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The role of reactive oxygen species in the mechanisms of local and remote myocardial ischemic preconditioning

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Introduction

The phenomenon of ischemic myocardial preconditioning (IPC) for the first time described by C.E. Murry et al. in 1986, consists of robust increase of myocardial tolerance to ischemia/reperfusion injury after several brief bouts of occlusion/reperfusion of the coronary artery [5]. Now it is shown, that IPC is capable of reducing the infarct size formed as a result of the subsequent long-term ischemia, and also in reducing the extent of ischemic arrhythmias and to a degree postischemic contractile dysfunction [1]. In the recent years, some authors have revealed that myocardial tolerance to ischemia significantly increases not only after several bouts of myocardial ischemia/reperfusion, but also after ischemia in organs remote to the heart, e.g. kidney, small intestine, skeletal muscle [3, 6, 7]. This phenomenon was termed remote myocardial preconditioning (RPC) in contrast to the local mode described earlier [1].

I Mechanisms of local myocardial preconditioning (LPC) according to modern conceptions consist of three sequential phases: a trigger phase, a phase of intracellular signal transfer, and a phase of realization of protective effect [1, 18]. The trigger phase is characterized by myocardial accumulation of molecular triggers, e.g. adenosine, bradykinin, catecholamines, etc., that bind with specific sarcolemmal receptors and launch the intracellular signal transfer cascade. Intracellular signal transfer is performed by activation of G protein-coupled protein kinases. Finally, the realization of protective effects assumes implementation of an hypothetical cellular energy-sparing program with unknown molecular mechanisms.

Key words: ischemic myocardial preconditioning, reactive oxygen species, N-2-mercaptopropionylglycine.

Editor's Column

Welcome to the Kopf Carrier for 2007! The past year was a very good one for David Kopf Instruments as the company introduced several new products and continued to improve the already extremely high quality of its instruments. We appreciate the continued support that the Neuroscience Community has shown to Kopf Instruments during the trying times of the past two years and you can rest assured that the company will continue to support neuroscientists with the highest quality and diversity of stereotaxic and accessory instruments available anywhere.

In Florida, we came through the last hurricane season with essentially no storms of any consequence. We needed that breathing room this past season because of all the damage around the state from the past two active hurricane seasons. As I drive around the Ft. Lauderdale area, I still see blue tarps on roofs and the effects on the trees of several damaging storms. When one realizes that it had been 40 years since this area had been hit by a real hurricane, you can imagine the “pruning” that the storm (Wilma) here did to the area. It is equally amazing how the trees come back. Many were stripped of all leaves and most branches during the October 2005 storm. Now they are bushing and filling out, with the canopy looking perhaps even better than before. The big palms were an amazing story. When I first say the 20 huge Royal Palms around our community pool, I thought we would have to replace all of them. They had no fronds at all, only the bowl at the top. They are wonderfully evolved for hurricanes; as long as the central bowl does not snap off, they regrow. They are made to lose all their fronds and then present little wind resistance. It make one realize how truly amazing the power of evolution is.

The article in this Carrier number 64 is again from our Russian colleagues and is a very interesting one. They have done a series of studies on a very hot topic in neuroscience and medicine, reactive oxygen. I think you will enjoy the article and find it useful in many ways.

I hope that all of you had a very good New Year and are really looking forward to this year. As usual, we will hope to see you at the Society for Neuroscience meetings early in November and hope you plan to be there in San Diego. It will be a great meeting.

If there is anything we can help you with, please email or phone either me or the Kopf Company.

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Reperfusion of ischemic myocardium is accompanied by the formation of high concentrations of reactive oxygen species (ROS) [13]. Formation of injurious concentrations of ROS has pathogenetic consequences and may lead to myocardial reperfusion injury, e. g. after prolonged ischemia [13]. At the same time, many authors consider a moderate ROS generation after a brief ischemia during IPC as one of the major mechanisms of this phenomenon [18]. There are some papers about triggering and mediating mechanisms of LPC, however these data remain quite contradictory [8, 12, 16, 17]. On the other hand, participation of ROS in realization of cardioprotective effects of RPC has practically not been investigated. Thus, aim of this study was to examine ROS role in the mechanisms of LPC and RPC experimentally.

Materials and methods

All experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (publication No. [NIH] 85-23) and were approved by the institutional ethical committee of the Pavlov State Medical University of St. Petersburg.

Experiments were performed on 47 male Wistar rats (250-350 g body wt) anesthetized by pentobarbital sodium (60 mg/kg ip with subsequent maintaining iv infusion) with mechanical ventilation through the tracheal incision (respiration rate of ~50 breaths/min, tidal volume of 3.0 ml/100 g body wt). Blood pressure (BP) was measured by the pressure transducer (Baxter, USA) through a catheter inserted into aorta through the common carotid artery, and monitored by IBM-PC computer by software PhysExp 2.0 (developed by St. Petersburg State Polytechnic University) during the experiment. Also we monitored electrocardiogram in standard leads and heart rate (HR). Access to the heart was performed by a left thoracotomy in the fourth intercostal space. We bluntly opened the pericardium and defined localization of the common trunk of the left coronary artery (LCA) under which we passed a thin polypropylene ligature by atraumatic needle (6-0). Then we produced an occluder for creation of reversible myocardial ischemia. RPC was simulated by formation of single bout of small intestinal ischemia for 20 min with subsequent reperfusion for 15 min. For this purpose, we performed a median laparotomy, and isolated the superior mesenteric artery (SMA) under which we passed a ligature using an operative microscope. Then we performed a reversible occlusion of SMA by clipping with small-type Dieffenbach clamp. For the investigation of ROS role we used pharmacological inhibition of its formation by synthetic antioxidant N-(2-mercapto-propionyl)-glycine (NMPG, Sigma, USA). NMPG in solution was infused intravenously in dosage of 1 ml (pH=7.38) through a catheter inserted into the left femoral vein by syringe pump (Razel, USA).

Experimental protocol:

The rats were randomized to one of six groups at the beginning of the study:

1). Control (nonpreconditioned) group (10 animals). The LCA was occluded for 30 min followed by 90 min of reperfusion without preconditioning.
2). Control + NMPG group (6 animals). A dosage of NMPG (90 mg/kg per 60 min, iv) was infused before the LCA occlusion.
3). LPC group (8 animals). The LPC protocol included four 3 min bouts of occlusion each followed by 6 min periods of reperfusion and then a 30 min of LCA occlusion.
4). LPC + NMPG group (8 animals). An infusion of NMPG (90 mg/kg per 25 min, iv) was started 25 min before the first bout of preconditioning and terminated before 30 min of LCA occlusion.
5). RPC group (7 animals) A 20 min SMA occlusion with the subsequent 15 min reperfusion followed by a 30 min LCA occlusion.
6). RPC + NMPG group (8 animals). Simulation of RPC was accompanied by infusion of NMPG (90 mg/kg, iv) started 25 min before the SMA occlusion and terminated before30 min LCA occlusion. Measurements of heart rate (HR) and mean blood pressure (BP) were made in all groups 60 and 30 min before LCA occlusion, immediately after occlusion, 15 and 30 min after occlusion, and at the end of experiment, i.e. after 90 min of LCA occlusion.

Infarct size assessment. Assessment of myocardial infarct size was done by double macroscopic staining with Evans blue solution and 2,3,5-triphenyltetrazolium chloride (TTC) (MP Biomed., USA). After termination of 90 min reperfusion, the LCA was reoccluded by ligature followed by intravenous administration of 0.5 ml of 5% Evans blue solution. After identification of border between non-ischemic and ischemic areas of the heart, it was quickly excised and cut into four slices (2.5 mm thick) parallel to the atrioventricular groove. The images of the basal surface of each slice were photographed using digital camera Camedia Olympus 2020 for the following identification of the size of anatomic area at risk (Evans-negative sites) and the size of non-ischemic myocardium (Evans-positive sites). Computer processing of images was performed by software VideoTest 4.0 (ISTA Ltd., St.-Petersburg, Russia). Total size of the area at risk for the given heart has been calculated by summation of multiplications of the area of Evans-negative site of each slice on its thickness by all four slices.

The heart slices were then incubated in 1% TTC solution (37°C, 15 min). After incubation in TTC, images were again fixed and processed by the above described way, after which we calculated the total size of the infarct zone. Data were represented in the form of the relation of the volume of risk zone to the total heart volume, and also in the form of the relation of the volume of infarct zone to the volume of zone at risk.*Statistics.* Statistical significance of the hemodynamic data at each time point, and also the sizes of the anatomic zone at risk and the infarct zone was estimated by software SPSS (ANOVA analysis, Scheffe test). All functional data were represented in the form of means ± SD. P = 0.05 was accepted as significant.

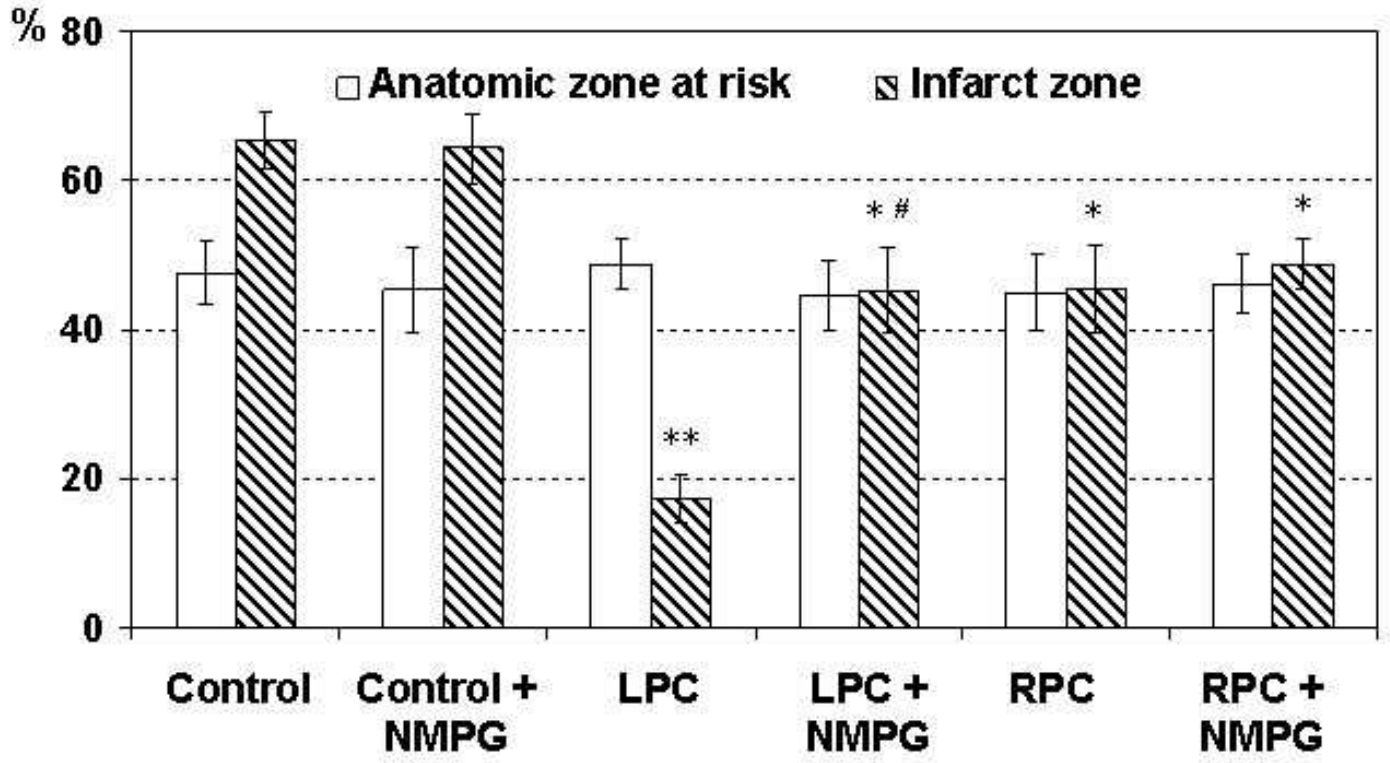
Results and discussion

Hemodynamic data (mean values of BP and HR) did not change significantly during the experiments in every group and did not differ in the experiments by various groups (Table 1). It is important to note that LCA occlusion both during LPC simulation and during long-term ischemia induced temporary dropping of BP on the average of 15-20% from the initial values, however this decrease was transitional and restoration of BP already occurred within 40-60 sec after coronary occlusion. Moreover, a transitional hypotension (BP decrease of 15-20% from initial values within 2-3 min) occurred together with an initial reperfusion phase of the RPC simulation in small intestine.

Table 1. Hemodynamic data in the experimental groups. Data were represented in the form of "mean ± standard deviation". BP – blood pressure; HR – heart rate; NMPG – N-(2-mercapto-propionyl)-glycine; LPC – local myocardial preconditioning; RPC – remote myocardial preconditioning.

Hemodynamic data	Group N 1 (control, n=10)	Group N 2 (control+NMPG, n=6)	Group N 3 (LPC, n=8)	Group N 4 (LPC+NMPG, n=8)	Group N 5 (RPC, n=7)	Group N 6 (RPC+NMPG, n=8)
BP, mm Hg						
60 min. before ischemia	117 ± 12	109 ± 10	111 ± 6	110 ± 9	111 ± 13	122 ± 5
30 min. before ischemia	114 ± 8	111 ± 8	115 ± 11	120 ± 12	115 ± 10	118 ± 9
Immediately before ischemia	116 ± 15	115 ± 10	118 ± 9	122 ± 7	110 ± 9	119 ± 10
15 min. after ischemia	110 ± 7	102 ± 14	100 ± 8	105 ± 10	106 ± 12	109 ± 7
30 min. after ischemia	105 ± 9	106 ± 13	105 ± 10	100 ± 14	101 ± 11	110 ± 15
90 min. after reperfusion	104 ± 10	99 ± 12	107 ± 13	102 ± 8	105 ± 12	103 ± 7
HR, beats/min						
60 min. before ischemia	392 ± 19	384 ± 30	385 ± 37	361 ± 28	398 ± 22	383 ± 31
30 min. before ischemia	398 ± 27	401 ± 35	398 ± 32	372 ± 25	392 ± 26	384 ± 28
Immediately before ischemia	371 ± 25	394 ± 21	381 ± 29	369 ± 21	380 ± 18	376 ± 20
15 min. after ischemia	380 ± 21	399 ± 32	375 ± 29	369 ± 19	382 ± 23	378 ± 22
30 min. after ischemia	388 ± 24	391 ± 27	384 ± 20	378 ± 23	376 ± 22	370 ± 27
90 min. after reperfusion	395 ± 18	382 ± 20	376 ± 19	370 ± 18	369 ± 16	372 ± 22

Figure 1. The anatomical area at risk and infarct size. * – p<0.05 vs. controls; ** – p<0.01 vs. controls; # – p<0.01 vs. LPC; NMPG – N-(2-mercapto-propionyl)-glycine; LPC – local myocardial preconditioning; RPC – remote myocardial preconditioning.



The size of the anatomic zone of risk, i. e. the volume of a myocardium subjected to ischemia after LCA occlusion, did not differ in experiments by various groups (Figure 1). At the same time, the size of the infarct zone, i. e. volume of a myocardium within an anatomic zone at risk, which was subjected to a necrosis during an ischemia, considerably varied depending on conditions of experiment (Figure 1). For example, the size of the infarct was 65.4±3.8 % from the size of the anatomic zone at risk at the group 1. Infusion of NMPG did not lead to significant changes in the infarct size (64.3±4.8 % in group 2, $p > 0.05$). LPC led to significant limitation of the infarct size, which was decreased more than 3.5 times and averaged 17.3±3.6 % ($p < 0.01$ in comparison with the control group). Infusion of NMPG led to partial inhibition of infarct-limiting LPC effects: the infarct size in group 4 was 45.2±5.8 %, $p < 0.01$ in comparison with LPC (group 3). RPC (group 5) promoted a reduction of the infarct size on the average of 20 % in comparison with the control group (45.4±5.9 and 65.4±3.8 %, respectively, $p < 0.05$). However, the infarct-limiting RPC effect was not inhibited by infusion of NMPG, and the infarct size at group 6 was 48.7±3.3 %, i. e. statistically did not differ from those in group 5 ($p > 0.05$). Thus, these results suggest that intravenous infusion of NMPG led to inhibition of the infarct-limiting LPC effects, but did not affect the RPC effects.

NMPG is a low-molecular synthetic glutathione analogue that contains thiol groups in its structure [10]. In experiments in vitro and in vivo it was shown that NMPG is potent antioxidant scavenger which inactivates mainly hydroxyl radical (OH·) and a peroxynitrite-radical (ONOO·). Low molecular weight and special characteristics of chemical structure provide easy diffusion of NMPG through biological membranes and, hence, its penetration inside of cells [10]. These properties provide benefits for NMPG above traditionally used extracellular enzymatic antioxidants, such as superoxide dismutase and catalase. Inhibition of infarct-limiting LPC effects after NMPG infusion in our experiments allows us to confirm an existing hypothesis about participation of ROS in realization of LPC. It is necessary to note, however, that inhibition of LPC effects after infusion of NMPG was not shown in all studies. In

particular, the negative results have been obtained by some authors on LPC simulation by performing several bouts of ischemia/reperfusion and infusing NMPG in a dosage of 20 mg/kg [8, 16]. At the same time, in the above-mentioned papers it is reported that this dosage of NMPG has led to inhibition of infarct-limiting LPC effects induced by a single bout of ischemia/reperfusion. These data imply that ROS are important for initiation of cardioprotective response only within the first

or in the mechanisms of intracellular signal transfer (Figure 2). It is suggested that ROS formed within short bouts of ischemia/reperfusion can lead to posphorylation of intracellular protein kinases, e.g. protein kinase C, tyrosine kinases and mitogen-activated protein kinases (MAPK) [2]. Activated kinases, in turn, can lead to opening of mitochondrial ATP-sensitive potassium (KATP) channels which have been considered for a long time as final LPC effectors 18]. Now, however, it

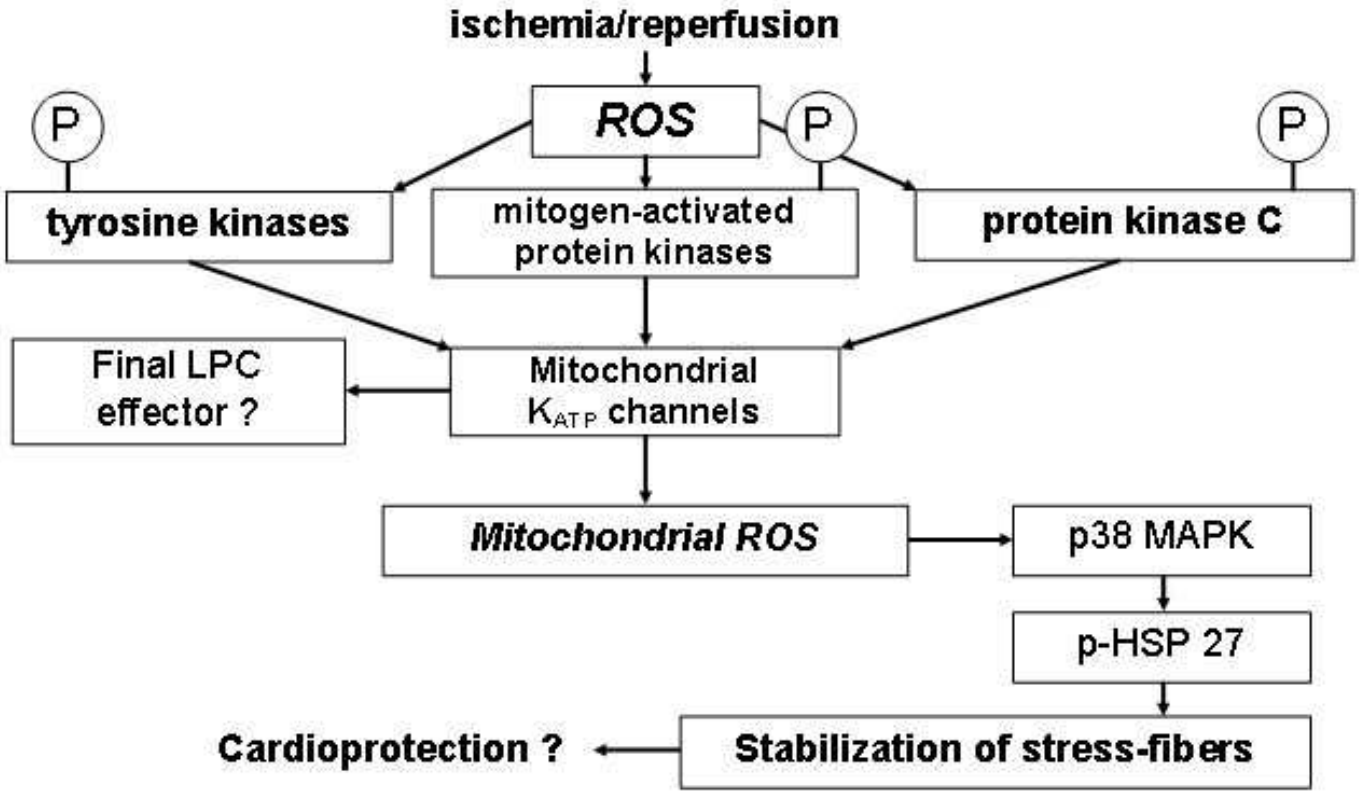


Figure 2. The hypothetical mechanisms of local myocardial ischemic preconditioning.
LPC – local myocardial preconditioning; ROS - reactive oxygen species;
MAPK – mitogen-activated protein kinase; HSP – heat shock protein; P – phosphate residues.

bout of LPC whereas at the subsequent bouts other triggers get higher importance [8, 12, 16]. However, Yamashita et al. with use of LPC protocol similar to ours (3 min ischemia and 10 min reperfusion) showed that NMPG in a dosage of 90 mg/kg/hr have led to more complete inhibition of the infarct-limiting LPC effects [17]. It seems that the simple increase of NMPG dosage from 20 up to 90 mg/kg leads to more complete inhibition of ROS formation and to exhibition of effects of the drug at recurrent bouts of LPC. ROS can play a key role either in the initiation of the cardioprotective LPC response,

is suggested [that the opening of KATP channels during a long-term ischemia may lead to formation of additional ROS due to their "leakage" from electron transport chain which mediates the cardioprotective response [18]. The particular mechanism of protection still remains unknown, but last data indicate that mitochondrial ROS may lead to activation of p38 MAPK, phosphorylation of a heat shock protein 27, and stabilization of stress fibers, i.e. the components of cardiomyocyte cytoskeleton that provides increased

resistance to an intracellular osmotic edema developing during an ischemia [19]. Thus, role of ROS in LPC mechanisms is double: on the one hand, ROS act as LPC triggers; on the other hand, they participate in the final phases of an intracellular transfer of cardioprotective signal.

It has already been shown that the mechanisms presumably underlying RPC may be different according to an organ in which the ischemia/reperfusion (Figure 3) is simulated. Thus, activation of visceral afferents accumulating during ischemia/reperfusion by

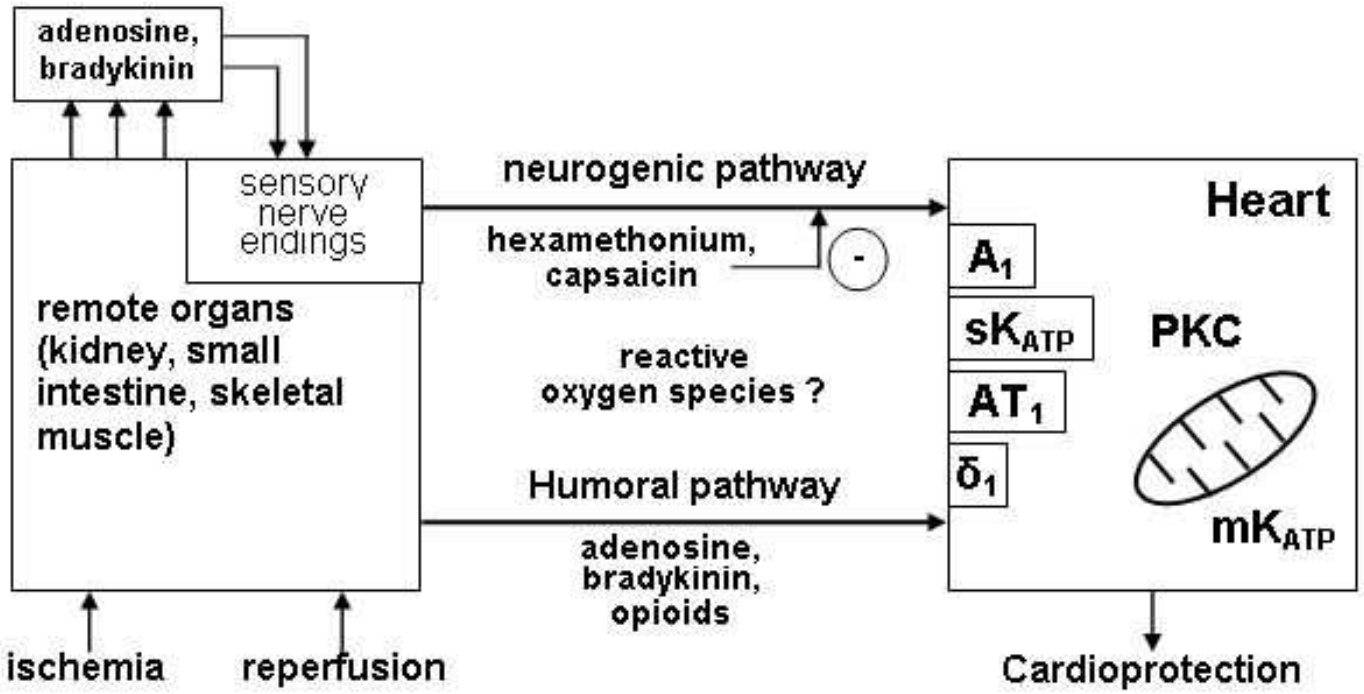


Figure 3. Proposed mechanisms of remote myocardial ischemic preconditioning.
PKC – protein kinase C, A1 – adenosine (A1) receptors, AT1 – angiotensine (AT1) receptors, δ_1 – opioid δ_1-receptors; sK_{ATP} – sarcolemmal ATP-sensitive potassium channels; mK_{ATP} - mitochondrial ATP-sensitive potassium channels.

Currently, there is only one paper about the investigation of the role of ROS in RPC mechanisms [16]. In this paper concerning experiments on rats, ischemia/reperfusion of a hindquarter induced by occlusion/reperfusion of an abdominal aorta below an origin of renal arteries was used as stimulus of RPC. Authors observed an inhibition of infarct-limiting RPC effects after infusion of NMPG in a dosage of 20 mg/kg and suggested that ROS contribute to realization of RPC. In our investigation an intravenous infusion of NMPG in a dosage of 90 mg/kg did not lead to inhibition of RPC effects induced by an ischemia/reperfusion of a small intestine. Such varied results can be explained by the differences in the experimental protocol, in the used dosage of a drug, and, the most important, in the technique of RPC simulation.

adenosine and bradykinin has a great importance in the organs with a high-level sensory innervation. According to B. C. Gho et al., this can lead to activation of the viscero-visceral reflex having as an efferent part the sympathetic nerves of heart [3]. Activation of these nerves can lead to stimulation of cardiomyocyte alpha1-adrenoceptors by endogenous catecholamines and to subsequent realization of the cardioprotective response. So, effects of RPC caused by ischemia in an intestine can be inhibited by administration of ganglion blockers (hexamethonium) [3] and capsaicin [11]. At the same time, the protective effects of RPC caused by occlusion/reperfusion of an abdominal aorta are not inhibited by ganglion blockers [15].

Taken together, these results indicate that RPC mechanisms may have the essential characteristics related to the specificity of an organ in which the short-term ischemia/reperfusion is performed. In particular, there are reasons to consider that the protective RPC effects are "transferred" to heart from such organs, as a kidney or a skeletal muscle, mainly by humoral pathway. Some data indicate that adenosine, bradykinin and opioid peptides act as humoral factors released in a systemic blood flow during reperfusion of these organs [15, 16]. However, this does not completely exclude a participation of the above described neurogenic mechanism. RPC effects induced by an ischemia/reperfusion of a kidney and a hindquarter are inhibited by intravenous infusion of antagonists of adenosine (A1) [4], opioid (delta1) [16] and angiotensin (AT1) receptors [9]. Besides, there are some published data indicating that protective RPC effects can be inhibited by mitochondrial and sarcolemmal KATP channel blockers and protein kinase C inhibitors [14]. These data show that intracellular signaling mechanisms of RPC are at least partly similar to mechanisms of LPC.

Thus, our results confirm the role of ROS in realization of cardioprotective effects of local myocardial preconditioning, but, on the other hand, it does not seem likely that ROS are involved in the mechanisms of remote preconditioning induced by an ischemia/reperfusion of a small intestine.

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Summary

The phenomenon of ischemic myocardial preconditioning describes increased myocardial tolerance to ischemia which occurs after brief episodes of myocardial ischemia-reperfusion (local preconditioning) or ischemia in the remote to the heart organ (remote preconditioning). This study focuses on the investigation of the role of reactive oxygen species (ROS) in the mechanisms of local (LPC) and remote preconditioning (RPC). For this purpose, pharmacological inhibition of ROS with synthetic antioxidant N-2-mercaptopropionylglycine was used. In the in vivo rat experiments administration of N-2-mercaptopropionylglycine resulted in partial abolishment of the infarct-limiting effect of LPC. In contrast, the infarct size reduction secondary to RPC elicited as a 20 minute intestinal ischemia with subsequent 15 minute reperfusion was unchanged. Therefore, ROS involvement in the mechanisms of RPC seems to be unlikely as opposed to their role in LPC. Possible molecular mechanisms of RPC are discussed.