Involvement of nitric oxide and reactive oxygen species in the early and delayed antiarrhythmic effects of preconditioning

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Summary of the Ph.D. Thesis

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1 Introduction

One of the most important consequences of the reduction in coronary blood flow due to acute myocardial ischemia is the occurrence of severe ventricular tachyarrhythmias, which remain one of the major causes of sudden cardiac death (SCD) in modern societies. Although the underlying mechanisms of these acute arrhythmias are many and varied, it is certain that the ischemia-induced rapid ionic alterations and the subsequent inhomogeneous conduction may play an essential role.

In the past few decades many attempts have been made on protecting the myocardium and thus preventing the heart from the severe consequences of ischemia/reperfusion injury. One of these attempts was made by Murry and colleagues[1] in 1986 where they observed that the heart responds to brief ischemia/reperfusion insults with an increased tolerance, rather than with cumulating ischemic damage, during a subsequent, more prolonged episode of ischemia. This phenomenon was termed as „ischemic preconditioning". The fact, that the heart can be protected against severe, often fatal injuries by similar, but sub-lethal ischemic stress has raised considerable interest both in experimental and also in clinical cardiovascular research. The short periods of ischemia are now commonly applied to protect the heart from ischemic injury during human coronary by-bass surgery[2]. There is also evidence that not only the short ischemic episodes but other stimuli, such as rapid cardiac pacing[3] and vigorous physical exercise[2] can also provide protection. However, the exact mechanisms by which the exercise-induced protection functions are still not fully elucidated.

Oxidative phosphorylation and mitochondrial respiration are porogressively uncoupled during hypoxia or ischemia/ reperfusion[4]. This uncoupling increases the production of free radicals: reactive oxygen and nitrogen species. These reactive oxygen and nitrogen species (ROS; NOS) play an essential role in the generation of the reperfusion arrhythmias. It seems that free radicals not only damage the myocardium cells but also has a role in the PC processes. The ROS and the nitric oxide (NO) were identified as non receptorial triggers in the protection against the ischemia/reperfusion injury in the PC process[5].

Numerous studies showed that ROS have a protective role in the delayed cardioprotection. The ROS activate the redox-sensitive molecules such as endogenous antioxidant enzymes like manganese-superoxide dismutase (MnSOD)[6] or thioredoxin[7]. In the PC heart it seems, that the redox-signal induced protein synthesis can contribute to cell survival in the myocites[7]. However, the role of ROS regarding the early protection against arrhythmias still remains unclear.

NO has a large vasodilatation effect with an extreme short half life. It is released from the endothelium by the effect of several endogenous molecules or physical stimuli. Three different

isoforms of NO synthase (NOS) exist in the human body. Two out of three of these are present in the heart: endothelial NOS (eNOS) and inducible form of NOS (iNOS). NOS oxidize the guanidine group of L-arginine by consuming NADPH and molecular oxygen to produce NO.

Numerous articles suggested that NO has a role in the late phase of PC protection against myocardial infarction or stunning. In rabbits and rodents exogenous NO is also sufficient to provoke the protective effect against stunning and infarction [8]. Their protective role against arrhythmias in late phase of protection still remains unclear. Bolli et al. have suggested the function of NO in the delayed PC: 'NO mediated NO release'[9]. However there is no evidence whether the NO has a trigger and or mediator role in the exercise induced late PC protection against life-threatening arrhythmias[10].

Babai et al. (2004) showed that a single period of treadmill exercise in conscious dogs suppressed the life-threatening ventricular arrhythmias that happened 24h after the treadmill exercise, when a major coronary artery was occluded, and then re-opened[11]. This protection was abolished by the prior administration of aminoguanidine. The aminoguanidine is not a selective inhibitor of NOS enzymes. It powerfully inhibits a number of other enzymes such as histaminase, mitogene-activated proteine kinase, catalase, SOD and malondialdhyde[12 - 15].

The main purpose of the studies, described in the present thesis, was to explore the mechanisms involved in the early and the delayed antiarrhythmic effects of preconditioning induced by brief coronary artery occlusions and by physical exercise, with particular attention of the role of nitric oxide and reactive oxygen species (ROS).

2 Purpose of the research

There were two main objectives of this research.

1. **To examine the role of reactive oxygen species in the early antiarrhythmic effect of ischemic preconditioning (1/a)**. In this case a highly diffusible and low molecular weight ROS scavenger N-2-mercaptopropionylglycine (MPG) [6, 16 - 17] was used. In this experiment PC was induced by two 5 min coronary artery occlusion 20 min prior to the 25 min occlusion of the LAD.

   In this topic the following questions were further investigated:

   - In non PC dogs the arrhythmic effect of ROS was examined during myocardial ischemia **(1/b)**. The possibilities to reduce the arrhythmias with an exogenous ROS scavenger were researched.
   - The demonstration of ROS formation in the ischemia induced preconditioning process, the initial and terminal time of the prolonged 25 min occlusion (test) period were planned **(1/c)**. The lucigenin-induced chemiluminescence method was used to investigate the ROS formation in vivo.
   - The MPG scavenging activity on phorbol 12-myristate 13-acetate (PMA) induced ROS generation was examined using the ferro-cytochrome-c reduction in vitro assay **(1/d)**. This was implied by the fact that in vitro activation of canine peripheral mononuclear cells by PMA generates ROS formation.

2. **To obtain further evidence that nitric oxide is involved in the delayed phase of preconditioning induced by heavy physical exercise (2/a).** In these experiments the role of NO was examined in the delayed antiarrhythmic effect of exercise, using

   - a non-selective NOS inhibitor ω-nitro-L-arginine-methyl-ester (L-NAME) **(2/b)** and
   - a relatively selective iNOS inhibitor S-(2-aminoethyl)-methyl-isothiourea (AEST) **(2/c)**.

   L-NAME was given prior to physical induction. AEST was administered immediately before the 25 minute test occlusion period.

3 The applied methods of the research

3.1 Surgical preparation of dogs

Under light anaesthesia (Sigma, 20 mg kg-1 intravenous sodium pentobarbitone) the right femoral artery of the dogs (for the measurement of systolic, diastolic blood pressure - SABP, DABP, and heart rate - HR) was catheterised. The right femoral vein was also catheterized through which a mixture of chloralose (60 mg kg-1) and urethane (200 mg kg-1) was administered to maintain anaesthesia. The left common carotid artery was prepared and a catheter was inserted into the left ventricle cavity to measure the left ventricle systolic (LVSP) and end-diastolic (LVEDP) pressures. A thoracotomy was performed at the left fifth intercostal space and the pericardium was excised. The main anterior descending branch of the left coronary artery (LAD) was prepared for occlusion just proximal to the first main diagonal branch. A side branch of this artery was also catheterised for the local, intracoronary administration of saline or of MPG. The circumflex branch of the left coronary artery (LCX) was prepared and an electromagnetic flow probe (diameter 4 mm) was positioned on the coronary artery. Myocardial blood flow (MBF) was measured in $ml \cdot min^{-1}$ using a blood flow-meter (Spectramed, Hugo Sacs Electronics, Germany). A composite electrode was sutured on the surface of the potentially ischemic area. This was used to assess changes in the degree of inhomogeneity of electrical activation.

3.2 Preconditioning procedures

In regarding to the early protection the beneficial effect of PC was produced by two 5 min coronary artery occlusions with 20 min reperfusion interval (ischemic PC). The 25 min test occlusion was carried out after 20 min of the second PC occlusion.

In the second part of the study, the delayed PC effect was evoked by treadmill exercise. The slope and the speed of the treadmill were increased every 3 min reaching the maximum during the final 3 min period[11]. Heart rate (HR) was measured during the exercise by a chest lead electrocardiogram. Data were collected by a computer assisted system and analyzed with the Advanced CODAS Analyzing System using the Windaq Waveform Browser playback and analysing software (DATAQ Instruments, USA). The test occlusion was performed 24h after the exercise.

3.3 Investigation of haemodynamic parameters and ventricular arrhythmias

In the right femoral artery the systolic (SABP), and the diastolic blood pressure (DABP) and also the heart rate (HR) were measured. The mean arterial blood pressure (MABP) was

calculated from the SABP and DABP. Left ventricular systolic (LVSP) and end diastolic pressure (LVEDP) were measured from a catheter inserted into the left common carotid artery. The maximum and the minimum of the first derivate of LVP over time (LVdP/dt) were calculated from these parameters. The coronary blood flow was measured in the left circumflex coronary artery using an electromagnetic flow probe

Ventricular arrhythmias during a 25 min (LAD) coronary artery occlusion and following reperfusion were assessed as outlined in the 'Lambeth Conventions'[18]. During occlusion we assessed the total number of ventricular ectopic (premature) beats (VPB's) and the incidence and number of episodes of ventricular tachycardia (VT; defined as a run of four or more VPB's at a rate faster than the resting sinus rate). The incidence of ventricular fibrillation (VF) during both occlusion and reperfusion were also determined. Those dogs were considers survivors whose heart beat was in sinus rhythm for 10 minutes after the combined ischemia-reperfusion insult.

3.4 Determination of ROS production during preconditioning absence and presence of MPG

The free radical production was examined with the lucigenin-enhanced chemiluminescence (CL) assay[19]. At each time instant two ml blood samples were collected from the coronary sinus in EDTA containing micro-tubes. The samples were centrifuged for 5 min, at 200 g at room temperature. Plasma aliquots (100 μl) were added to 250 μM lucigenin in DPBS solution (final volume 1 ml). After a 2 minute equilibration period in darkness, measurements were commenced. CL was measured with a liquid scintillation counter (Packard Tri-Carb 2100 Model) with a single photomultiplier tube positioned in out-of-coincidence mode.

3.5 In vitro studies for the determination of superoxide anion generation by canine leucocytes

Superoxide anion generation was assayed by measuring the superoxide-dependent reduction of ferro-cytochrome-c, as described by Guarnieri and colleagues[20]. 2 mLs of blood were collected from the control dogs in tubes containing 100 mM EDTA and 100 mM dextran. PMNs were isolated from blood by Ficoll-Hypaque gradient method, then centrifuged at 200 g for 20 min at room temperature. After the centrifugation it was re-suspended in 40 μM ferricytochrome-c containing Dulbecco phosphate-buffered saline (DPBS) solution (1.5×10^6 cells/sample/cuvette). The cells were allowed to equilibrate for 2 minutes at 37 ^{0}C, and then were stimulated with 0.3 μM phorbol myristate acetate (PMA). Superoxide radical production was recorded graphically as the increase in absorbance at 550 nm.

4 Conclusion

4.1 The role of ROS in the early antiarrhythmic effect of preconditioning

In canine, as in other species, the severity of the ventricular arrhythmias is reduced when a prolonged period of occlusion is preceded by one or more brief (preconditioning) ischemia and reperfusion insults. This pronounced antiarrhythmic effect of ischemic preconditioning was clearly demonstrated in this study. None of the preconditioned dogs fibrillated during the occlusion compared with a high incidence (82%) in the control group. The number of VPBs and VT episodes were also much less in dogs that underwent a PC procedure. Moreover the epicardial ST segment elevation and the degree of inhomogeneity of electrical activation were less pronounced in the PC dogs than in the non-PC controls.

(1/a) It was demonstrated that the involvement of ROS is unlikely in this marked antiarrhythmic protection. This was proved by the use of MPG, a well known scavenger of ROS. MPG was applied in a dose (equivalent to a total intravenous dose of 90 mg kg−1) similar to that used in previous studies[6, 16 - 17]. It was given directly into a side branch of the coronary artery that was later occluded. The results show that such a locally administered MPG failed to modify the antiarrhythmic effects of preconditioning. The incidences of VF and VT, and the distribution of ventricular ectopic beats were similar in the preconditioned dogs irrespective of whether or not the scavenger had been given (Figures 2 and 3). It can be concluded that in this species under *in vivo* conditions ROS, and in particular superoxide, are not necessary for inducing early protection against life-threatening arrhythmias. The protection is present even when a highly diffusible and effective scavenger had been administered.

(1/b) MPG given to control dogs reduced the phase Ia arrhythmias (Figures 2 and 3), a phenomenon that deserves further investigation. Nevertheless, the total number of VPBs in this group was not significantly different from the saline administered controls. Furthermore, the reduction of the two measured parameters of ischemia severity by preconditioning was not significantly modified by MPG, at least, during the first 5 to 8 minutes of ischemia.

(1/c) In this study the superoxide production during the PC procedure was also determined both in the absence and in the presence of MPG. The results clearly show that after the first PC occlusion there was no detectable superoxide formation. This could mean two things: either a single 5 minute occlusion is not sufficient to produce superoxide, or the superoxide production induced by the short PC occlusion was effectively scavenged by superoxide dismutase.

(1/d) In an *in vitro* experiment the scavenging activity of MPG was demonstrated in PMCs using a ferricytochrome-c reduction assay. It was proven that the MPG can decrease the ROS

formation by a concentration dependent manner. The applied *in vitro* doses of MPG are comparable with an *in vivo* dose of MPG.

Tanaka[21] and Baines[22] have found, that in rabbits the administration of MPG, in doses lower than those used in the present study, prior to and during a single PC (5 min) occlusion, completely abolished the infarct size reducing effect of PC. However, Baines and colleagues[22] found that MPG failed to modify the preconditioning effect of four 5 minute occlusions. They proposed that other mediators (adenosine, bradykinin), that are released when multiple occlusions are used, may act together with ROS, thus inhibition of ROS formation only attenuates but not completely abolishes the protection. More ROS were generated by reperfusion after many occlusions compared to just one. There is increasing evidence that the intensity of the preconditioning stimulus determines the signalling pathways that leads to cardioprotection.

4.2 The role of NO in the delayed antiarrhythmic effect of preconditioning induced by physical exercise

The results of this part of the study confirm the previous observations of Babai and colleagues11 that vigorous treadmill exercise, which is sufficient to increase the heart rate over 200 beats min^{-1} can protect the heart against life-threatening arrhythmias.

(2/a and 2/b) The aim of the present research was to obtain further evidence that nitric oxide is involved in the delayed phase of preconditioning induced by heavy physical exercise. In this case two NOS inhibitors (L-NAME and the AEST) were used. A non-selective NOS inhibitor (L-NAME) was used before the exercise to examine the trigger activity of NO·in the process. A relatively selective iNOS inhibitor AEST was administrated before a prolonged 25 min occlusion period (24 h after the exercise trigger) to examine NO as a mediator in the antiarrhythmic mechanisms. The single period of exercise induced beneficial effect against arrhythmias (such as: VPBs and VT reduction, the rise of survival numbers, the reduced ST segment elevation) were abolished by both NOS inhibitors.

The results from the application of two different NOS inhibitors suggest that NO plays both a trigger and a mediator role in this protection. Since the marked antiarrhythmic effects of exercise, as well as the reduced ischemic and haemodynamic changes that were apparent after exercise, were significantly attenuated by these inhibitors. Further it was supposed that NO is most likely derived from the inducible form of NOS enzyme, since the iNOS inhibitor AEST was as effective in reducing the protection as was L-NAME. The experiment revealed that the administration of L-NAME, given prior to the exercise stimulus, abolishes the delayed

antiarrhythmic protection. It can be suggested that NO is generated as a result of exercise that acts as a trigger for the subsequent activation of iNOS (NO stimulated NO release).

5 Summary of New Scientific Results

The aim of this thesis was to investigate the non-receptor triggers and mediators in the early and late phase of PC protection against arrhythmias.

(1/a) The role of reactive oxygen species were examined in the early phase of protection induced by two five minute coronary occlusion, by the use of the ROS scavenger MPG. It was found that scavenging of ROS failed to modify the antiarrhythmic effect of PC, indicating that ROS does not play an essential role in the IPC induced early protection against arrhythmias. It can be proposed that this two five minute PC triggers the release of other mediators and stimulates simultaneous protecting pathways which predominate over ROS in the induction of the protection.

(1/b) The ROS formation at different times was investigated during the experiment. It was found that after the second PC stimuli, there is a burst of detectable ROS. In the presence of MPG this burst of ROS disappeared.

(1/c) The MPG treatment blocks the appearance of phase Ia arrhythmias during the 25 minute of test occlusion but not the phase Ib. These were observed from the MPG treated control group (without PC stimuli).

(1/d) With an *in vitro* experiment it was proved that ROS could be activated in dog PMNs by PMA. The activated ROS scavenged by MPG was shown using a chemiluminescence detection method.

The other part of the research was to obtain further evidence for the involvement of nitric oxide in the delayed phase of the preconditioning-induced protection against arrhythmias. For this purpose two inhibitors of NOS were used. The PC stimulus was induced by physical exercise, 24 h before a 25 min occlusion and reperfusion insult. A single 21 min period of exercise 24 h prior to ischemia resulted in a marked protection against ventricular arrhythmias.

(2/a) It was found that the beneficial, antiarrhythmic effect of treadmill exercise is abolished by the use of the eNOS inhibitor L-NAME before the 21 minutes of exercise.

(2/b) The applied iNOS inhibitor, AEST, can block the protecting effect of the exercise if it is applied immediately before the test occlusion.

This investigation with the applied two NOS inhibitors clearly demonstrates the role of NO as a trigger and a mediator in the exercise induced cardiac preconditioning against ventricular ectopic beats.

6 Related articles and abstracts

6.1 Full papers

Hajnal Á, Nagy O, Litvai A, Papp JGy, Parratt JR, Végh Á
Nitric oxide involvement in the delayed antiarrhythmic effect of treadmill exercise in dogs
LIFE SCIENCES 77:(16) pp. 1960-1971. (2005)
IF: 2.512

Hajnal Á, Nagy L, Parratt JR, Papp JGy, Végh Á
N-2-mercaptopropionylglycine, a scavenger of reactive oxygen species, does not modify the early antiarrhythmic effect of ischaemic preconditioning in anaesthetised dogs
CARDIOVASCULAR DRUGS AND THERAPY 18:(6) pp. 449-459. (2004)
IF: 1.486

6.2 Abstracts in journals with impact factor

Hajnal Á, Csillik A, Litvai Á, Parratt J R, Végh Á
Protection against arrhythmias by exercise; role of nitric oxide: -p. A27.
JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY (2002) Volume 34; Issue 6, Page A27.
International Society for Heart Research (ISHR) European Section Meeting, 2002, Szeged

Hajnal A, Nagy L, Parratt JR, Vegh A
The role of free radicals in the early anti-arrhythmic effects of ischaemic preconditioning
JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY 36:(5) p. 51. (2004)
International Society for Heart Research (ISHR) European Section Meeting, 2004, Dresden

6.3 Other abstracts

Hajnal Á., Litvai Á., Dr. Papp Gy., J.R. Parratt, Dr. Végh Á.
A nitrogén-oxid szerepének vizsgálata a fizikai terheléssel kialakított prekondicionálás késői antiarrhythmiás hatásában.
A Magyar Kísérletes és Klinikai Farmakológiai Társaság V. Kongresszusa
Debrecen, 2002. december 12-14.

Hajnal Á., J.R. Parratt, Végh Á.
A reaktív oxigén gyökök (ROS) szerepének vizsgálata az ischaemiás prekondícionálás korai antiarrhythmiás hatásában altatott kutyában.
Magyar Kardiológusok Társasága 2003. évi Tudományos Kongresszusa,
Balatonfüred, 2003. május 14- 17.

Á. Hajnal, J.R. Parratt, Á. Végh
Possible role of ROS in the antiarrhythmic effects of ischaemic preconditioning in anaesthetised dogs.
International Society for Heart Research (ISHR) European Section Meeting, 2003. Strasbourg

Á. Hajnal, J.R. Parratt, Á. Végh
Possible role of ROS in the antiarrhythmic effects of ischaemic preconditioning in anaesthetised dogs.
Magyar Élettani Társaság Vándorgyűlése, 2003, Pécs

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