

THE NUCLEAR FACTOR κ B IN THE SURVIVAL
SIGNALS OF THE ENDOGENOUS ADAPTATION OF
ISCHAEMIC CARDIOPROTECTION

PhD Thesis

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Pécs 2004

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ABBREVIATIONS

ADP	adenosine diphosphate
AP-1	activation protein-1
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
COX	cyclooxygenase
DAG	diacylglycerol
DNA	deoxyribonucleic acid
EMSA	electrophoretic mobility shift assay
ECG	electrocardiograph
FV	ventricular fibrillation
GSH	reduced glutathione
HSP	heat shock protein
iNOS	inducible nitric oxide synthase
Iκ-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	mitogen activated protein kinase
MDA	malondialdehyde
MnSOD	manganese superoxide-dismutase
MPG	N-2-mercaptopropionylglycine
NF-κB	nuclear factor-kappaB
NO	nitric oxide
NSAID	non-steroid anti-inflammatory drugs
OFR	oxygen free radicals
ONOO	peroxynitrite
PC	preconditioning
PIP2	phosphatidyl/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	tyrosin kinase
TTC	triphenyltetrazolium chloride

1. INTRODUCTION

It is now eighteen years since the phenomenon termed "ischaemic preconditioning" was formally recognised. 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.

1.2. 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. 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, rapid cardiac pacing, thermal stress, stretch, 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. Ischaemic preconditioning also reduces the extent of apoptosis. 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. However, with longer reperfusion ischaemic preconditioning diminishes adverse left ventricular remodeling following infarction and improves long-term functional recovery in chronically instrumented rabbits. Ischaemic preconditioning protects against arrhythmias in mice, rats, rabbits, and dogs. 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.

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. 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 1994 published a study that positively confirmed

delayed protection against reperfusion arrhythmias in the canine heart, using ventricular rapid pacing to globally precondition the heart.

Signaling 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. 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, 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 , and that this can be blocked with chelerythrine.

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 signaling cascade since administration of genistein during preconditioning in rabbits abrogates the delayed anti-infarct effect. Interestingly, delayed protection induced by adenosine A₁ 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). The interactions of these complex signaling systems and their involvement or interaction with membrane channels or new protein synthesis still needs to be evaluated.

The transcription factor nuclear factor-kappaB (NF-kB) appears to be a critical regulator for gene expression induced by diverse stress signals including mutagenic, oxidative and hypoxic stresses. NF-kB 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 (I κ B) from NF-kB. Activation of NF-kB 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.

2. OVERALL AIM OF THE PROJECTS

In the first series of our investigations we aimed to monitor the time fluctuation 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 series of our investigations we focused on the role of oxygen free radicals (OFR) in the signaling cascade of this adaptive process leading to the induction of NF-kB. 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 NF-kB activation after repeated cycles of preconditioning ischaemia-reperfusion 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.

We also aimed to define the activation level of NF-kB after repeated number of preconditioning stimuli. Our question was to determine the number of cycles to induce preconditioning with or without antioxidant. 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 hypothesized 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.

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

3. DYNAMISM OF ACTIVATION OF NUCLEAR FACTOR- κ B AND ACTIVATION PROTEIN-1 IN THE SIGNALING OF DELAYED MYOCARDIAL PRECONDITIONING

(First series of investigations)

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) - κ B and Activation Protein-1 (AP-1) is a key component in the signal transduction mechanism of ischaemic preconditioning.

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- κ B and AP-1 levels in the preconditioned myocardium. We measured the DNA binding activity of NF- κ B and AP-1 at various times after brief ischaemic episode in reperfused myocardium.

3.2. MATERIALS AND METHODS

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.

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 2.)

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.

3.3. RESULTS

Time course of NF-κB activation. Specificity of the signal was verified in a competition assay wherein the signal detected by labeled NF-κB was abolished, when the protein homogenate was preincubated with excess unlabeled NF-κB oligo before the addition of labeled NF-κB. The signal was not abolished, when the competition assay contained excess unlabeled non NF-κB binding oligonucleotide. Low and consistent levels of NF-κB 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-κB in preconditioned myocardium.

Time course of AP-1 activation. 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.

3.4. DISCUSSION

The results of our experiments show that there is a biphasic activation of NF-κB 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-κB activation after ischaemic PC. Through receptor-dependent triggers (Adenosine A₁ agonists, opioid δ₁ agonists, 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). 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. 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-κB.

Another possible way of NF-κB activation in ischaemic PC is came off through the increased production of nitric oxide (most likely via eNOS) and ·O₂- (leading to formation of secondary reactive oxygen species (ROS)) after a brief episode of myocardial ischaemia/reperfusion. Both NO and ·O₂- derived ROS could directly activate the ε isoform of PKC via nitrosylation and oxidative modification, respectively; alternatively, NO and ·O₂- 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.

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. They argued that during reoxygenation, increased intracellular ROI activate existing NF-kB by dissociation from its inhibitor IκB, 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 TNFα, 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. Whether such interaction exists in preconditioned myocardium is not known.

Conclusion. From our study we conclude, that after the ischaemic preconditioning stimuli the activation of NF-kB 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 NF-kB and AP1 have a specific time curve in the signaling of endogenous cardioprotection.

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

(Second series of investigations)

4.1. INTRODUCTION

Since oxidative stress is known to induce the synthesis of cardioprotective proteins, such as antioxidant enzymes and HSPs, 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 NF- κ B 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, thus through phosphorylation of Inhibitor Kappa Kinase- β could induce NF- κ B activation and translocation to the nucleus.

One possibility, that has not been tested, is that the free radicals may act in concert with the other triggers of delayed preconditioning in the induction of the transcription factor NF- κ B. 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 NF- κ B 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-mercaptopropionylglycine (MPG), to attenuate the induction of NF- κ B in ischaemic preconditioning induced by either a single or multiple episodes of ischaemia-reperfusion in *in situ* rabbit hearts.

4.2. MATERIALS AND METHODS

Surgical procedure. (see at 3.2.1.)

Experimental protocol. In the *first part* of our study we aimed to measure the NF- κ B activation after repeated cycles of ischaemia-reperfusion. The animals were selected in five groups, in each group there were 6 rabbits. For NF- κ B 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- κ B 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 reperfusion (2x5 IPC). In the fourth and fifth group all animals underwent three (3x5) and four cycle of 5 min of regional ischaemia (4x5 IPC).

In the *second part* of this study we sought to block OFR by administering an antioxidant: N-2-mercaptopropionylglycine (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.

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.

4.3. RESULTS

Measurement of NF-kB activation. 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 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 NF-kB was still statistically significant. Adding MPG, however, abolished the previously observed NF-kB induction with either 2x5 or 1x5 IPC.

4.4. DISCUSSION

Our results demonstrated that oxygen radicals produced in the process of the ischaemic preconditioning represent an important trigger for activation of NF-kB in the signaling mechanism of ischaemic preconditioning to act parallel with adenosine, bradykinin and the others. 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 NF-kB activation in preconditioned myocardium.

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.

The authors have investigated in this study, in an animal model, that OFR are important triggers of delayed ischaemic preconditioning; examining NF-kB 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.

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 NF-kB 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.

5. EFFECT OF ASPIRIN ON NF- κ B ACTIVATION AND ON LATE PRECONDITIONING AGAINST INFARCTION IN PRECONDITIONED MYOCARDIUM

(Third series of investigations)

5.1. INTRODUCTION

Recent studies have demonstrated, that the NF- κ B 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 I κ B- α .

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.

The aim of this study was to investigate the effect of aspirin on the NF- κ B 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- κ B in the preconditioned myocardium. We aimed to demonstrate whether ASA could block the activation of NF- κ B, and consequently inhibit the development of the cardioprotection in these doses - antithrombotic (5 mg/kg), analgetic, antirheumatic (25 mg/kg) and maximal, subtoxic (130 mg/kg) doses -, which are based on clinical therapy.

5.2. MATERIALS AND METHODS

Surgical preparation. (see at 3.2.1.)

Experiments for NF- κ B 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. Between the ischaemic cycles the heart was allowed to reperfuse for 5 min. 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 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- κ B analysis with EMSA and enzyme immunoassay.

Experiments for SWOP investigation. After the surgical preparation the threads were pulled through a reinforced tube (2.5 mm internal diameter, Mallinckrodt Medical, Athlone, Ireland), which was tunneled subcutaneously to the interscapular space. 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 hydrochlorid 0,1 mg/kg intravenous), and recovery in an isolated pen.

Rabbits were allowed to recover for 10-13 days. Then, under anaesthesia we made a small incision above the peripheral end of the tube, and in preconditioned groups the suture was tightened, thereby inducing occlusion of the coronary artery. 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.

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 rabbits were then subjected to 30 min of coronary artery occlusion by tightening the snare. After 30 min of occlusion the snare was released and 2 h of reperfusion was allowed.

After the reperfusion period the area of infarction within the area at risk was determined by double staining of the heart. 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.

Protocol I: Effect of ASA on the activation and translocation of NF- κ B. 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 antithrombotic 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.

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.

5.3. RESULTS

Protocol II: Effect of ASA on delayed preconditioning against myocardial infarction. Low and consistent levels of NF- κ B 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- κ B transcription factor. In contrast, the high dose (130mg/kg group V) ASA arrested the activation of NF- κ B. There was no change in NF- κ B activation in the nonischaemic region among the five groups.

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. Ischaemic risk zone

volume was similar in all experimental groups at around 19% to 23% of the left ventricle mass. Sham-operated control rabbits (group VI.) had a mean infarct size of $61,3 \pm 12,3\%$ of the risk zone. Preconditioning (PC) with four 5-min coronary occlusion episodes limited the infarction to $32,7 \pm 8,6\%$ ($p < 0,05$) 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 influence the protective effect of late PC, and resulted in $34,6 \pm 8,7\%$ and $36,4 \pm 9,3\%$ infarct/risk ratio. In contrast, pre-treatment with 130 mg/kg ASA (group X.) prior to PC abolished the cardioprotection and led to an infarct size of $59,1 \pm 11,6\%$. 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.

5.4. DISCUSSION

In clinical practice ASA is used at three different dosage levels, with each dose reflecting the relative ASA sensitivity of different target cells. 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. We found that this dose of ASA inhibited platelet aggregation (a COX-1-dependent phenomenon) but had no effect on NF- κ B 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 NF- κ B 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.

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 NF- κ B activation, can deprive the heart of its innate defensive response.

6. CLINICAL RELEVANCES

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- κ B 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 NF- κ B and AP-1 has a specific time curve after ischaemic-reperfusion stimulus, and suggest that the regulation of these two transcription factors in the signaling of ischaemic preconditioning are different.

We have demonstrated, that one cycle of ischaemia induced a significant increase of NF- κ B and AP-1 activation in the preconditioned myocardium. Further repetition of ischaemia-reperfusion cycles has not resulted in further elevation in activation of NF- κ B 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 NF- κ B 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 NF- κ B 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 NF- κ B 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 NF- κ B, and completely blocks the cardioprotection afforded by late PC.

8. ACKNOWLEDGEMENT

I would like to take this opportunity to express my thanks for the overwhelming support I have received from my supervisor Professor Erzsébet Róth in completing this work. I would also like to acknowledge the help and assistance of all the staff at the Department of Experimental Surgery in Pécs, and of my colleagues Mohammad Taghi Jaberansari, Balázs Borsiczky, Zalán Szántó, László Benkő, Balázs Gasz, Andrea Ferencz, and János Lantos to carrying out the investigations and giving me the inward support over the years. Special thanks Dr Katalin Kiss and Prof. József Szeberényi for giving me the chance to carry out the biochemical analyses in the laboratory of the Department of Medical Biology.

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