been examined with conflicting results[83 84 85 86], yet it is now generally acknowledged that oxygen free radicals can indeed induce early protection. Their role in delayed preconditioning however remains ambiguous.

Since oxidative stress is known to induce the synthesis of cardioprotective proteins, such as antioxidant enzymes[87 88 89 90] and HSPs[91 92 93], and since these proteins could theoretically mediate the protection observed 24 hours after the initial ischaemic challenge, we hypothesized that the molecular adaptations, that lead through transcription factor NFkB activation to the late preconditioning, are initiated by the exposure to increased levels of reactive oxygen species during the preconditioning ischaemia. Low levels of free radicals can activate protein kinase C (PKC) directly[94], thus through phosphorylation of Inhibitor Kappa Kinaseβ could induce NFkB activation and translocation to the nucleus. In addition, reactive

oxygen species may stimulate phospholipase D[95], and the consecutive production of diacylglycerol could then also lead to the activation of PKC and to the induction of NFkB.

4.1.2.AIMS

One possibility, that has not been tested, is that the free radicals may act in concert with the other triggers of delayed preconditioning (adenosine, bradykinin etc.) in the induction of the transcription factor NFkB. If that were the case, then elimination of the free radical component following a single cycle preconditioning protocol, which is close to the threshold for protection, would cause a subthreshold stimulation for NFkB activation and loss of protection. On the other hand, if multiple cycles of preconditioning were employed then loss of only the free radical component would not be missed, because enough additional adenosine and bradykinin and other triggers would be released to reach threshold. In the present study we tested this hypothesis by examining the ability of the potent, cell-permeant radical scavenger, N-2-mercaptoproprionylglycine (MPG), to attenuate the induction of NFkB in ischaemic preconditioning induced by either a single or multiple episodes of ischaemia-reperfusion in in situ rabbit hearts.

In the *first series* of this study we aimed to investigate the activation rate of NF-κB in cases of repeated cycles of ischaemic PC. In the *second series* we examined the NFkB activation after repeated cycles of PC in the presence of MPG.

4.2. MATERIALS AND METHODS

All procedures were approved by the Local Committee on Animal Research, and were in accordance with recommendations by International Guiding Principles for Animal Research[96] (48) and was approved by the local institutional Committee on Animal Research of Pécs University (BA02/2000-29/2001).

Adult New Zealand White rabbits (60 animals) of either sex with body weight ranging from 2,6-3,3 kg (mean 2,8kg) were used. The rabbits were subjected to overnight fast prior to the experiments. Animals were pre-medicated with droperidol (1,5mg/kg) and atropine (1mg). Anesthesia was induced by intravenous (iv.) xylazine (6mg/kg), ketamine (6 mg/kg) and propofol (10mg/kg). Anesthesia was maintained with isoflurane (2-4 Vol.%) and nitrous oxide:oxygen (7:3) gaseous mixture.

4.2.1. SURGICAL PROCEDURE

After anesthesia we opened the chest by midline sternotomy. 5-0 prolene (Ethicon 5/0, 1-metric, TF) ligature was circled around the left anterior descending (LAD) coronary artery. In general the site of vessel encirclement was on the long axis of the left ventricle towards the apex approximately one-fourth of the distance from the atrioventricular groove to the left ventricular apex. Fifteen minutes after completion of surgical preparation animals were heparinized with 500 U of heparin sodium.

In the ischaemic preconditioned (PC) groups the snare was tightened for inducing occlusion of the coronary artery. At the end of the ischaemic period, the suture was released and removed to ensure proper reperfusion, which was verified by the disappearance of the ECG changes within 5 min in every animal.

In the *second series* of the study the animals N-2-mercaptopropionylglicine (MPG) was universally administered as a continuous infusion to block oxygen free radicals during the ischaemia- reperfusion period of preconditioning cycles.

4.2.2. EXPERIMENTAL PROTOCOL

In the *first series* of our study we aimed to measure the NF-kB activation after repeated cycles of ischaemia-reperfusion. The animals were selected in five groups, in each group there were 6 rabbits. For NF-kB investigations the animals were subjected to either 1x-, 2x-, 3x-, or 4x-5 min LAD occlusion with an intermittent 5 min reperfusion, and after 30 min R (NF-kB showed the activation maximum at 30 min R) tissue samples were taken from the ischaemic zone of the heart. In the control group (group 1) animals were subjected to thoracotomy and LAD isolation, however no ligature was applied. In the second group the preconditioning stimuli comprised of a single cycle of ischaemia and reperfusion (1x5 IPC). In the third group animals were subjected to two cycles of ischaemia and reperfusion with intermittent 5-min reperfusions (2x5 IPC). In the fourth and fifth group all animals underwent three (3x5) and four cycle of 5 min of regional ischaemia (4x5 IPC). (figure 5.)

In the *second series* of this study we sought to block OFR by administering an antioxidant: N-2-mercaptopropionylglicine (1.5 mg/kg/min) as a continuous infusion to any protocol. In the first group, acting as drug control, MPG was administered 30 min before sham thoracotomy. In the others MPG was infused 30 min prior to 4x5, 2x5, 3x5 or 1x5 IPC. All groups were administered normal saline (vehicle) infusion, starting 30 min before the procedures. (figure 6.)

For NF-kB investigation (because NF-kB showed the activation maximum at 30 min R) we lasted 30 min reperfusion after the last cycle of PC and tissue samples were taken from the ischaemic zone of the heart for analysis.

After the experimental period the heart was rapidly excised and rinsed in ice-cold physiological saline. The ischaemic zone was excised on the basis of the previously defined landmarks. The tissue was snap frozen in liquid N2, and stored for not more than 3 days at -82°C before EMSA analysis.

4.2.3. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The exact method of EMSA analysis we applied in this study was described in the previous part of this thesis (3.2.3.).

4.2.4. HAEMODYNAMICS, ARRHYTHMIAS AND FIBRILLATIONS

Following a control measurement, ECG, heart rate and systemic blood pressures were registered and recorded every 15 minutes during the two-day procedure. Furthermore occurrence of any ventricular tachycardia and fibrillation were recorded automatically. In case of fibrillations, cardioversion was immediately attempted. Hearts that needed more than 3 consecutive cardioversions to convert ventricular fibrillation were excluded from the study.

4.2.5. STATISTICAL EVALUATION

For statistical analysis EMSA pictures were appreciate with Colim 2000 Color Image Measuring (Pictron Kft, Hungary) densitometry software. The data (mean ± SEM) for levels of expression of individual protein (EMSA) were subjected to analysis of variance with post hoc Dunnett's 1-tailed t-tests (NF-κB, and AP-1 EMSA), and two-sample Student's t-test for

significance. Haemodynamic data were analysed using repeated measures ANOVA. The null hypothesis was rejected when $P < 0.05$.

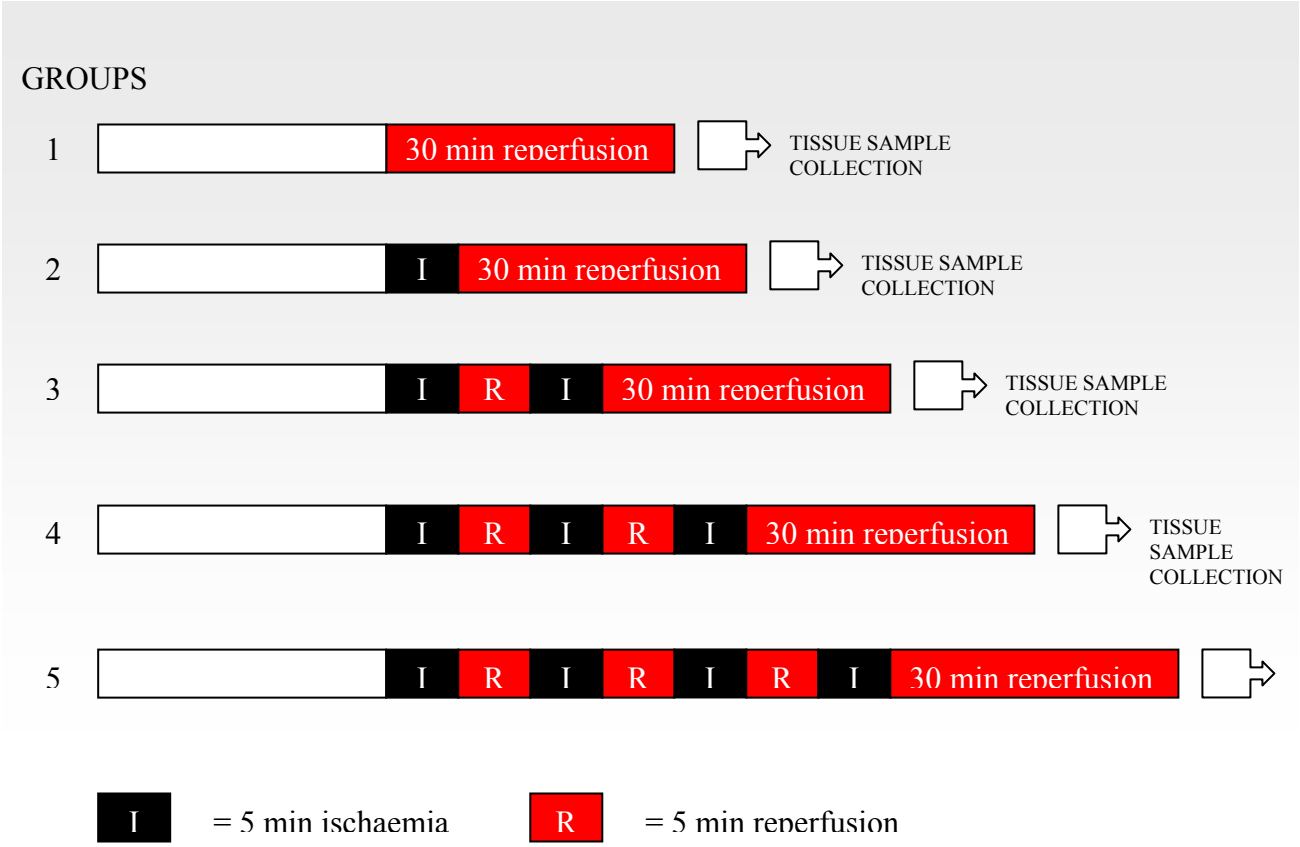


Figure 5. Experimental protocol to first series of the study. (I= ischaemia, R= reperfusion)

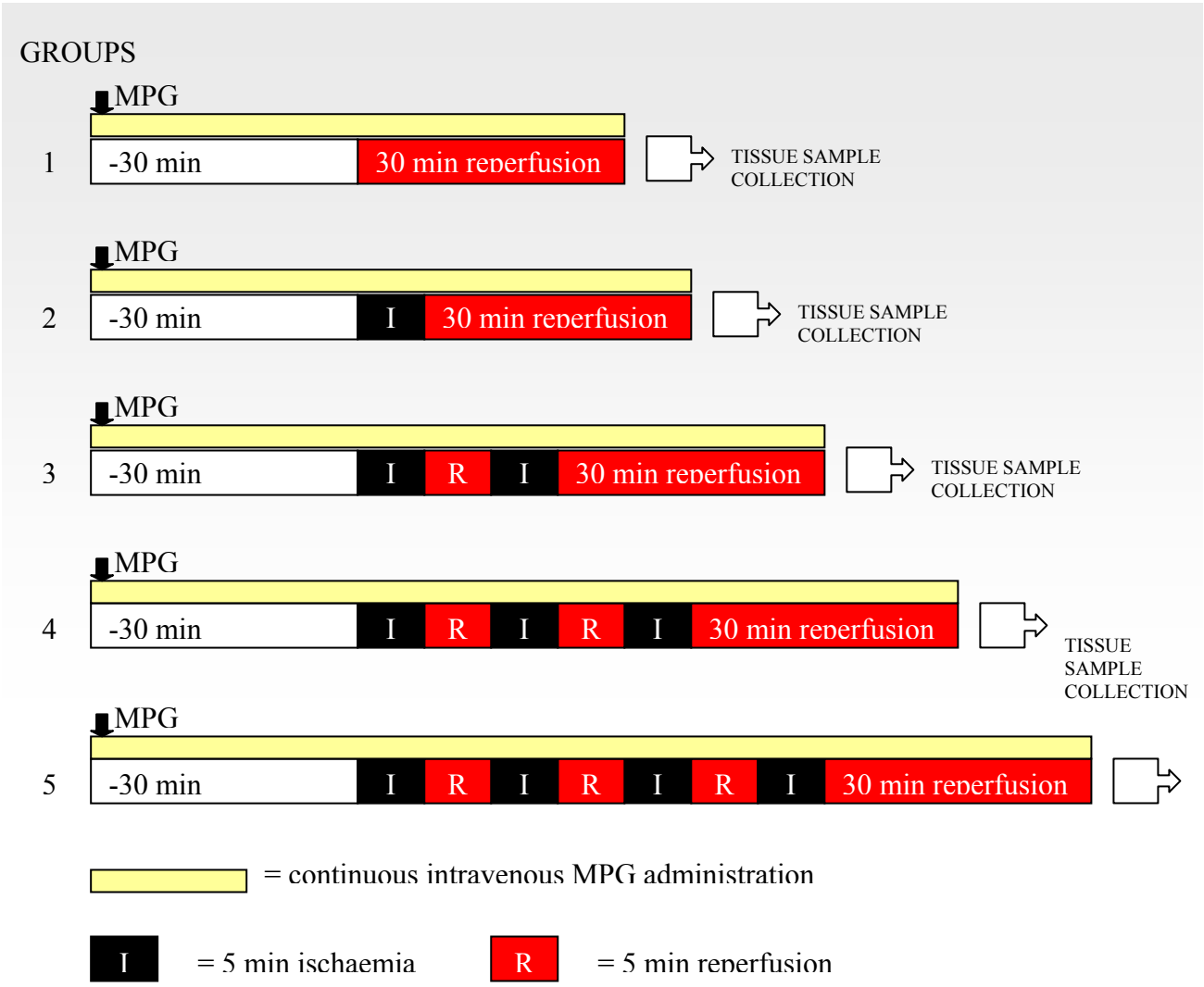


Figure 6. Experimental protocol to the second series of the study. (I= ischaemia, R= reperfusion)

4.3. RESULTS

4.3.1. EXCLUSIONS FROM THE STUDY

In total 6 animals were excluded from the statistical analysis of NFkB activation, leaving 60 animals who completed the protocol. Three animals were disregarded from the 4x5 ischaemic preconditioning (IPC) group. Two developed intractable ventricular fibrillation (VF) during the last ischaemic cycle of preconditioning, and one during the early reperfusion. Two animals were excluded from the 3x5 IPC group due to VF that was terminal. Similarly one animal was excluded from the 2x5 IPC group due to persistent fibrillation.

4.3.2. HAEMODYNAMICS

Changes in heart rate, systolic and diastolic blood pressures, and rate-pressure products are shown in table I. The pre-ligation (control), ischaemic preconditioning (during the end of the ischaemic cycles), as well as early reperfusion (rep.5') and late reperfusion (rep.30') mean values are shown. Values were checked for significant variation during the time course of the experiment within any given group, as well as with the control group. Changes in heart rate did not show any significant fluctuations, even though most frequent rhythm disturbances were noted during early reperfusion (rep.5'). Similarly systolic and diastolic blood pressures only showed slight variations, however none of the changes were statistically significant. (table I.)

Although MPG has been associated with a slight fall in mean arterial pressure in some models[97], this fact is not of relevance in this study. (table II.)

The rate pressure product, an indicator of myocardial oxygen consumption, was also fairly constant through out the experiments. Although few values were either significantly different within a group or when compared with the control group, no set pattern of change could be established between preconditioned and non-preconditioned animals, in either phases of the

study. In short, haemodynamic parameters were comparable across the groups and it is unlikely that changes in infarct size could be attributed to haemodynamic variations.

Table I. Haemodynamic variations in the different groups of the first series.

CONTROL GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	170.7±14.0	101.3±4.3	81.3±5.6	17,2±9,3
PC ischaemia	165.3±8.4	98.0±7.5	73.4±7.4	16,1±9,4
Reperfusion 5’	157.5±5.7	96.3±15.9	78.6±17.2	15,1±6,7
Reperfusion 30’	168.0±13.3	103.0±14.8	87.7±25.0	17,3±4,0

1x5 IPC GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	176.7±17.0	104.3±8.3	76.3±7.5	18,4±2,9
PC ischaemia	169.4±9.4	88.0±7.3	64.4±3.8	14,9±7,2
Reperfusion 5’	162.6±6.7	104.3±13.9	80.6±15.2	16,9±5,9
Reperfusion 30’	171.9±11.3	109.0±9.7	92.7±14.8	18,7±3,7

2x5 IPC GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	169.4±12.6	99.3±7.3	68.3±1.7	16,7±8,1
PC ischaemia	164.0±13.4	93.0±7.2	74.4±3.4	15,2±5,2
Reperfusion 5’	152.7±8.7	106.3±7.9	79.6±6.2	16,2±3,2
Reperfusion 30’	158.0±7.3	103.0±11.8	86.7±5.0	16,3±7,4

3x5 IPC GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	167.7±18.0	102.3±13.3	72.3±1.4	17,1±5,4
PC ischaemia	162.5±11.4	82.0±14.5	64.4±3.7	13,3±2,5
Reperfusion 5’	169.6±5.6	97.3±15.9	80.6±17.2	16,5±2,0
Reperfusion 30’	153.3±7.3	106.0±7.8	92.7±14.0	16,2±9,8

4x5 IPC GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	180.7±22.0	104.3±7.3	76.3±1.7	15.0±1.6
PC ischaemia	153.0±13.4	88.0±7.5	64.4±3.4	11.2±1.6
Reperfusion 5’	154.6±5.7	104.3±15.9	80.6±17.2	13.8±2.2
Reperfusion 30’	154.0±13.3	109.0±14.8	92.7±14.0	15.7±2.9

Table II. Haemodynamic variations in the different groups treated with MPG (second series).

MPG GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	175.5±2.7	85.3±6.3	69.3±7.7	14,9±7,0
PC ischaemia	147.3±5.4	78.6±7.3	76.4±3.8	11,5±7,7
Reperfusion 5’	154.6±5.7	86.7±11.9	85.6±17.6	13,2±9,5
Reperfusion 30’	173.4±8.3	107.0±9.5	78.7±8.0	18,5±5,3

MPG + 1x5 IPC GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	175.7±14.6	99.5±3.2	81.3±5.8	17,4±8,2
PC ischaemia	168.0±8.3	88.8±5.7	66.7±7.4	14,9±8,4
Reperfusion 5’	159.6±5.2	103.7±11.7	70.8±17.2	16,5±5,2
Reperfusion 30’	163.0±7.7	97.4±22.5	86.1±22.3	15,8±7,6

MPG + 2x5 IPC GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	169.6±4.8	93.3±4.3	81.3±4.7	15,8±6,8
PC ischaemia	166.0±21.4	76.0±1.5	67.4±7.3	12,6±6,1
Reperfusion 5’	197.6±5.4	106.3±11.9	84.6±11.2	21,0±8,4
Reperfusion 30’	183.0±17.3	103.0±22.8	93.7±17.0	18,8±4,9

MPG + 3x5 IPC GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	158.7±7.6	108.3±7.3	85.3±1.7	17,1±7,2
PC ischaemia	172.7±2.9	104.0±7.5	71.4±3.4	17,9±6,8
Reperfusion 5’	173.6±7.7	111.3±15.9	76.6±17.2	19,3±8,6
Reperfusion 30’	168.0±11.8	96.0±17.3	74.7±14.0	16,1±2,8

MPG + 4x5 IPC GROUP	**Heart rate (beat/min)**	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**	**Rate pressure product (mmHg/min x 10^3)**
Control reading	173.7±3.0	112.3±5.2	88.3±5.7	19,3±7,6
PC ischaemia	184.0±7.4	63.0±7.5	61.4±8.4	11,5±9,2
Reperfusion 5’	166.4±5.7	111.3±6.1	85.6±11.2	18,5±3,2
Reperfusion 30’	181.7±5.3	97.0±4.4	83.7±4.0	17,6±4,9

4.3.3. MEASUREMENT OF NFkB ACTIVATION

Figure 7. (left side) shows the changes in NF-kB levels in preconditioned myocardium after different cycles of preconditioning ischaemia *(first series* of investigation). Specificity of the signal was verified in a competition assay wherein the signal detected by labelled NF-kB was abolished, when the protein homogenate was preincubated with excess unlabeled NF-kB oligo before the addition of labelled NF-kB. The signal was not abolished, when the compatition assay contained excess unlabeled non NF-kB binding oligonucleotide.

Low and consistent levels of NF-kB were detected in normal myocardium (untreated: control group) at steady state. Significantly higher levels were detected after one cycle of 5 min ischaemia (group 1x5 IPC) compared to control (2,35 fold; $p<0.05$). In case of further repeated cycles (group 2x-, 3x-, 4x5 IPC) the NF-kB levels were significant elevated according to the control, but did not resulted in additional significant accretion of NF-kB rate compared to one cycle PC.

The mean levels of NFkB in the *second series* of the study are depicted in Figure 7. (right side). The drug control group had a mean level comparable to that of the controls in series one. The addition of the antioxidant during the IPC protocol had little effect on the group preconditioned with 4 and three cycles of 5 min ischaemia, as the level of NFkB was still statistically significant. Adding MPG, however, abolished the previously observed NFkB induction with either 2x5 or 1x5 IPC.

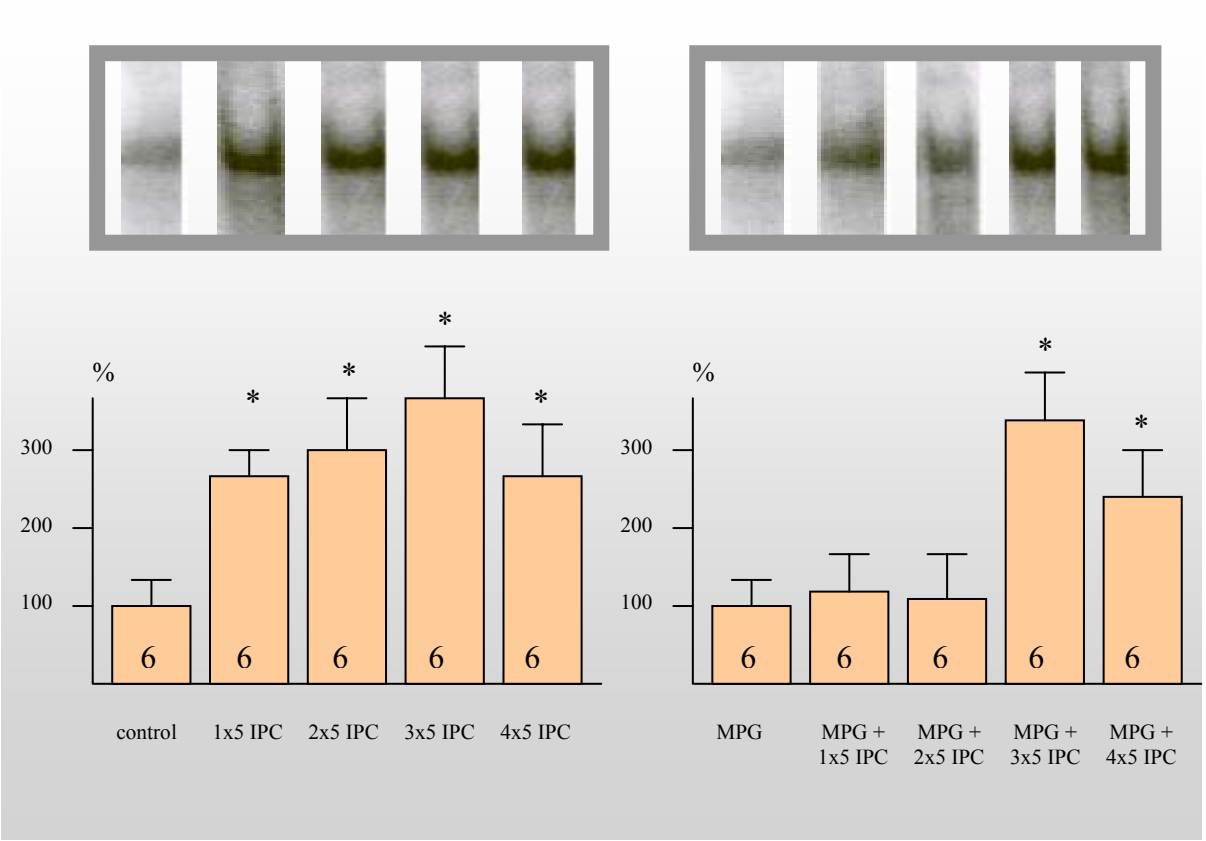


Figure 7. NFkB activation rates in the first (left) and in the second (right) series of the study. Representative EMSA blots are showed. (upper panel) In the graphs the results of the densitometry measurements are expressed as percent of control. Data are means ± SEM. (* means $p<0,05$) The values in the columns show the number of the samples.

4.3.4. ARRHYTHMIA AND FIBRILLATION

Figure 8. demonstrates the tolerance to ventricular fibrillation rendered by IPC. Although we also monitored the incidence of arrhythmia and premature ventricular beats in every experiment, it can be safely claimed that no specific pattern, or significant difference was noted between the groups. However a distinct pattern of reduced susceptibility to VF was seen in most preconditioned groups.

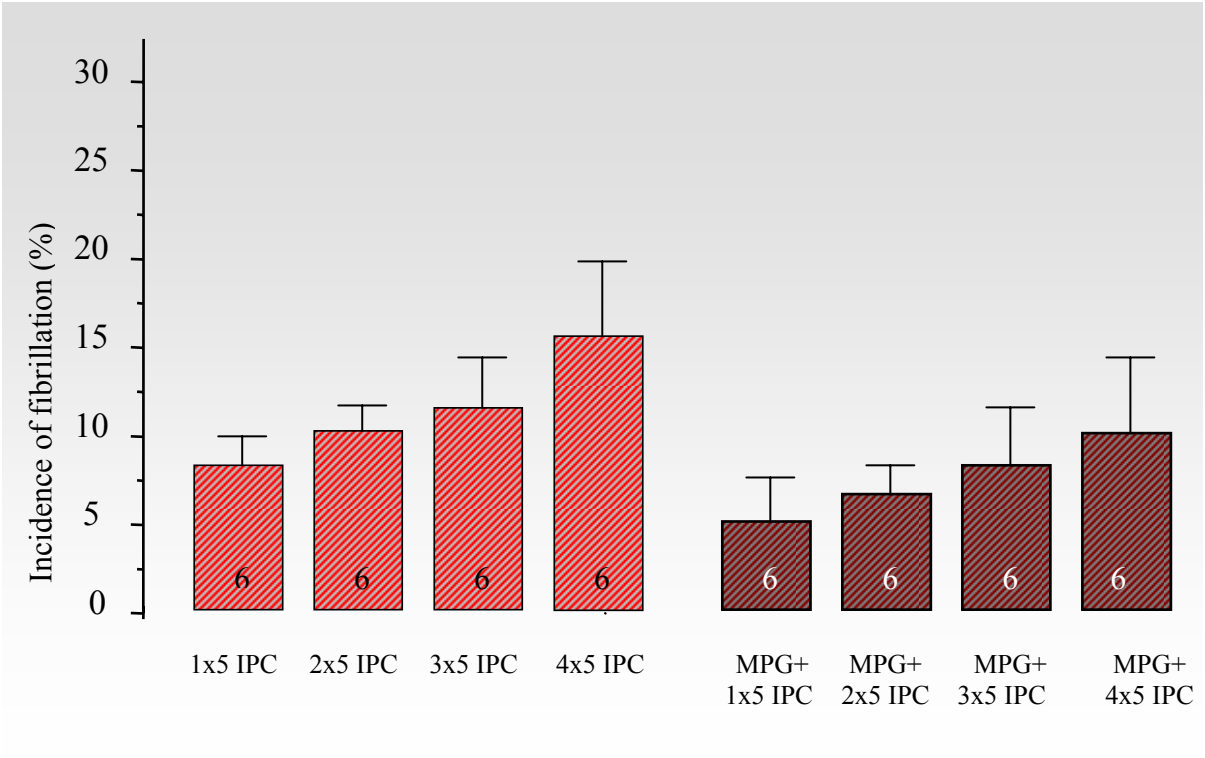


Figure 8. Incidence of ventricular fibrillation during the experiments in the different groups. . Data are means ± SEM. (* means p<0,05) The values in the columns show the number of the samples.

4.4. DISCUSSION

4.4.1. FREE RADICALS IN THE SIGNALING CASCADE OF PRECONDITIONING

Our results demonstrated that oxygen radicals produced in the process of the ischaemic preconditioning represent an important trigger for activation of NFkB in the signaling mechanism of ischaemic preconditioning to act parallel with adenosine, bradyknin and the others. The hypothesis that oxygen free radicals may also be involved in preconditioning was first proposed by Murry *et al*[98]. In their experiments protection against infarction afforded by four cycles of ischaemic preconditioning in dogs was partially limited by the combined administration of the free radical scavengers Superoxide-dismutase (SOD) and catalase. In a subsequent study Iwamoto failed to show any alteration of protection using SOD and catalase in anesthetized rabbits preconditioned with four cycles of 5 min ischaemia[99]. In direct contrast to the study of Iwamoto Tanaka reported that the limitation of infarct size in in situ rabbit hearts could be abolished by either SOD or MPG[100]. The model of Tanaka was identical to that of Iwamoto, except for the preconditioning protocol. Tanaka used a single cycle of ischaemic preconditioning, whereas Iwamoto utilized four cycles. The present study clearly reveals that the preconditioning protocol was the cause of the divergent results.

Consequently, our experiments in rabbits confirmed that the production of oxygen free radicals during the brief ischaemia-reperfusion is an important contributor to the triggering the signaltransduction cascade leading to NFkB activation in preconditioned myocardium.

There are several possible mechanisms whereby reactive oxygen species could induce NFkB activity. Oxygen radicals could stimulate the protein kinase C (PKC) enzyme directly and Gopalakrishna and Anderson have documented that hydrogen peroxide could oxidatively modify the regulatory domain of PKC leading to its activation in a manner independent of either phosphatidylserine or calcium ions[101]. Consequently, - as it was described in the

discussion of the previous study – PKC induce the activation of NFkB through the phosphorylation of Inhibitor Kappa Kinase-epsilon (IKK-ε). Indirect mechanisms also exist. It was previously believed that mediators of preconditioning were activating PKC via the stimulation of phospholipase-C and hence phosphatidylinositide hydrolysis. However, recent evidence suggests that phospholipase-D, and not c, may be involved in the upregulation of PKC[102]. Von Ruecker *et al.* have shown that membrane binding of PKC in hepatocytes is markedly increased following exposure to oxidative species suggesting that oxygen radicals can stimulate the translocation and hence activation of PKC[103]. It is unknown which, if any, of these influences on PKC accounts for the induction of NFkB transcription factor in the preconditioning action of oxygen radicals.

However, when multiple cycles are employed, other mediators such as adenosine and bradykinin appear to be released in sufficient quantities to trigger the signaling, even in the absence of oxygen radicals. A similar redundant mode of action has been postulated for bradykinin in pigs[104].

4.4.2. CONCLUSION

In our experiment we demonstrated the DNA binding activities of NF-kB after different number of ischaemia-reperfusion (I/R) cycles. Our results show, that after one cycle of I/R – which was previously shown to exert powerful cardioprotective effects for ischaemic hearts – the activation of NF-kB increased progressively and steadily. But further clone of I/R cycles has not resulted in further elevation in activation of NF-kB compared to the one cycle. These findings correlate with Goto's threshold hypothesis, he found that ischaemic PC is an "all or nothing" response to slight ischaemic-reperfusion injury. If the PC stimulus is strong enough to reach a "threshold" level, a full signaling cascade and protection will be induced, but in

cases of subthreshold stimulus the whole process will be failed. Above this threshold the strenght of the PC stimulus does not influence the volume of the signaling cascade and the degree of the evoked cardioprotection[105]. (figure 9.)

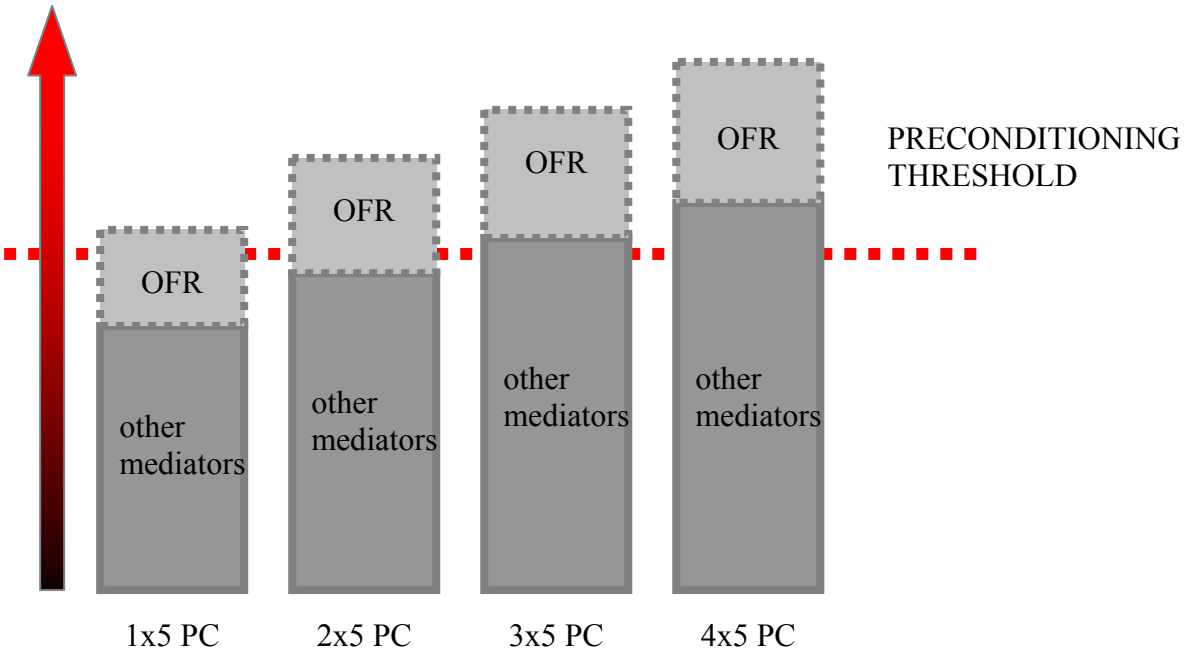


Figure 9. Additive interaction of the triggers in ischaemic preconditioning. The triggers together could reach the threshold of protection and start up the signalling of the delayed adaptation.

The authors have investigated in this study, in an animal model, that OFR are important triggers of delayed ischaemic preconditioning; examining NFkB induction as to show the start up of the signaltransduction of ischaemic preconditioning. The robust 4x5 IPC protection, even in the presence of MPG, as opposed to loss of protection with fewer cycles under the same conditions, indicates that generation of OFR is essential in triggering delayed cardioprotection in rabbits only when a less rigorous preconditioning stimulus is used. In other words, multiple cycles (4x5 IPC) may lead to the release of numerous mediators so that

eliminating a single trigger (in this case OFR) would not diminish the overall stimulation to a subthreshold level. (figure 10.)

Nuclear Factor kappa-B activation induced by one and two cycles of ischaemic preconditioning was abolished by the oxygen radical scavenger MPG in *in situ* reperfused rabbit hearts, suggesting that oxygen radicals are involved in the triggering of the signaling cascade of ischaemic preconditioning. However MPG failed to abort NFkB induction by three and four cycles of ischaemic preconditioning in which accumulation of other substances could be sufficient to trigger the signaltransduction in the absence of oxygen radicals.

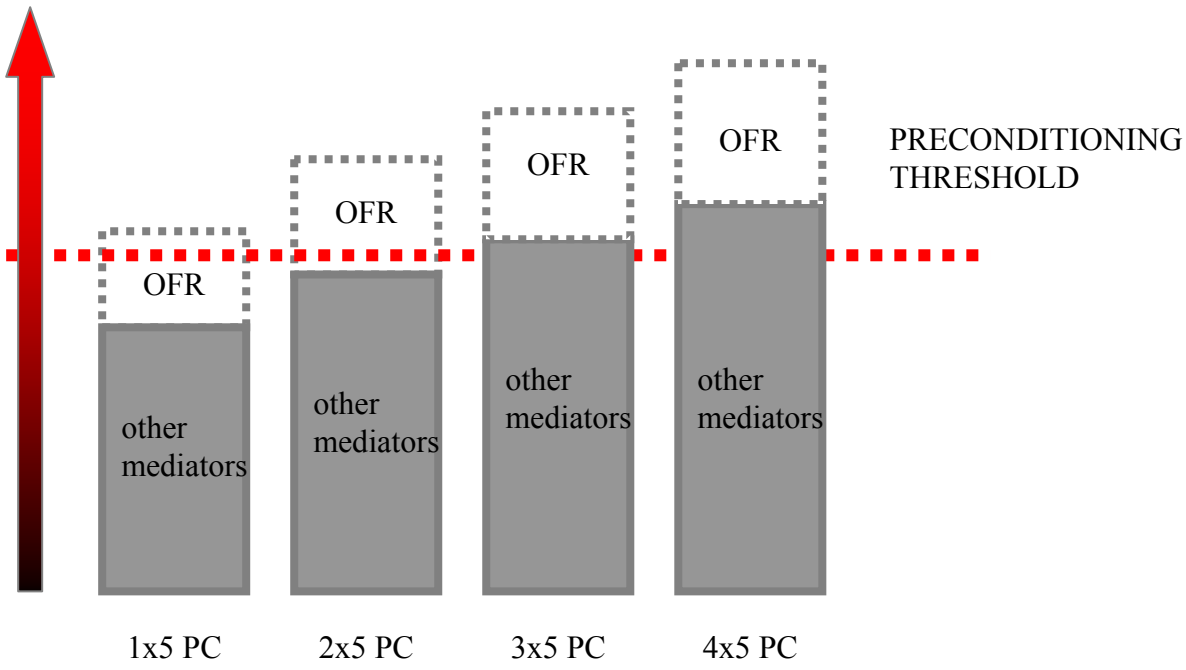


Figure 10. After MPG administration the triggers of one and two cycles PC were not enough to induce cardioprotection withouth the OFR-s.

5. EFFECT OF ASPIRIN ON NUCLEAR FACTOR-kappa B ACTIVATION AND ON LATE PRECONDITIONING AGAINST INFARCTION IN PRECONDITIONED MYOCARDIUM

5.1. INTRODUCTION

5.1.1. GENERAL BACKGROUND

Recent studies have demonstrated, that the NF-kB dependent gene activation can be blocked by sodium salicylate and by acetyl-salicylate (aspirin) in lymphoid and endothelial cells, through preventing phosphorylation and the subsequent proteosomal degradation of the inhibitor IkappaB-alpha[106 107 108 109 110].

Acetylsalicylic acid (ASA) is one of the most often used nonsteroidal anti-inflammatory drugs applied against acute pain, fever, inflammatory diseases, and it is an important additional therapy for patients with ischaemic heart disease, through ASA significantly inhibits platelet aggregation in vivo. Thus ASA is useful in coronary artery sclerosis preventing the generation of thrombus on the scleroid lesions of coronary artery wall[111]. Otherwise it has been demonstrated by Shinmura *et al.* that ASA is able can to block late preconditioning in a dose dependent way. These authors presumed that this retardant effect could be explained by the inhibition of COX-2 activity [112]. In contrast to these assumptions there are clinical evidences that in patients with prolonged ASA treatment significant delayed cardioprotection can be evoked[113].

5.1.2. AIMS

The aim of this study was to investigate the effect of aspirin on the NF-kB activation in the endogenous adaptation response of the myocardium. Accordingly, we aimed to investigate the effect of three different doses of acetylsalicylic acid (ASA) on the late phase of ischaemic preconditioning (PC) against myocardial infarction, and on the activation and nuclear translocation of NF-kB in the preconditioned myocardium.

5.2. MATERIALS AND METHODS

The present study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and was approved by the local institutional Committee on Animal Research of Pécs University (BA02/2000-29/2001).

5.2.1. SURGICAL PREPARATION

A marginal ear vein was cannulated in 60 New Zealand White rabbits weighing 2,6-3,3 kg (mean 2,8kg), after local anaesthesia was induced using lidocaine cream. The animals were anaesthetized with intravenous (iv.) xylazine (6mg/kg), ketamine (6 mg/kg) and propofol (10mg/kg). The trachea was intubated (tube 3 mm internal diameter) and the lungs were ventilated (Sulla 808, Drager, Lübeck, Germany) at a frequency of 30-35 breaths/min and a tidal volume of 15-20 ml. Anaesthesia was maintained by inhalation of isoflurane (2-4 Vol.%) and nitrous oxide (50 Vol.%).

All surgical procedures were performed under sterile conditions, and intravenous antibiotic profilaxis (cephazolim, 35mg/kg) was given. Temperature was measured inside the pericardial cradle (Siemens Sirem, Digital Thermometer, Düsseldorf, Germany) and maintained between 38,3˚C and 38,7˚C by adjusting a heating pad and an infrared lamp.

The chest was opened by midline sternotomy, and a small incision in the pericardium was made. The left anterior descending (LAD) coronary artery was encircled with a 5-0 prolene suture (Ethicon 5/0, 1-metric, TF). In general the site of vessel encirclement was on the long axis of the left ventricle towards the apex approximately one-fourth of the distance from the atrioventricular groove to the left ventricular apex. Fifteen minutes after completion of

surgical preparation baseline measurements were performed and animals were heparinized with 500 U of heparin sodium.

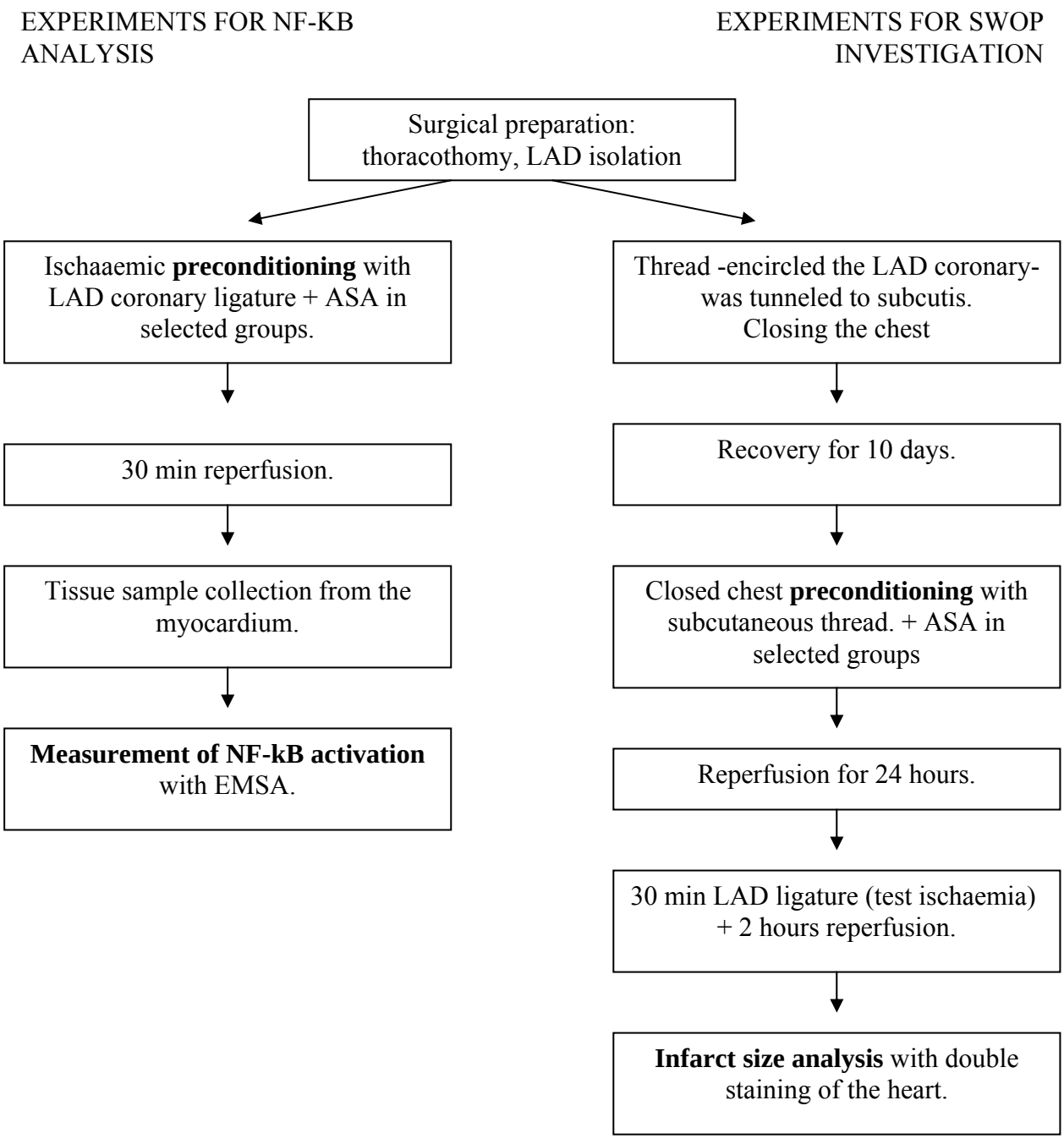


Figure 11. Graphic algorithm of the different steps of the experiment.

5.2.2. EXPERIMENTS FOR NF-KB ANALYSIS

Following the surgical preparation in the ischaemic preconditioned (PC) groups the snare was tightened for 4 times 5 min, thereby inducing occlusions of the coronary artery, this caused ischaemic preconditioning (PC) in the concerned myocardium. Myocardial ischaemia was readily discernible by the development of a dusky, bulging region of myocardium (careful note was made of anatomic landmarks of this region), and by the immediate occurrence of ST-segment elevations in surface electrocardiogram (ECG) (Siemens Sirecust 1260, Düsseldorf, Germany). Between the ischaemic cycles the heart was allowed to reperfuse for 5 min. At the end of every 5-min period of coronary artery occlusion the suture was released to ensure proper reperfusion. The effectiveness of this manoeuvre was verified by the disappearance of the ECG changes within 2 min in every animal. 10 min before preconditioning in selected groups the animals were treated with intravenous acetylsalicylic acid (ASA) (Aspisol,Bayer AG, 51368 Leverkusen, Germany) following the experimental protocol. After the four cycles of ischaemia/reperfusion the animals were assigned to 30 min reperfusion period, and the heart was rapidly excised and rinsed in ice-cold physiological saline. The right ventricle and atria were trimmed away, and the left ventricle (LV) was divided into ischaemic and non-ischaemic zones on the basis of the previously defined landmarks. The tissue samples taken from the previously ischaemic region and from the posterior wall (nonischaemic region) were snap frozen in liquid N2, and stored for not more than 3 days at -82C before NF-kB analysis with EMSA and enzyme immunoassay.

5.2.3. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The exact method of EMSA analysis we applied in this study was described in the previous part of this thesis (3.2.3.).

5.2.4. EXPERIMENTS FOR SWOP INVESTIGATION

After the surgical preparation (described above) the threads were pulled through a reinforced tube (2.5 mm internal diameter, Mallinckrodt Medical, Athlone, Ireland), which was tunneled subcutaneously to the interscapulare space. The central end of the tube was placed close to the sutures around the coronary artery and fixed at the pericardium. The chest wound was then closed in layers and air aspirated from the thorax. Postoperative care included analgeticum (piritramide, 2 mg/kg, subcutaneously), mucolysis (bromhexin hydroclorid 0,1 mg/kg intravenous), and recovery in an isolated pen.

5.2.5. LATE PRECONDITIONING

Rabbits were allowed to recover for 10-13 days. Then, under anaesthesia we made a small incision above the peripherial end of the tube, and in preconditioned groups the suture was tightened, thereby inducing occlusion of the coronary artery. The outgrowth myocardial ischaemia was verified by the ST-segment aberrations in the ECG. In selected groups animals were treated with intravenous acetylsalicylic acid (ASA) 20min before the experimental protocol, and were subjected to four 5-min coronary occlusion / 5-min reperfusion cycles. For reperfusion the suture was released, and the complete reperfusion of the myocardium was verified by the disappearance of the ECG changes within 2 min in every animal.

5.2.6. INFARCT SIZE ASSESSMENT

24 hours later animals were anesthetized again with the above mentioned method. After median thoracotomy the suture around the coronary artery was dissected free. The animals were then heparinized with 500 U of heparin sodium. The rabbits were then subjected to 30 min of coronary artery occlusion by tightening the snare. Ventricular fibrillation during coronary artery occlusion was treated by electrical defibrillation (5 J, DCS261 Defibrillator,

Piekser, Ratingen, Germany). After 30 min of occlusion the snare was released and 2 h of reperfusion was allowed.

After the reperfusion period, the LAD was briefly reoccluded and patent blue dye (2ml of 1% cc, Byk Gulden, Konstanz) was injected into the left auricle at a constant pressure. After allowing for 1 more minute of circulation, the heart was excised and washed twice in cold saline. The atria and right ventricle were then cut away, leaving only the left ventricle. Total left ventricular mass was recorded. Then using fine surgical scissors the bluish non-ischaemic myocardium was excised from the area at risk. Area at risk –or risk zone- is the blood-supply area of the LAD coronary distal from the ligature. During LAD occlusion area at risk stays red, because Patent blue can not perfuse in this myocardium. The isolated area at risk was then weighed, sliced (1 mm thick) and incubated in 1% triphenyltetrazolium chloride (TTC) for 20 min at 37^{o}C. The slices were then immersed in 10% formalin to enhance the contrast between viable (deep red) and pale, infarcted myocardium. The percentage of infarction within the area at risk was determined by planimetry of each slice. The volume of each zone was then calculated by multiplying each area by the thickness of the slice and summed up as a total size of infarction and area at risk in individual hearts.

5.2.7. EXPERIMENTAL PROTOCOLS

PROTOCOL I: EFFECT OF ASA ON THE ACTIVATION AND TRANSLOCATION OF NF-kB.

Rabbits were randomly assigned into five groups. In the control group (group I, shame operation, 6 rabbits) animals underwent the whole surgical procedure, but the coronary artery was not closed. In preconditioned groups rabbits underwent 4 cycles of 5 min ischaemia / 5 min reperfusion, inducing ischaemic preconditioning (PC) in the myocardium. In group II (6

rabbits) animals were subjected to 4x5 min PC without acetylsalicylic acid (ASA) treatment. In group III (6 rabbits) animals were treated with 5mg/kg intravenous ASA 20 min before 4x5 min PC. 5 mg/kg is the dose of clinical antythrombotic profilaxis: significantly inhibits in vivo thrombocyte aggregation. In group IV (6 rabbits), before 4x5 min PC, animals were treated with 25 mg/kg ASA. 25 mg/kg is a high dose for analgesia and antirheumatic therapy. In group V (6 rabbits) animals were treated with 130 mg/kg ASA before 4x5 min PC. 130 mg/kg is the maximum, subtoxic dose of ASA that can be used in clinical practice. (figure 12.)

PROTOCOL II: EFFECT OF ASA ON DELAYED PRECONDITIONING AGAINST MYOCARDIAL INFARCTION.

Rabbits were randomly assigned into further five groups. The experiments in this protocol lasted for two days. On the first day animals underwent a shame operation (group VI, 6 rabbits), or in the preconditioned group a four cycles of 5 min ischaemia / 5 min reperfusion (4x5 min PC, group VII, 6 rabbits). 20 min before PC rabbits were pretreated with 5mg/kg ASA (in group VIII, 6 rabbits), 25 mg/kg ASA (in group IX, 6 rabbits), or 130 mg/kg ASA (in group X, 6 rabbits). On the second day all of the animals were subjected to 30 min coronary occlusion and 2 hours reperfusion before infarct size analysis. (figure 13.)

PROTOCOL III: EFFECT OF ASA ON PLATELET AGGREGATION.

To prove the inhibitory effect of ASA on the platelet aggregation in rabbits we measured platelet function before and after administration of 5 mg/kg ASA in group III (6 rabbits). Three milliliters of blood were collected from the mid-dorsal ear artery into a syringe containing sodium citrate (Becton Dickinson Vacutainer Systems 9NC, Plymouth, UK) before and 60 min after the intravenous administration of 5 mg/kg ASA. After determination of

circulating blood cell counts, in vitro platelet aggregation was measured in stimulated whole blood in presence of adenosine diphosphate (ADP) and collagen with platelet aggregometer (Chronolog Lumi-Aggregometer M560-VS, Havertown, USA)[114].

5.2.8. STATISTICAL ANALYSIS

Data are reported as mean ± SEM. Intragroup comparisons, levels of expression of individual protein (NF-kB EMSA) were subjected to analysis of variance with post hoc Dunnett's 1-tailed t-tests, and one-sample Student's t-test for significance. Infarct size data were analyzed with one-way analysis of variance (ANOVA) followed by unpaired t test with Bonferroni's correction for multiple comparisons. Hemodynamic data were analyzed using repeated-measures ANOVA. Changes were considered significant when the *P* value was less 0,05.

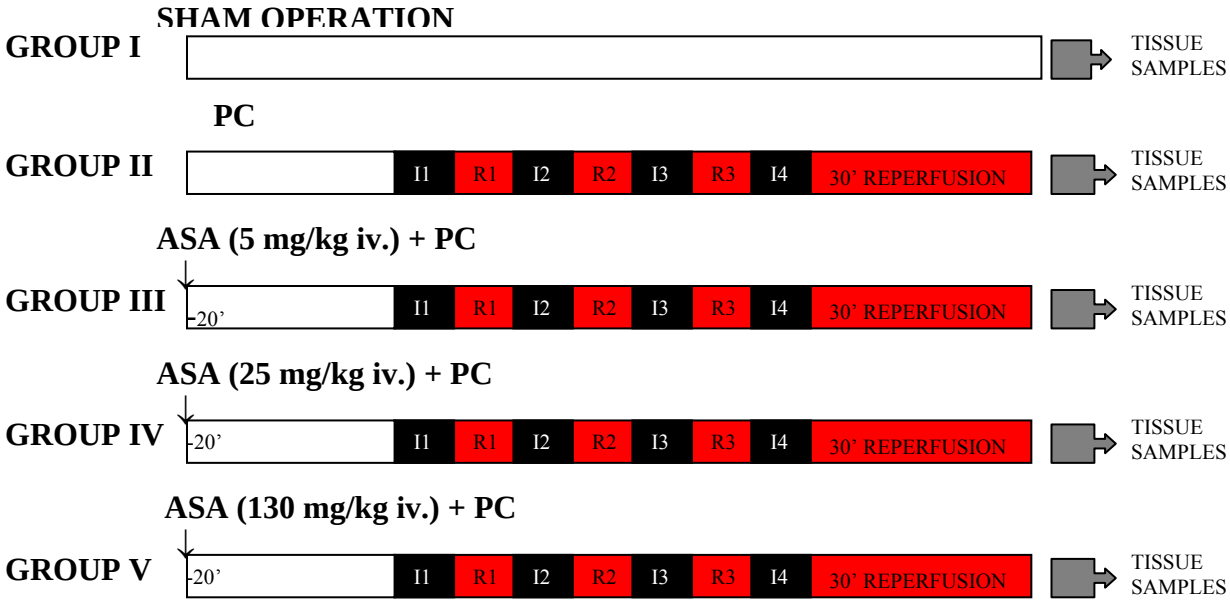


Figure 12. Experimental protocol I. (PC = preconditioning; I = Ischaemia; R = Reperfusion; iv = intravenous administration.)

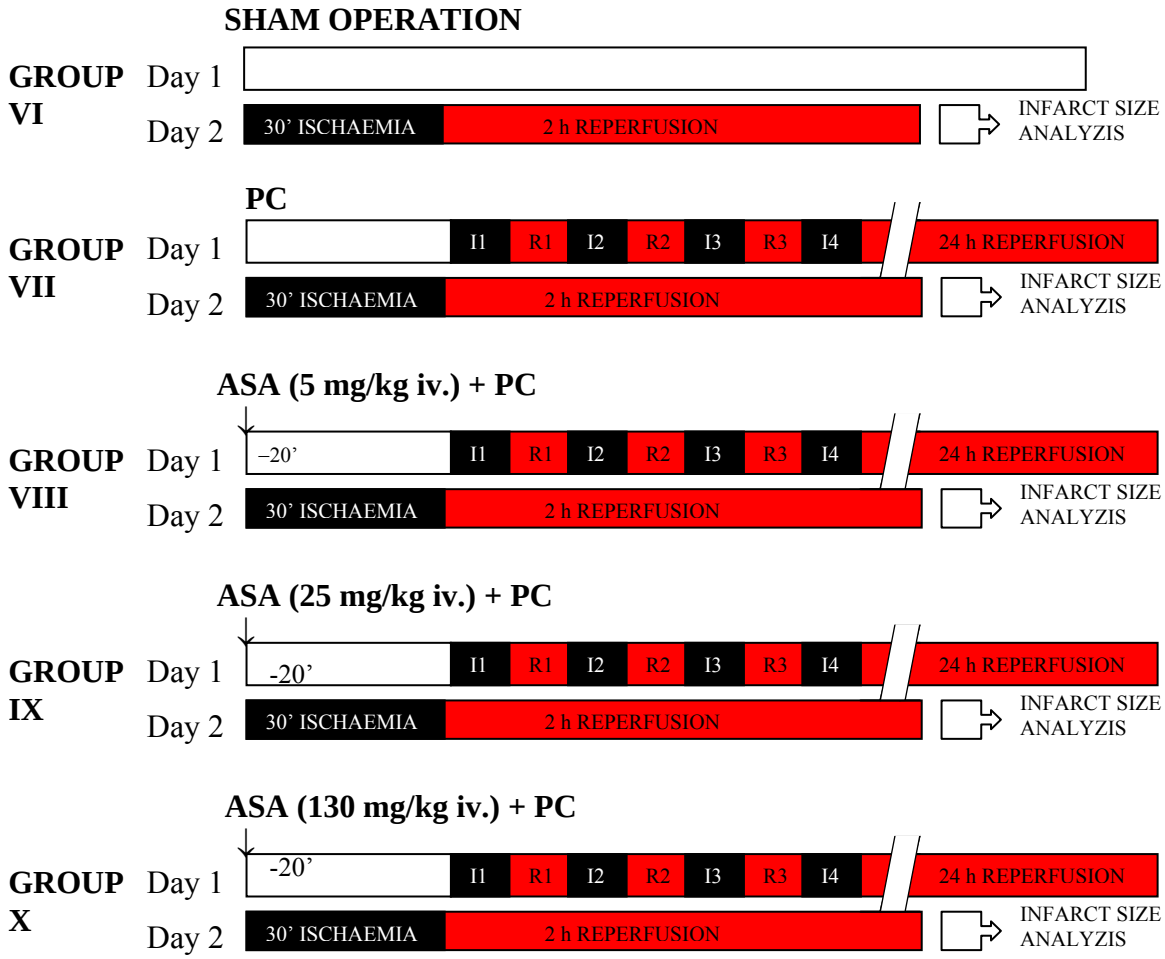


Figure 13. Experimental protocol II. (PC = preconditioning; I = Ischaemia; R = Reperfusion; iv = intravenous administration.)

5.3. RESULTS

5.3.1. PROTOCOL I: EFFECT OF ASA ON THE ACTIVATION AND TRANSLOCATION OF NF-KB.

Representative examples of electrophoretic mobility shift assay of NF-kB in nuclear fraction are illustrated in figure 14. The diagram shows changes in NF-kB levels in preconditioned myocardium after using various doses of ASA. Specificity of the signal was verified in a competition assay wherein the signal detected by labeled NF-kB was abolished, when the protein homogenate was preincubated with excess unlabeled NF-kB oligo before the addition of labeled NF-kB. The signal was not abolished, when the compatition assay contained excess unlabeled non NF-kB binding oligonucleotide.

Low and consistent levels of NF-kB were detected in normal myocardium (untreated, control: group I) at steady state. Significantly higher levels were detected, when rabbits were preconditioned with four 5-min occlusion/5-min reperfusion cycles (group II) in all 6 animals (densitometry: 2,35-fold; $p<0.001$ vs. controls). The administration of low (5mg/kg) and medium (25mg/kg) dose ASA before ischaemic preconditioning failed to abolish the activation of NF-kB transcription factor. In contrast, the high dose (130mg/kg group V) ASA arrested the activation of NF-kB (Fig.14, left graph). There was no change in NF-kB activation in the nonischaemic region among the five groups (Fig.14 , right graph).

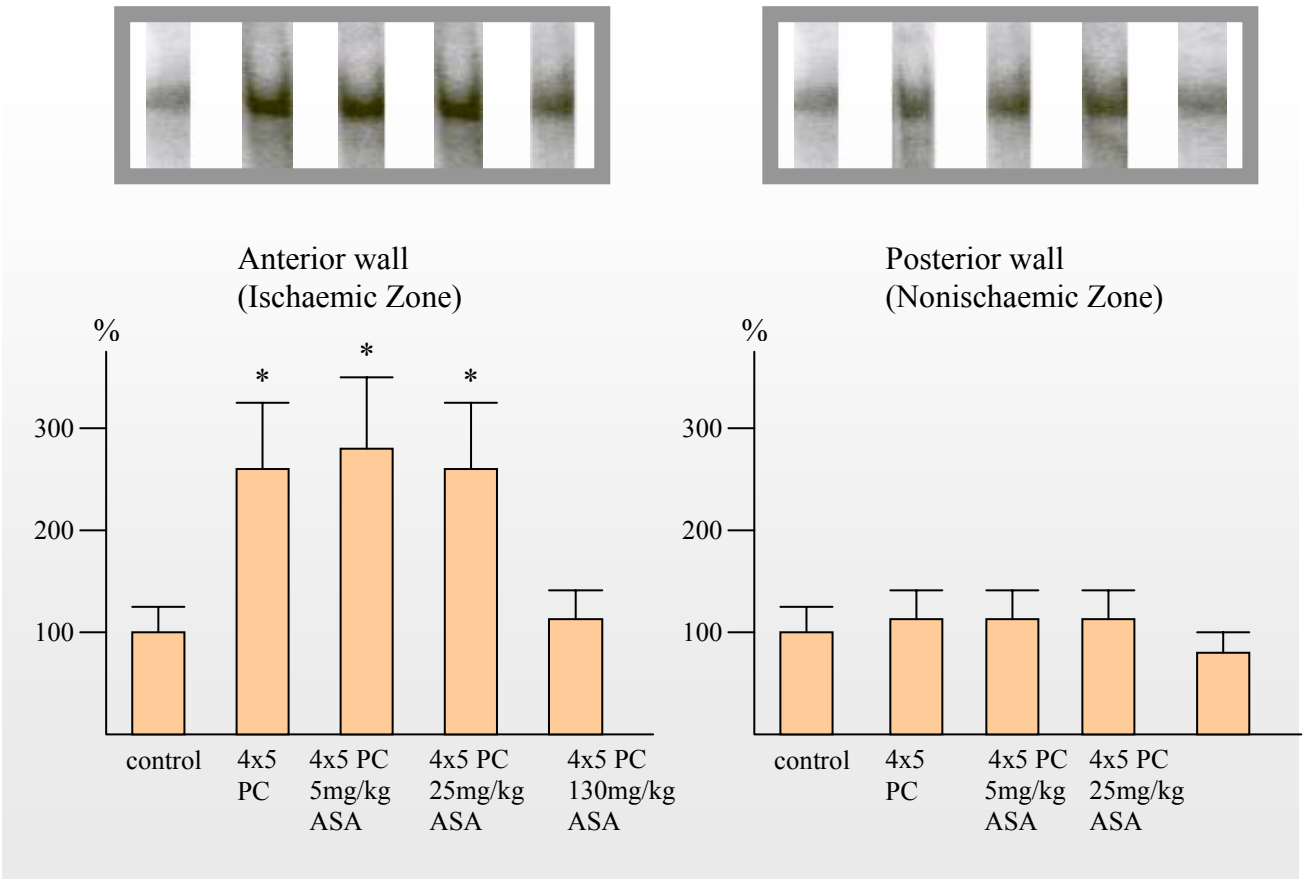


Figure 14. Representative EMSA pictures showing the level of activated and translocated NFkB in the different groups. Densitometric measurements of NFkB on the EMSA pictures were expressed as a percentage of the average value measured in the control rabbits. The two comparisons performed in each panel were adjusted by the Bonferroni correction. Data are means ± SEM. (ASA=acetylsalicylic acid, PC= preconditioning)

5.3.2. PROTOCOL II: EFFECT OF ASA ON DELAYED PRECONDITIONING AGAINST MYOCARDIAL INFARCTION.

There were no significant differences in the heart rate, systemic blood pressures or rate/pressure product (not shown) among the five (VI.-X.) groups. Table III. summarizes changes in heart rate at selected intervals during both first and second day of the experiments. Ischaemic risk zone volume and infarct size are shown in table IV. Ischaemic risk zone volume was similar in all experimental groups at around 19% to 23% of the left ventricle mass. Infarct size as a percentage of the risk zone is demonstrated in figure 15. Sham-operated

control rabbits (group VI.) had a mean infarct size of 61,3 ± 12,3% of the risk zone. Preconditioning (PC) with four 5-min coronary occlusion episodes limited the infarction to 32,7 ± 8,6% ($p<0,05$) in group VII. Pre-treatment of the animals before PC with 5 mg/kg ASA (group VIII.) and with 25 mg/kg ASA (group IX.) did not influenced the protective effect of late PC, and resulted in 34,6 ± 8,7% and 36,4 ± 9,3% infarct/risk ratio. In contrast, pre-treatment with 130 mg/kg ASA (group X.) prior to PC abolished the cardioprotection and lead to an infarct size of 59,1 ± 11,6%. Thus, protection against infarction was observed 24 h after PC with 4x5min coronary artery occlusion, and this protective effect was significantly blocked with a high dose (130 mg/kg) of ASA treatment prior to the PC stimuli.

Arrhythmias during the PC procedure on the first day were rare and minor in nature. Although the majority of animals in all groups experienced some form of ventricular premature beats and/or ventricular tachycardia (VT) (data not shown), their extent was highly variable, and no specific pattern could be determined. Fewer animals had ventricular fibrillation (VF) during the ischaemia-reperfusion period, overall, there were no statistically significant differences in VT or VF across the groups.

Table III. Heart Rate (beats/min) during the PC on first day and ischaemia-reperfusion on second day. Values are means ± SEM.
(Baseline = before administration of ASA; Before PC = just before the first ischaemic period of preconditioning; PC1 = end of the first ischaemic cycle of preconditioning; PC4 = end of the fourth ischaemic cycle of preconditioning; second day control = on the second day before surgical procedur;e ASA = acetylsalicylic acid)

Heart rate (Beats/min)	Baseline	Before PC	PC 1	PC 4	Second day control	Ischaemia 30 min	Reperfusion 2 h
Group 6 (control)	-	237±9	252±5	248±9	226±11	247±9	234±8
Group 7 (PC)	-	245±6	257±5	251±9	237±4	252±11	236±14
Group 8 (PC+5ASA)	244±9	232±12	239±7	238±11	228±9	249±12	231±11
Group 9 (PC+25ASA)	253±6	241±5	251±15	247±13	234±13	237±6	228±7
Group 10 (PC+130ASA)	248±7	229±7	236±8	235±14	233±8	241±15	232±13

Table IV. Size of ischaemic-reperfused (risk) zone. Values are means ± SEM. (ASA = acetylsalicylic acid; PC = preconditioning; LV = left ventricle; *= significant)

	Group 6 (control) (6 animals)	**Group 7 (PC)** (6 animals)	**Group 8 (PC+5ASA)** (6 animals)	**Group 9 (PC+25ASA)** (6 animals)	**Group10 (PC+130ASA)** (6 animals)
Body weight (g)	2869 ± 97	2765 ± 125	2925 ± 89	2874 ± 65	2831 ± 139
LV weight (g)	3,77 ± 0,25	4,13 ± 0,17	4,06 ± 0,21	3,92 ± 0,15	4,26 ± 0,28
Area at risk (g)	0,81 ± 0,08	0,79 ± 0,07	0,87 ± 0,05	0,91 ± 0,09	0,84 ± 0,10
Risk-to-LV ratio (%)	21,5 ± 2,8	19,1 ± 3,1	21,4 ± 2,9	23,2 ± 3,2	19,7 ± 3,5
Infarct size-to risk zone ratio (%)	61,3 ±12,3%	32,7 ± 8,6%*	34,6 ± 8,7%*	36,4 ± 9,3%*	59,1 ± 11,6%

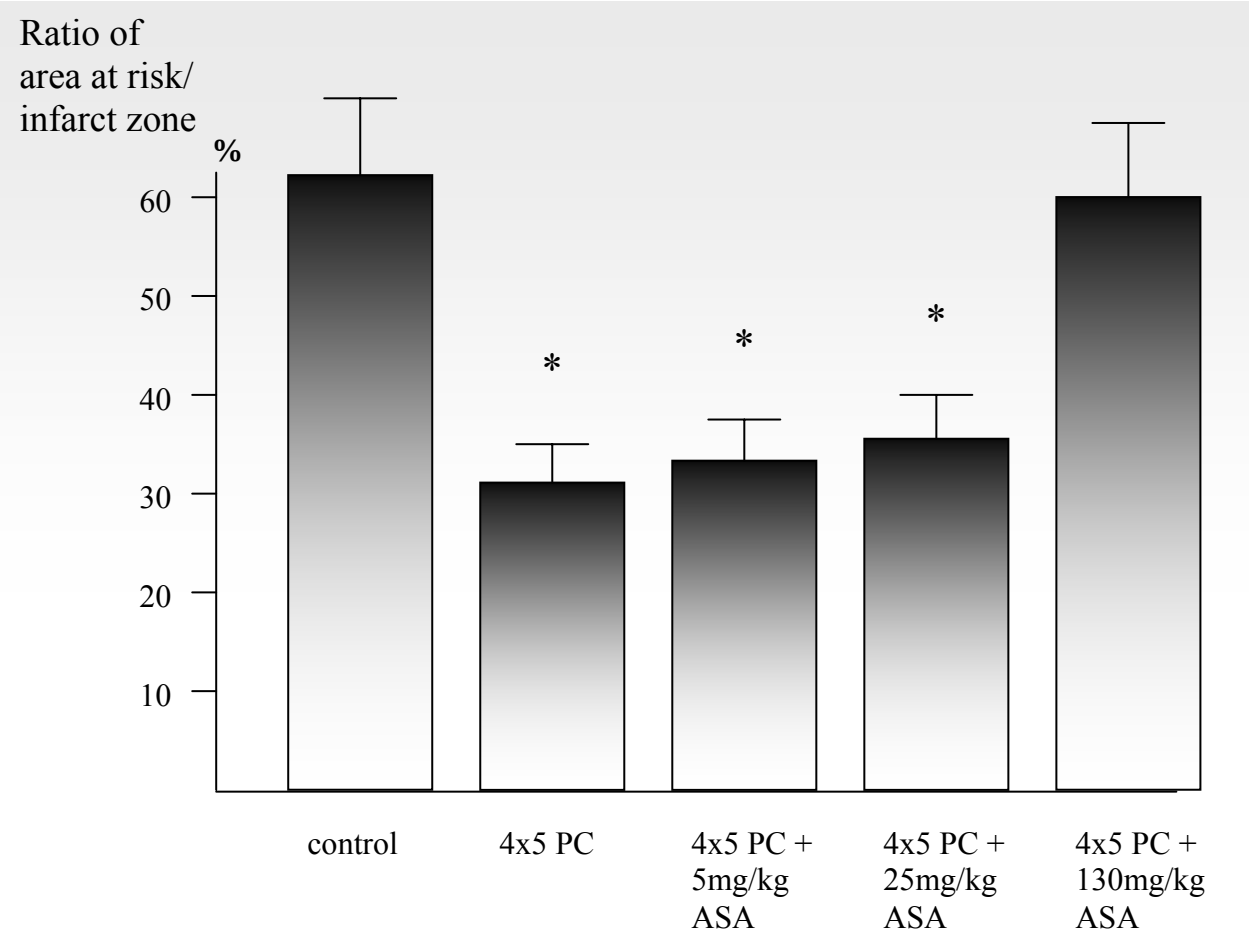


Figure 15. Infarct size: infarct size as a percentage of the area at risk. * p<0,05. (PC = preconditioning; ASA = acetylsalicylic acid)

5.3.3. PROTOCOL III: EFFECT OF ASA ON PLATELET AGGREGATION.

There were no significant differences in the number of white blood cells, platelets and red blood cells before and after the administration of 5 mg/kg ASA (table V). After ASA treatment the values decreased slightly, but not significantly in all categories, probably due to the diluting effect of the intravenous infusion and the blood sampling. With collagen induction the platelet aggregation was suppressed by low dose (5 mg/ kg) ASA. In contrast, platelet aggregation in response to ADP was unchanged after low dose ASA. That demonstrates, that the inhibitory effect of ASA (5mg/kg) on platelet aggregation is similar in rabbits and in humans. (table V.)

Table V. Complete blood count and platelet aggregation before and after ASA treatment in rabbits of group III.
Values are means ± SEM. ASA = acetylsalicylic acid; ADP = adenosine diphosphate.

	Baseline	After ASA (5 mg/kg iv)	Units	P Value
Complete Blood Count				
White blood cells	7,3 ± 0,5	7,0 ± 0,4	10^3/ml	NS
Red blood cells	5,52 ± 0,06	5,19 ± 0,06	10^6/ml	p<0,05
Hemoglobin	12,0 ± 0,4	11,5 ± 0,4	G/dl	NS
Hematocrit	35,4 ± 0,8	34,1 ± 1,1	%	NS
Platelets	231 ± 22	217 ± 23	10^3/ml	NS
Platelet Aggregation Test				
ADP (at 6 min)	9,3 ± 0,46	8,2 ± 0,38	Ω	NS
Collagen (at 6 min)	21,3 ± 2,4	14,6 ± 1,9	Ω	p<0,05

5.4. DISCUSSION

5.4.1. THE QUANDARY: ASPIRIN, COX-2 AND CARDIOPROTECTION

An obvious question is, whether ischaemic preconditioning is a clinically relevant phenomenon, that is, whether brief bouts of ischaemia evoke a protective phenotype in patients at risk of suffering acute coronary events. In this regard, current evidence, although not definitive, favors the conceptthat, as in the experimental laboratory, brief episodes of antecedent ischaemia may confer both an early and delayed phase of endogenous cardioprotection in the human heart[115] [116] [117]. However, if the conclusions regarding the crucial role of COX-2 in delayed PC derived from experimental models can be explorated to the clinical arena, this raises a disturbing possibility: the benefits of delayed PC may, in concept, be compromised in patients using the COX inhibitor acetylsalicylic acid (ASA; aspirin) for relief of fever, pain, and inflammation and, perhaps of greatest concern, in the countless patients prescribed ASA for the prophylactic prevention of acute myocardial infarction and stroke[118] [119] [120]. Moreover, as the protective role of prostanoids is not limited to the delayed second window of preconditioning – there is experimental evidence implicating the involvement of endogenous prostaglandins in infarct size reduction in some models[121] of early, first window of PC, in the protection afforded by angiotensin-converting enzyme (ACE) inhibitors against postischaemic myocardial stunning[122] [123], and in the reduction of infarct size seen with angiotensin A1 receptor blockers[124] – ASA therapy may adversely effect these other cardioprotective modalities. In fact, retrospective analyses of large clinical trials have suggested that the prophylactic use of ASA may deprive the postmyocardial infarction patient[125]. It is not, however, clear from these analyses whether the potential loss of benefit related to *antithrombotic, analgesic or antirheumatic doses of ASA*.

5.4.2. EFFECT OF ESCALATING DOSES OF ASA ON THE ACTIVATION OF NFkB AND ON THE LATE PC AGAINST MYOCARDIAL INFARCTION

In clinical practice ASA is used at three different dosage levels, with each dose reflecting the relative ASA sensitivity of different target cells[126]. ASA acts as an antithrombotic (60 to 325 mg per day), as an analgesic/antipyretic (650 mg), or as an antirheumatic agent (3000 to 6000 mg). We chose 5 mg/kg as the low dose because this dosage is comparable to that used to prevent cardiovascular events in patients[127]. We found that this dose of ASA inhibited platelet aggregation (a COX-1-dependent phenomenon) but had no effect on NFkB induction followed the brief ischaemic-reperfusion episodes of PC (COX-2-dependent phenomenon and had no effect on late PC against myocardial infarction. We also evaluated the antirheumatic dose of aspirin (25 mg/kg) and nor NFkB activation neither late cardioprotection was blocked. Taken together, these results indicate that doses of ASA commonly given to patients (5 to 25 mg/kg) do not interfere with late PC.

In a recent report of Shinmura et al. extend their previous work and address the question of ASA therapy and cardioprotection in a conscious rabbit model of late PC against myocardial stunning[128]. Using a multigroup and multidisciplinary study design, the primary end point of late PC against stunning was quantified by measurement of systolic wall thickening, and myocardial COX-2 levels were determined by Western immunoblotting. Shinmura reported that a single, low-dose administration of ASA (5mg/kg), designed to mimic clinical antithrombic therapy (typical daily dose of 75 to 325 mg) and confirmed by the authors to inhibit in vitro platelet aggregation, attenuated the increase in COX-2 activity seen with brief antecedent PC ischaemia. Most importantly, however, low dose ASA, despite its partial inhibition of COX-2 activity, did not block the favorable, delayed PC response[129].

The ability of ASA to prevent platelet aggregation and, thereby, prevent cardiac and cerebral ischaemia, results from inhibition of COX-1 due to irreversible acetylation of the protein at serine 530[130 131]. ASA also inhibits COX-2 in a similar manner but exhibits less potency for COX-2 than for COX-1[132] because the substrate channel of COX-2 is larger and more flexible than that of COX-1[133]. These considerations provide a plausible explanation for our finding that 5mg/kg of ASA inhibited platelet aggregation but failed to affect late PC.

In contrast with the effect of antithrombotic and antirheumatic doses of ASA, administration of the subtoxic dose of ASA (4-8000 mg per day; maximum subtoxic serum level: 150-300 microg/ml), 130 mg/kg, blocked the induction of the transcription factor NFkB and completely ablated the beneficial action of late PC on myocardial infarction, suggesting that they should be used with caution in patients with atherosclerotic cardiovascular disease because they may deprive the hearts endogenous adaptation capability. Given the ubiquitous use of ASA and other NSAIDs and the increasing use of selective COX-2 inhibitors, the present findings have potential clinical reverberations. Recent studies indicate that COX-2 inhibitors increase the incidence of cardiovascular events[134 135], possibly because they inhibit late PC (a COX-2 dependent phenomenon) without inhibiting platelet aggregation (a COX-1 dependent phenomenon)[136]. Because many NSAIDs, such as ibuprofen and indomethacin, are less COX-1 selective than ASA[137], they may interfere with late PC at relatively lower doses. Our result suggest that the actions of NSAIDs in patients with atherosclerosis are more complex than heretofore appreciated, and that when NSAIDs are given in dosis sufficient to block COX-2, inhibition of the PC response may offset the benefits deriving from inhibition of platelet aggregation.

5.4.3. CONCLUSIONS OF OUR STUDY

Our study provides the first experimental insight into the consequences of nonsteroidal anti-inflammatory therapy on the efficacy of the delayed PC against myocardial infarction, and yields three important observations:

The administration of a low dose of ASA (5mg/kg), which is sufficient to inhibit platelet aggregation, does not block NFkB activation and does not ablate the cardioprotective effect of late PC.

Higher doses of ASA, in the range used for analgesic/antipyretic and antirheumatic effects (25 mg/kg), also do not block NFkB activation and late PC.

In contrast, a very high dose of ASA (the subtoxic, maximally allowed daily dose: 130 mg/kg) abrogates the activation and nuclear translocation of transcription factor NFkB, and completely blocks the cardioprotection afforded by late PC.

These results suggest that, in patients taking ASA, the ability of the myocardium to shift to a preconditioned phenotype is not impaired so long as these drugs are given in low and medium doses, however, high doses of ASA that completely block NFkB activation, can deprive the heart of its innate defensive response.

5.5.4. EXPERIMENTAL AND CLINICAL CONSIDERATIONS

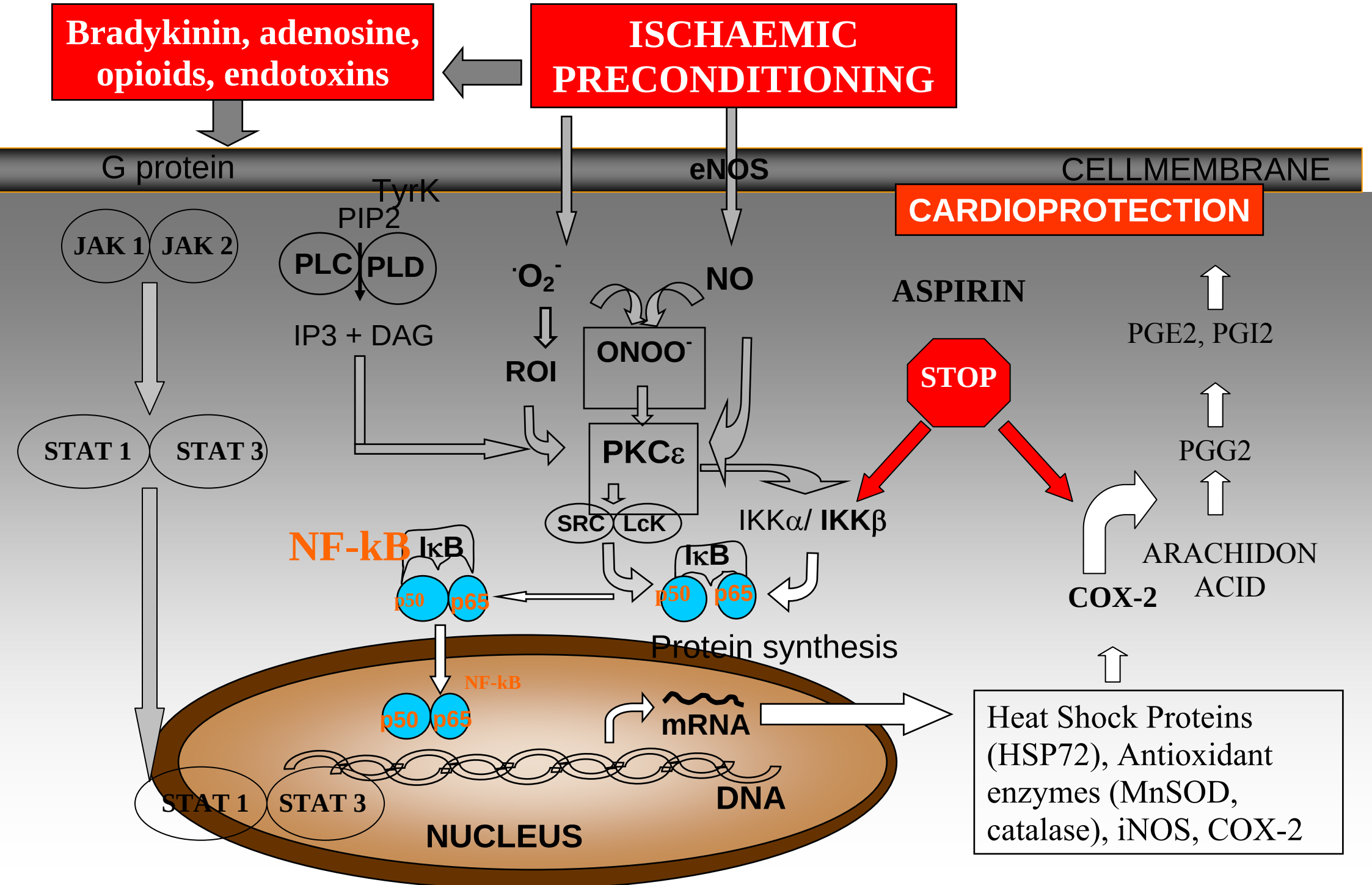
Despite the comprehensive protocol design there are two caveats that warrant consideration in the interpretation of our data. First, in addition to the care that must always be exercised in the extrapolation of experimental studies to the clinical setting, it must further be acknowledged that the single, low-dose administration of aspirin in our rabbit model does not fully mimic the long-term, daily aspirin therapy prescribed to patients for the primary or secondary

prevention of cardiovascular events. Second, although the age of the rabbits was not specified, our study was conducted in adult animals. The effect of increasing age on the delayed, "second window" of PC are at present unknown, however, concerns have emerged that, in some models, the efficacy of the early protection may wane, or be lost in senescent cohorts[138] [139] [140] [141], while, in other species (i.e., rabbit), recent evidence suggest that the cellular mechanisms responsible for early PC may differ in adult versus old animals[142] [143]. As the aging cohort is, without question, precisely the population in which the incidence of acute ischaemic events is greatest and, thus, cardioprotection by any means (including both PC and prophylactic aspirin therapy) is most germane, future studies focusing on delayed PC in old animals, with versus without aspirin therapy, would be of considerable interest and relevance.

In addition to these aforementioned issues, the current result raises several other compelling questions. For example, the widespread clinical use of other nonsteroidal anti-inflammatory agents with greater COX-2 specificity (i.e., ibuprofen, naproxen) and growing popularity of recently developed COX-2.specific inhibitors (celexocib, rofexocib)[144] begs the question: do agents that more closely target COX-2 undermine the endogenous, late phase of cardioprotection conferred by brief antecedent ischaemia? This concept may, again, be of particular relevance in aging cohorts. Finally, although prospective clinical evaliation of these issues would be daunting, a retrospective analysis of surrogate indexes of delayed, "second window" preconditioning, incorporating use of nonsteroidal anti-inflammatory agents as a covariate, may provide a more feasible approach to explore the clinical implications of the "COX-2 hypothesis of late PC". All of these concepts would build upon our observations, and represent fruitful lines of future investigation.

Figure 15. (next page) Effect of ASPIRIN on the intracellular signaling mechanism of ischaemic preconditioning.

(NO-nitric oxide; ROI-reactive oxygen intermediers; ONOO-peroxinitrit; PLC and PLD-phospholipase C and D; PIP2-phosphatitil/inositol/ diphosphate; Ip3-inositol triphosphate; DAG-diacilglicerol; PKC-protein kinase C; IKK-inhibitor kappa kinase; MAP kinase-mitogén aktivated protein kinase; TyrK-tyrozin kinase; NFkB-nuclear factor-kappaB; iNOS-inducible nitric oxide synthase; MnSOD- manganese superoxide-dismutase)


Bradykinin, adenosine, opioids, endotoxins
ISCHAEMIC PRECONDITIONING
G protein
eNOS
CELLMEMBRANE
CARDIOPROTECTION
TyrK
PIP2
JAK 1
JAK 2
PLC
PLD
IP3 + DAG
·O2-
NO
ROI
ONOO-
ASPIRIN
PGE2, PGI2
STOP
PGG2
STAT 1
STAT 3
PKCε
SRC
LcK
IKKα/ IKKβ
NF-kB
IκB
p50
p65
ARACHIDON ACID
COX-2
Protein synthesis
mRNA
DNA
NUCLEUS
Heat Shock Proteins (HSP72), Antioxidant enzymes (MnSOD, catalase), iNOS, COX-2

6. DISCUSSION AND CLINICAL RELEVANCES

6.1 PRECONDITIONING THE HUMAN HEART

The evidence in support of the occurrence of the preconditioning phenomenon in human myocardium arises from laboratory and clinical experiments. Studies in isolated human ventricular myocytes[145], and isolated atrial trabeculae[146] both suggest that protection can be induced in vitro using metabolic and functional end-points respectively. In the clinical setting there is some evidence to suggest that preconditioning may occur naturally. Patients suffering angina prior to a myocardial infarction have a better in-hospital prognosis, a reduced incidence of cardiogenic shock and congestive cardiac failure, and smaller infarcts as assessed by release of cardiac enzymes[147]. The phenomenon of warm-up angina, in which patients complain that their anginal symptoms are worse in the morning but improve during the course of the day has been studied[148]. There is evidence of increased efficiency of myocardial metabolism during a second episode of exercise in terms of reduced oxygen consumption at a given work load as well as less anginal symptoms and ST segment changes. PTCA studies, in which the effect of serial balloon inflations can be examined, have provided further support[149] but, as with all of the above examples, results may be confounded by the effects of collateral recruitment despite efforts to control for this effect[150].

More direct evidence for preconditioning in man has emerged from a study in patients undergoing cardiac surgery in which resistance to global ischaemia was assessed[151]. In this situation changes in collateral flow do not play a role. Intermittent application of the aortic cross clamp was used to deliver repeated episodes of global ischaemia to provide the preconditioning stimulus. Patients subjected to this protocol had better preservation of ATP

levels in myocardial biopsies during a subsequent 10 minute global ischaemic period. These metabolic changes were almost identical to those seen in dogs by Jennings group[152]. However, as discussed later, total myocardial ATP content may not reflect local turnover within subcellular compartments, and certainly does not provide information about the efficiency of cellular metabolism in terms of ATP requirements. In a more recent study[153], involving a larger group of patients, serum levels of troponin-T were used as an indicator of myocardial cell necrosis. Using this end-point, patients subjected to the same preconditioning protocol suffered less necrosis as determined by release of troponin-T. Of considerable interest, however, was the finding that the ATP levels did not differ between preconditioned and control groups. This emphasises the need for multiple end-points to be used, especially in studies where small differences in myocardial viability without overt clinical effects are expected.

6.1.1. WHO SHOULD WE TREAT WITH THERAPEUTIC APPROACHES BASED ON PRECONDITIONING?

It would appear from the evidence outlined above that human myocardium is amenable to preconditioning and that preconditioning may occur as a natural feature of some ischaemic syndromes. However, even with the development of pharmacological agents that can mimic or evoke the protection of ischaemic preconditioning, *the timing of administration will be critical*. Prompt reperfusion will always remain the most effective method of limiting ischaemic injury and is, therefore, the most important determinant of prognosis. However, there are certain situations in which the timing of treatment before the onset of ischaemia can be controlled to some extent.

Patients presenting with unstable angina are at high risk of myocardial infarction and would form a reasonably well-defined group for pre-emptive treatment. A therapy that stimulated or

augmented the cellular preconditioning mechanisms over a period of several days or weeks could keep the myocardium protected. In the event of the patient suffering an acute myocardial infarction the treatment would enhance tissue tolerance and slow the rate of necrosis. Such a treatment would `buy time' for the administration of revascularisation therapies. A major theoretical hurdle is maintaining myocardium in a protected state by preconditioning. Experiments in Downey"s laboratory suggest that continuous adenosine A_1 receptor activation with high dose chronic infusion of CCPA leads to down-regulation of the signalling mechanism and loss of protection[154]. However, more encouraging data have been obtained recently using a different dosing schedule. CCPA was administered to rabbits by intermittent dosing over a 10 day period, and the persistence of myocardial protection assessed 48 hours after the final dose. The expected down-regulation of adenosine A_1 receptors was not observed (since the haemodynamic responses to administration of the agonist were preserved) and infarct size remained significantly reduced in the drug treated group[155].

Preconditioning strategies might also be applied prior to a planned procedure involving a potentially injurious ischaemic insult. An example is coronary artery bypass graft (CABG) surgery. Highly effective strategies for myocardial preservation have already been developed including the use of various cardioplegic solutions, topical and systemic hypothermia, and intermittent aortic cross-clamping with ventricular fibrillation. In general, the rationale behind the use of cardioplegic techniques includes rapid diastolic arrest, membrane stabilisation, hyperosmolarity (to prevent intracellular oedema), acid buffering, and hypothermia. Additional strategies such as continuous coronary perfusion, warm instead of cold cardioplegia (to avoid cold injury), and the use of blood instead of crystalloid solutions (to improve oxygen delivery) have all added to the choices available to the cardiac surgeon. Having said that present cardioprotective measures are highly effective at minimising

irreversible injury that might occur during these periods of imposed ischaemia, they are not without their limitations. Even with carefully controlled intra-operative ischaemic periods and hypothermia, sensitive markers of tissue injury such as troponin-T indicate that discrete necrosis occurs[156 157 158 159]. Moreover, as surgeons undertake more complex and higher risk operations, so the need for better preservation methods increases. In a situation like CABG, the administration of an agent prior to surgery that could enhance myocardial defences would reduce susceptibility to focal necrosis during surgery and permit the extension of the intra-operative ischaemic period. High risk patients with poor pre-operative left ventricular function might certainly benefit if the degree of protection could be improved by invoking endogenous cellular adaptive mechanisms. The possibility that organ preservation prior to transplantation might be amenable to the same improved protection is also of significant interest.

6.1.2. CLINICAL ENDPOINTS FOR ASSESSMENT OF EFFICACY

Any clinical trial involving the use of a potential pharmacological agent designed to mimic the protection of ischaemic preconditioning will have to demonstrate its value in terms of relevant clinical end-points such as preservation of left ventricular function, attenuation of stunning, need for inotropic or balloon support, incidence of clinically detectable infarction, left ventricular failure, and post-operative death. However, studies so far have concentrated on low risk patients with good pre-operative status that would be expected to do well in any event. The benefit derived from ischaemic preconditioning in this group of patients is likely to be marginal. The end-points used presently are relatively insensitive; they provide us with indirect information on myocardial viability and are no substitute for direct measurement of infarct size. Measurement of total myocardial ATP content is not universally accepted as a sensitive marker of cell viability and the concept of a critical tissue concentration of ATP,

below which cell death occurs, is now known to be incorrect[160]. If it were possible to measure sub-cellular levels of ATP within different compartments (such as the mitochondrial fraction), and thereby assess local turnover, then more useful information might be available. End-points of clinical outcome are more likely to demonstrate a difference in studies conducted in a group of patients at higher risk, but these can only be performed once safety and tolerability have been established.

6.1.3. REMOTE PRECONDITIONING AND STRETCH-INDUCED PRECONDITIONING

Stimuli other than ischaemia of the myocardial risk territory may confer cardioprotection against damage by subsequent coronary artery occlusion. Brief circumflex coronary occlusions in canine myocardium induce protection of remote myocardium subtended by the left anterior descending coronary artery, suggesting that unidentified diffusible factors or neuronal mechanisms may influence remote tissue[161]. A further, and more intriguing, example of remote preconditioning is that brief renal ischaemia or mesenteric artery occlusion leads to protection against coronary artery occlusion[162]. The mechanisms of `inter-organ' preconditioning are unknown but it is possible that there is a neuronal component. Transient stretch of myocardium by acute volume overload has been shown to confer protection against myocardial ischaemia by Ovize and co-workers[163]. This phenomenon is abolished by gadolinium, a stretch activated ion channel blocker, and by a PKC blocker[164]. These investigations expand the strictly defined concept of ischaemic preconditioning.

6.2. CLINICAL CONCLUSION

Direct activation of the cellular pathways involved in ischaemic preconditioning by pharmacological manipulation would allow improved myocardial protection without the need for an ischaemic preconditioning insult. A clear understanding of the mechanisms involved in either form of protection (early or late) is essential to allow a reasoned approach to drug design.

There are several classes of pharmacological agents that may be able to mimic the protection conferred by ischaemic preconditioning and provide some basis for optimism that a beneficial and clinically detectable improvement in myocardial protection may be possible.

In conclusion, we feel that exploitation of endogenous cardioprotective mechanisms may be possible in the context of carefully conducted clinical studies. There have been significant advances in our understanding of the mechanisms underlying ischaemia-reperfusion injury as a result of preconditioning research and potential pharmacological approaches to protection seem feasible. However further development of pharmacological therapies should be based on sound experimental investigation and assessed in the context of other effective therapeutic strategies.

7. NOVEL FINDINGS

Our result shows a biphasic increase of Nuclear Factor-kB activation, with peak levels at 30 min and at 3 hour of reperfusion in preconditioned myocardium. Induction of Activation Protein-1 increased monophasically, with peak level at 1 hour of reperfusion. Our results shows that the activation of NFkB and AP-1 have a specific time curve after ischaemic-reperfusion stimulus, and suggest that the regulation of these two transcription factors in the signalling of ischaemic preconditioning are different.

We have demonstrated, that one cycle of ischaemia induced a significant increase of NFkB and AP-1 activation in the preconditioned myocardium. Further repetition of ischaemia-reperfusion cycles has not resulted in further elevation in activation of NF-kB and AP1 compared to the one cycle. These findings demonstrate that the activation of these transcription factors in the signaltransduction of ischaemic PC is an “all or nothing” response. If the PC stimulus is strong enough to reach a “threshold” level, a full signaling cascade and protection will be induced. Above this threshold the strenght of the PC stimulus does not influence the volume of the signaling cascade and the degree of the evoked cardioprotection.

In our experiments we were able to demonstrate that oxygen radicals are involved in the triggering of the signaling cascade of ischaemic preconditioning and in the induction of the transcription factor NFkB in the preconditioned myocardium in an *in vivo* rabbit model. Oxygen free radicals act in concert with the other triggers of ischaemic preconditioning confirming the additive interaction between the triggers of the endogenous cardioprotection.

Our results demonstrated firstly that the administration of a low dose of ASA (5mg/kg), which is sufficient to inhibit platelet aggregation, does not block NFkB activation and does not ablate the cardioprotective effect of late PC. Higher doses of ASA, in the range used for analgesic/antipyretic and antirheumatic effects (25 mg/kg), also do not block NFkB activation and late PC. In contrast, a very high dose of ASA (the subtoxic, maximally allowed daily dose: 130 mg/kg) abrogates the activation and nuclear translocation of transcription factor NFkB, and completely blocks the cardioprotection afforded by late PC.

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