Dynamism of NF- κ B and AP-1 Activation in the Signal Transduction of Ischaemic Myocardial Preconditioning

G. Jancso J. Lantos B. Borsiczky Z. Szanto E. Róth

Department of Experimental Surgery, Medical Faculty, University of Pecs, Pecs, Hungary

Key Words

Nuclear factor- κ B · Activation protein-1 · Transcription factors · Ischaemic myocardial preconditioning · Signal transduction

Abstract

Nuclear factor (NF)- κ B and activation protein (AP)-1 transcription factors play an important role in the signal transduction of delayed ischaemic preconditioning (PC) leading to myocardial cytoprotection. Because the exact mechanism of the activation of these factors is still not clear, we aimed to monitor the time fluctuation of NF- κ B and AP-1 induction in an in vivo animal model. Furthermore, we measured the induction rate of these factors using repeated cycles of PC. Following median thoracotomy, anaesthetized animals (24 New Zealand White rabbits) were subjected to ischaemic PC by occlusion of the left anterior descending coronary artery for 5 min. After 10 and 30 min, and 1, 2, 3 and 4 h of reperfusion, tissue samples were taken from the ischaemic myocardium, and the DNA binding activity of the transcription factors was measured with electrophoretic mobility shift assay. A further 12 animals were subjected to 2 $\times$, 3 $\times$ or 4 $\times$ 5-min ischaemic PC, and after a 30-min or 1-hour reperfusion period, we investigated the possible modulation

of NF- κ B and AP-1 induction. Our results show significant, biphasically increased NF- κ B activity with peak levels at 30 min and 3 h of reperfusion in preconditioned myocardium. AP-1 increased monophasically, with the peak level at 1 h of reperfusion. Repeated PC stimuli enhanced the activity of both transcription factors analyzed, but there was no significant correlation between the number of cycles and the rate of activation. Our results show that the activation of NF- κ B and AP-1 have a specific time curve, and the induction of these factors is only slightly influenced by the number of PC cycles.

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Introduction

Ischaemic preconditioning (PC) is a polygenic defensive cellular adaptive phenomenon whereby brief ischaemic stimuli render the heart resistant to subsequent similar stress. The phenomenon was first described by Murry et al. [1], and it was the most intensively examined question in cardiovascular research in the last decade. Ischaemic PC protects against myocardial stunning, reperfusion arrhythmias and infarction, and thus has considerable clinical relevance in treatment of patients with ischaemic heart disease.

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Gabor Jancso
Department of Experimental Surgery, University of Pecs, Medical Faculty
Kodaly Zoltan St. 20
HU-7624 Pecs (Hungary)
Tel. +36 72 535 820, Fax +36 72 535 821, E-Mail jancsogabor@hotmail.com

Diverse signalling molecules released by a sublethal ischaemic stress initiate a complex signal transduction cascade that modulates the activation of the severe transcription factors, which lead to the expression of cardio-protective genes. Recent studies have assumed that the activation and translocation of nuclear factor (NF)- κ B and activation protein (AP)-1 are key components in the signal transduction mechanism of ischaemic PC [2, 3].

NF- κ B is a redox-sensitive transcription factor involved in transcription of proteins in response to mutagenic, oxidative and hypoxic stress [4]. Under normal physiologic conditions, NF- κ B is held inactive in the cytoplasm by the inhibitory subunit, inhibitor (I)- κ B α [5]. Under conditions of oxidative stress, NF- κ B disassociates from I- κ B α and translocates to the nucleus, where it initiates the transcription of pro-inflammatory, procoagulant and vasoactive genes [6]. In contrast to the role NF- κ B activation has in the destructive events of inflammation, NF- κ B also mediates the expression of cytoprotective proteins (heat shock proteins), which block apoptosis or inhibit inflammation in response to several types of cellular stress [7]. In a negative feedback manner, these cytoprotective proteins inhibit NF- κ B [8].

The NF- κ B pathway displays the capacity to activate, in a cell- and stimulus-specific manner, only a subset of the total repertoire of NF- κ B-responsive genes. The seemingly promiscuous nature of NF- κ B 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- κ B in particular, provides intriguing insights as to how this may occur.

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 oxygen intermediates – generated by ischaemia-reperfusion – induction of AP-1 is not known, and alteration of the 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- κ B, 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 PC induces either NF- κ B or AP-1 activation, and both of them activate pro- and anti-apoptotic signals in vivo in the myocardium.

To understand 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 episodes in reperfused myocardium. Furthermore, we aimed to investigate the activation rate of NF- κ B and AP-1 in cases of repeated cycles of ischaemic PC.

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).

Surgical Preparation

A marginal ear vein was cannulated in New Zealand White rabbits weighing 2.6–3.3 kg (mean 2.8 kg), after local anaesthesia was induced using lidocaine cream. The animals were anaesthetized with intravenous xylazine (6 mg/kg), ketamine (6 mg/kg) and propofol (10 mg/kg). The trachea was intubated (tube internal diameter, 3 mm) and the lungs were ventilated (Sulla 808, Dräger, 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 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 and 38.7 °C by adjusting a heating pad and an infrared lamp.

In the ischaemic PC groups, the snare was tightened for 5 min, thereby inducing occlusion of the coronary artery (5 min of ischaemia with 10 min of reperfusion was previously described – and in our pilot infarct size study conferred – as inducing both early and delayed PC in rabbit myocardium [9]). 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 for NF- κ B and AP-1 Analysis

In the first series of our experiment, animals (42 rabbits) were randomly divided into 7 groups. In control animals (group 1), the

heart was excised right after thoracotomy and a tissue sample was taken from the untreated heart. In the preconditioned groups, after a 5-min ischaemic period, animals were assigned to 10 min (group 2), 30 min (group 3), 1 h (group 4), 2 h (group 5), 3 h (group 6) or 4 h (group 7) of reperfusion before tissue samples were taken from the ischaemic zone of the heart (fig. 1).

In the second series of our study, we aimed to measure NF- κ B and AP-1 activation after repeated cycles of ischaemia-reperfusion. The animals (36 rabbits) were divided into 6 further groups. For NF- κ B investigations, the animals were subjected to either 2 $\times$, 3 $\times$ or 4 $\times$ 5 min of left anterior descending occlusion (groups 8, 9 and 10) with an intermittent 10-min reperfusion, and after 30 min of reperfusion (NF- κ B showed its activation maximum at 30 min of reperfusion), tissue samples were taken from the ischaemic zone of the heart. For AP-1, after 2 $\times$, 3 $\times$ or 4 $\times$ 5 min of ischaemia (in order, groups 11, 12 and 13), a 1-hour reperfusion period (AP-1 showed its activation maximum at 1 h of reperfusion in the first series) was applied, before tissue samples were taken from the ischaemic zone of the heart (fig. 2).

After the experimental period, the hearts were 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 electrophoretic mobility shift assay (EMSA) analysis.

Electrophoretic Mobility Shift Assay

100 mg of tissues were homogenized in 1 ml of TE buffer (1.5 mM EDTA, 0.01 M Tris base, pH 7.4) containing 10 μM PMSF. Nuclei were separated from the cytosol by centrifugation at 1,400 g for 20 min at 4°C , and this separation procedure was repeated 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 MgCl₂, 0.2 mM EDTA, 0.5 mM DTT and protease inhibitors (Complete Mini, Boehringer Mannheim, Germany) and placed on ice for 20 min. After 10 s of centrifugation, the supernatants were saved, aliquoted and stored.

The protein concentration was determined with the Bio-Rad Protein Assay kit. 5'-End labelling of oligonucleotides was performed using [γ -³²P]-ATP and T4 polynucleotide kinase (Amersham Pharmacia Biotech Inc., UK) according to the manufacturer's protocol. 20 μg of nuclear proteins were mixed with 1 μg of poly(dI-dC), 100 ng of non-specific single-stranded oligonucleotide and 4 μl of buffer containing 10 mM HEPES, pH 7.5, 10% glycerol, 1 mM EDTA and 100 mM NaCl. After 15 min of incubation at room temperature, the mixture was completed with 2 μl of ³²P oligonucleotide (approximately 100,000 cpm) and then incubated for another 30 min. DNA-protein complexes were electrophoresed in a 5% non-denaturing polyacrylamide gel using a Tris base-borate-EDTA buffer system (pH 8.3) for 2.5 h at 200 V. Gels were dried and quantitated using an image scanning densitometer (Cyclone PhosphorImager System, Packard I.C. USA).

Infarct Size Assessment

After the surgical preparation (described above), the threads were pulled through a reinforced tube (internal diameter 2.5 mm, Mallinckrodt Medical, Athlone, Ireland), which was tunnelled subcutaneously to the interscapular space. The chest wound was then closed in layers and air was aspirated from the thorax.

Rabbits were allowed to recover for 10–13 days. Then, under anaesthesia, we made a small incision above the peripheral end of the

Fig. 1. Experimental protocol of the first series of experiments, to monitor the NF- κ B and AP-1 activation time course in preconditioned myocardium. For details, see Materials and Methods.

Fig. 2. Experimental protocol of the second series of experiments. Examination of NF- κ B and AP-1 activation after repeated cycles of ischaemic PC. For details, see Materials and Methods.

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 the control animals, we performed a sham operation; the suture was not tightened. In preconditioned groups animals were subjected to either 1 $\times$, 2 $\times$, 3 $\times$ or 4 $\times$ 5 min of left anterior descending occlusion with an intermittent 10-min reperfusion. 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.

24 h later, 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, we measured the infarct size with double staining as a percentage of the risk zone. The method of double staining has been described in detail previously [10].

Fig. 3. The time course of NF- κ B activation in the myocardium after ischaemic PC. Activated NF- κ B bands (a p50/p65 heterodimer and a p50 homodimer) are shown. Left panel: EMSA analysis. Right panel: densitometry measurements expressed as a percentage of the control. * $p < 0.0001$ versus control.

Fig. 4. The time course of AP-1 activation in the preconditioned myocardium. Left panel: EMSA analysis. Right panel: densitometry measurements expressed as a percentage of the control. * $p < 0.001$.

Statistical Analysis

For statistical analysis, EMSA pictures were evaluated with Cyclone PhosphorImager System densitometry software. The data (mean $\pm$ SEM) for levels of expression of individual protein (EMSA) were subjected to analysis of variance with post hoc Dunnett's one-tailed t tests (NF- κ B, and AP-1 EMSA), and two-sample Student's t test for significance. Infarct size data were analyzed with one-way analysis of variance followed by an unpaired t test with Bonferroni's correction for multiple comparisons. $p < 0.05$ was considered significant.

Results

Time Course of NF- κ B and AP-1 Activation

Figure 3 shows changes in NF- κ B levels in preconditioned myocardium after various reperfusion times. Specificity of the signal was verified in a competition assay

wherein the signal detected by labelled NF- κ B was abolished when the protein homogenate was preincubated with excess unlabelled NF- κ B oligo before the addition of labelled NF- κ B. The signal was not abolished when the competition assay contained excess unlabelled non-NF- κ B-binding oligonucleotide. Low and consistent levels of NF- κ B were detected in normal myocardium (untreated, group 1) at steady state. Significantly higher levels were detected after 30 min of reperfusion (group 3) in all 6 animals (densitometry: 2.35-fold; $p < 0.0001$ vs. controls), and then fell to a lower level at 1 h of reperfusion. Again at 3 h of reperfusion (group 6), the levels were significantly higher (2.59-fold; $p < 0.0001$). At 4 h of reperfusion, the levels decreased to the basic rate, indicating a biphasic regulation (with an emphatic up- and down-regulation) of NF- κ B in preconditioned myocardium.

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

NF- κ B and AP-1 Activation after a Repeated Number of Ischaemic Cycles

One cycle of 5-min ischaemia (group 3) resulted in significant elevation of NF- κ B levels (2.35-fold) compared to controls (group 1) after 30 min of reperfusion. In the case of further repeated cycles of ischaemia (groups 8, 9 and 10), the NF- κ B levels were significantly elevated compared to the controls, but did not result in additional significant accretion of NF- κ B compared to 1 cycle of PC (group 3) (fig. 5).

At 1 h of reperfusion, 1 cycle of 5-min ischaemia (group 4) caused a significant rise in AP-1 level (5.43-fold; $p < 0.005$) compared to controls (group 1). After repeated PC cycles, AP-1 levels showed analogous results to NF- κ B. There was no significant difference between the AP-1 levels generated by repeated cycles of ischaemia (groups 11, 12 and 13) or 1 cycle of ischaemia (group 4) (fig. 6).

Infarct Size Measurement after a Repeated Number of Ischaemic Cycles

The ischaemic risk zone volume was similar in all experimental groups at around 19–23% of the left ventri-

cle mass. Sham-operated control rabbits had a mean infarct size of $61.3 \pm 12.3\%$ of the risk zone. PC with a 5-min coronary occlusion episode limited the infarction to $32.7 \pm 8.6\%$ ($p < 0.05$). In the case of further repeated cycles ($2 \times$, $3 \times$ or 4×5 min of ischaemia), the infarct sizes were significantly decreased compared with the controls, but did not result in further significant changes compared to 1 cycle of PC (in order: $29.4 \pm 6.2\%$, $30.9 \pm 4.8\%$, $33.1 \pm 7.5\%$).

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 a 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 PC – is mediated through the up-regulation of the heart's own cellular defence via the accumulation of intracellular mediators and reprogramming of gene expression. Recent studies suggest that the transcription factors NF- κ B and AP-1 have a possible role in the signal transduction pathways of this cytoprotection resulting from ischaemic adaptation.

A potential role for NF- κ B in the endogenous adaptation of ischaemic PC has recently been suggested [3, 11]. Myocardial protection by NF- κ B activation may be caused by induction of an NF- κ B-regulated mediator, such as manganese superoxide dismutase [12], inducible cyclooxygenase [13] and inducible nitric oxide synthase [14]. Heat shock proteins of the 70-kD family, which are upregulated during PC by other pathways, have been suggested as mediators of ischaemic adaptation [15]. Heat shock proteins modulate AP-1 and NF- κ B DNA binding activity [16, 17], and might reduce NF- κ B activation and thereby reduce inflammation during reperfusion.

The results of our experiments show that there is a biphasic activation of NF- κ B in the preconditioned myocardium, with increased levels at an early time point (30 min) and again at 3 h of reperfusion. It is presumed that there are two different pathways leading to early NF- κ B activation after ischaemic PC. Through receptor-dependent triggers (adenosine A_1 agonists [18], opioid δ_1 agonists [19], bradykinin, prostaglandins, norepinephrine, angiotensin, endothelin), the receptor is coupled through G proteins to, among others, phospholipase C (PLC) and D. PLC catalyzes the hydrolysis of membrane

Fig. 5. NF- κ B activation after repeated cycles of ischaemic PC. Left panel: EMSA analysis. Right panel: densitometry measurements expressed as a percentage of the control. * $p < 0.0001$.

Fig. 6. AP-1 activation after repeated cycles of ischaemic PC. Left panel: EMSA analysis. Right panel: densitometry measurements expressed as a percentage of the control. * $p < 0.001$.

inositol-containing phospholipids into inositol trisphosphate and diacylglycerol (DAG) [20]. DAG stimulates the translocation and activation of protein kinase C ϵ (PKC ϵ). The onset of the PLC reaction is typically very rapid, and DAG production is short-lived, peaking at 30 s [21]. PKC ϵ activation then triggers a complex signalling cascade that involves Src and/or Lck tyrosine kinases and probably other kinases, leading to phosphorylation of I- κ B α and to mobilization (nuclear translocation) and activation of the transcription factor NF- κ B [22].

Another possible pathway of NF- κ B activation in ischaemic PC is through the increased production of nitric

oxide [most likely via endothelial nitric oxide synthase (eNOS)] and $O_2^{\cdot -}$ (leading to formation of secondary reactive oxygen species) after a brief episode of myocardial ischaemia-reperfusion [23]. Both nitric oxide (NO) and $O_2^{\cdot -}$ derived reactive oxygen species could directly activate the ϵ isoform of PKC via nitrosylation and oxidative modification, respectively; alternatively, NO and $O_2^{\cdot -}$ are known to react to form $ONOO^-$, which in turn could activate PKC ϵ . Thus, PKC is thought to be a critical component in both pathways [24, 25].

Ischaemic PC has recently been found to activate Janus activated kinases 1 and 2 with a subsequent tyrosine phosphorylation and activation of STAT1 and STAT3, which is essential for inducible nitric oxide synthase (iNOS) up-regulation. Binding of NF- κ B and STAT1/3 to the iNOS promoter results in transcriptional activation of the iNOS gene and leads to synthesis of new iNOS protein (eNOS-dependent iNOS induction). iNOS-derived NO is supposed to create a second wave of PKC ϵ activation, leading to the late phase of NF- κ B activation and translocation [26].

The other possible explanation of the late increase in NF- κ B activation might be the feedback mechanism of NF- κ B-induced pro-inflammatory cytokines (e.g. TNF- α , IL-1 β). After ischaemia-reperfusion, even in the case of ischaemic PC, NF- κ B can induce TNF- α and IL-1 β gene expression [27, 28]. Through an unknown mechanism, these cytokines generate a mass of reactive oxygen intermediates in the myocardium, which can – via the above-mentioned pathway – newly activate NF- κ B in the cytoplasm and lead to a delayed wave of nuclear translocation of this transcription factor. TNF- α and IL-1 β can also start up a signalling pathway leading to I- κ B phosphorylation, and thus NF- κ B activation, through cell membrane receptors [29]. A number of signal transduction proteins have been identified as being associated with these receptors, including TNF receptor-associated factors 2 and 6, death domain-containing proteins (TRADD and FADD) and kinases associated with the IL-1 receptor (IRAK1 and 2, and MYD88) [30]. These kinases phosphorylate members of the I- κ B family at specific serines within their N-termini, leading to site-specific ubiquitination and degradation of NF- κ B by the 26S proteasome. This circle cascade (NF- κ B $\rightarrow$ TNF- α , IL-1 β $\rightarrow$ NF- κ B feedback) might also be an explanation of the biphasic activation of NF- κ B after ischaemic PC [30].

The second major observation from our study is the detection of increased AP-1 levels in the preconditioned myocardium. In contrast to NF- κ B, for which after an initial increase at 30 min, a second peak was observed at 3 h of reper-

fusion, AP-1 levels increased in a monophasic manner at 1 h of reperfusion. Though we did not measure the levels of AP-1 during the ischaemic period, in various in vitro systems, a substantial increase in AP-1 levels was demonstrated during hypoxic conditions. In a cancerous cell line (HeLa), Rupec and Baeuerle [31] showed increased NF- κ B activity within 15 min after initiation of reperfusion, while increased AP-1 was detected during hypoxia itself. They argued that during reoxygenation, increased intracellular reactive oxygen intermediates activate existing NF- κ B by dissociation from its inhibitor I- κ B, while low levels of free radicals during hypoxia, a condition similar to that observed during antioxidant treatment, induced AP-1.

Because both NF- κ B and AP-1 are activated by cytokines such as IL-1 and TNF- α , the positive synergy between NF- κ B and the subunits of AP-1 might have important implications for both immune and inflammatory responses. Stein et al. [32] showed functional cross-coupling of NF- κ B p65 and AP-1 families of transcription factors, resulting in increased DNA binding activity of NF- κ B. Both s-fos and c-jun synergised with NF- κ B by physically interacting with the p65 subunit [32]. Whether such interaction exists in preconditioned myocardium is not known.

In this report, we also demonstrated the DNA binding activities of NF- κ B and AP-1 after different numbers of ischaemia-reperfusion cycles. Our results show that after one cycle of ischaemia-reperfusion, which was previously shown to exert powerful cardioprotective effects for ischaemic hearts, the activation of NF- κ B and AP-1 increased progressively and steadily. However, further numbers of ischaemia-reperfusion cycles did not result in further elevation in activation of NF- κ B and AP-1 compared to the single cycle. These findings correlate with the threshold hypothesis of Goto et al. [33]; they 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 fail. Above this threshold, the strength of the PC stimulus does not influence the volume of the signalling cascade and the degree of the evoked cardioprotection [33].

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