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The Role of the Opioid System of the Myocardium in Mediating the Cardioprotective Effect of Postconditioning

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The involvement of endogenous myocardial opioid receptor agonists in mediating the cardioprotective effect of postconditioning was studied in a model of isolated Langendorff-perfused rat hearts. This phenomenon was found to occur using three sessions of reperfusion (30 sec) and ischemia (30 sec) at the end of a 45-min period of total ischemia. Studies using selective blockers of different opioid receptor subtypes, added to the perfusion solution at the beginning of the reperfusion period, showed that the resistance of the heart to the actions of ischemia-reperfusion in ischemic postconditioning are mediated by activation of δ_1 opioid receptors. It is suggested that the mechanism of the cardioprotective phenomenon of postconditioning involves a significant role for endogenous opioids synthesized in the myocardium.

Keywords: heart, ischemia, reperfusion, opioid receptors.

Studies of the molecular mechanisms of adaptation with the aim of developing new approaches to increasing the resistance of the heart to ischemia and reperfusional injury constitute an important problem in current physiology and pathophysiology. Recent years have seen progress in this direction. In particular, quite detailed studies of the phenomenon of "ischemic preconditioning" have been reported, the essence of this phenomenon being an increase in the adaptive resistance of the heart to the actions of prolonged ischemia after several prophylactic sessions of transient coronary occlusion and reperfusion [10]. Studies in 2003 showed that modeling of brief episodes of ischemia-reperfusion during the postischemic period after prolonged cardiac ischemia ends also has analogous cardioprotective actions. This phenomenon was termed "ischemic postconditioning" [19, 23]. The adaptive phenomenon of ischemic postconditioning occurs not only in vivo, but also in vitro, on both isolated hearts and in cultures of cardiomyocytes. It can be suggested that the mechanism of formation of the cardioprotective effect involves processes occurring in myocardial cells themselves. Later studies showed that some extracellular factors developed by the body play an important role in ischemic postconditioning. These include the actions of substances acting via receptor-mediated mechanisms, such as adenosine, bradykinin, opioid peptides, and urocortin [9, 14, 22]. However, the receptor mechanism of postconditioning thus far remain unknown. Analyzing published data, we note the not unimportant role of the opioid system in forming another adaptive phenomenon – myocardial preconditioning. In this case, the effects of opioids are mediated by δ and ϰ opioid receptors [1, 11]. Thus, the aim of the present work was to study the role of endogenous δ and ϰ opioid receptor agonists in the mechanism of the adaptive phenomenon of ischemic postconditioning of isolated perfused hearts.

Methods

Experiments were performed using a model of Langendorff-perfused rat hearts. The skin of the chest wall was removed from rats anesthetized with ether and the chest was opened with scissors, making three cuts: one transverse across the chest and two longitudinal. Spontaneous contractions of harvested hearts were terminated by transferring them to Krebs–Henseleit solution cooled to +4°C, after which they were placed in a moist incubator and a cannula

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was introduced into the ascending arch of the aorta for delivery of isotonic saline. Studies used isotonic Krebs–Henseleit solution containing 120 mM NaCl, 4.8 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM $NaHCO_3$, and 10 mM glucose. Perfusion solution was saturated with a gas mix containing 95% O_2 and 5% CO_2. Solution temperature was 37 ± 0.5°C, perfusion pressure was 52 mmHg, and pH was 7.5. Krebs–Henseleit solution was prepared using reagents from Sigma-Aldrich (St. Louis, USA) and MP Biomedicals (Irvine, USA). Krebs–Henseleit solution and all reagents were prepared using deionized water purified in a Millipore Simplicity apparatus (France).

The experiment was performed in two parts. The first part consisted of a series of experiments assessing the contribution of endogenous ligands to the formation of myocardial resistance to the actions of ischemia and reperfusion. After a 30-min stabilization period, total myocardial ischemia was modeled by complete termination of perfusion solution delivery for 45 min, after which perfusion was restarted and observations were continued for a further 30 min. During the first 10 min of reperfusion, hearts were perfused with Krebs–Henseleit solution containing one or another opioid receptor blocker.

The second part of the study consisted of a series of experiments performed using the same scheme, though the phenomenon of ischemic postconditioning was reproduced at the beginning of reperfusion using three sessions of ischemia (30 sec) and reperfusion (30 sec). This experimental algorithm was used because the results of our previous experiments showed that this protocol for reproducing phenomenon of ischemic postconditioning yielded the greatest level of cardioprotection [3]. Immediately after three adaptive cycles of ischemic postconditioning, hearts were perfused with Krebs–Henseleit solution containing opioid receptor antagonists during the first 10 min of reperfusion.

The following opioid receptor blockers were used:

naloxone, a nonselective antagonist of all types of opioid receptor (synthesized by Multiple Peptide Systems, San Diego, USA) at a final concentration of 300 nM [2];

naltrindole, a mixed antagonist of δ_1 and δ_2 receptors, at a final concentration of 30 nM [18];

7-benzylidenenaltrexone maleate (BNTX), a selective antagonist of δ_1 opioid receptors, at a final concentration of 1 nM [16] (synthesized by Tocris Bioscience, Bristol, UK);

naltriben, $C_{26}H_{25}NO_4.CH_4O_3S.H_2O$ (Naltriben), a selective antagonist of δ_2 opioid receptors, the a final concentration of 1 nM [16] (synthesized by Tocris Bioscience, Bristol, UK);

norbinaltorphimine hydrochloride, an antagonist of $\varkappa$ opioid receptors, at a final concentration of 3 nM [13] (synthesized by Sigma Aldrich, St. Louis, USA).

Cardioprotective effects were evaluated in terms of the level of creatine phosphokinase (CPK) activity in the perfusate flowing out of the heart. CPK activity in outflowing perfusate was assayed using a commercial Bioceon CK-nac enzyme kit (Marienhagen, Germany). This method was based on an increase in absorption at 340 nm, which was strictly proportional to CPK activity in the sample in the cuvette containing incubation mix. CPK activity was expressed in μmol NADH/min/g heart tissue at 30 min of reperfusion (U/g).

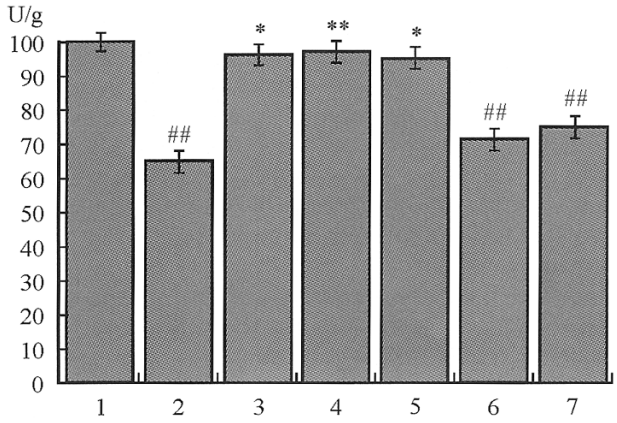

Fig. 1. Creatine phosphokinase activity (U/g) in perfusion solution after ischemic postconditioning and blockade of opioid receptors. Significant differences compared with controls: $^{\#\#}p < 0.05$; significant differences relative to postconditioning (IP): $^{*}p < 0.05$; $^{**}p < 0.01$. *1*) Controls; *2*) ischemic postconditioning (IP); *3*) IP + naloxone; *4*) IP + naltrindole; *5*) IP + BNTX; *6*) IP + naltriben; *7*) IP + norbinaltorphimine.

Data were analyzed in Statistica 6.0. Intergroup differences were identified using the nonparametric Mann–Whitney U test. Differences were regarded as statistically significant at $p < 0.05$.

Results

The initial task of these experiments was to identify the roles of endogenous opioid receptor agonists in mediating the phenomenon of ischemic postconditioning, which makes the heart resistant to injury initiated by ischemia and reperfusion. The cardioprotective effects of postconditioning were detected using three 60-sec cycles of reperfusion and ischemia. In this situation, the perfusate CPK level was 32% lower than that in controls (Fig. 1).

Use of selective antagonists of different opioid receptor subtypes showed the following. Figure 1 shows that on the background of prior blockade of opioid receptors with the nonselective antagonist naloxone at a final concentration of 300 nM, the cardioprotective effect of ischemic postconditioning was not seen. Creatine kinase activity in perfusate was identical in this situation to that in the control group. This indicates that opioid receptors are involved in mediating the cardioprotective effect of postconditioning.

Use of the mixed δ_1 and δ_2 receptor antagonist naltrindole at a final concentration of 30 nM also prevented the protective effect of postconditioning (Fig. 1). These data indicate that δ opioid receptors are involved in forming cardioprotection. There are two subtypes of δ opioid receptors: δ_1 and δ_2, and further experiments allowed assessment of

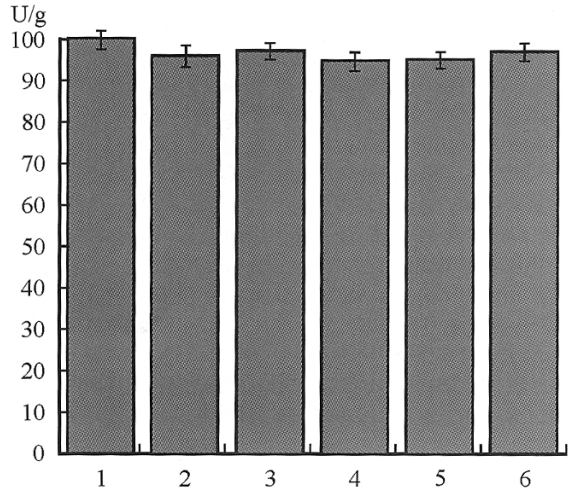

Fig. 2. Creatine phosphokinase activity (U/g) in perfusion solution in experiments on hearts subjected to ischemia-reperfusion on the background of blockade of different opioid receptor subtypes. *1*) Controls; *2*) naloxone; *3*) naltrindole; *4*) BNTX; *5*) naltriben; *6*) norbinaltorphimine.

the roles of each of these in mediating the protective postconditioning phenomenon.

In conditions of blockade of δ_1 receptors with the selective antagonist BNTX at a final concentration of 1 nM, hearts did not show any tolerance to the pathogenic action of reperfusion after postconditioning. At the same time, use of the selective δ_2 receptor antagonist naltriben at a final concentration of 1 nM had no effect on the cardioprotective effect (Fig. 1). These results provide evidence that δ_1 receptors play an important role in mediating the cardioprotective effect.

The role of endogenous κ agonists in mediating this phenomenon was assessed by selective blockade of this receptor subtype by addition of norbinaltorphimine to the perfusion solution, to a final concentration of 3 nM. The results of these experiments did not support the involvement of endogenous κ agonists in the protective effect of postconditioning, as blockade of these receptors had no effect on cardioprotection (Fig. 1). Thus, κ opioid receptors are not involved in the mechanism of the cardioprotective effect of ischemic postconditioning.

Thus, these experiments demonstrated that blockade of δ_1 receptors eliminated the cardioprotective effect of postconditioning, while blockade of myocardial δ_2 and κ receptors had no effect on the creatine phosphokinase level in the outflowing perfusate throughout the reperfusion period after postconditioning. Thus, activation of δ_1 opioid receptors by endogenous antagonists has a defining role in the mechanism of the adaptive increase in the resistance of the heart to the harmful action of reperfusion.

The task of the next series of experiments was to assess the role of endogenous opioid receptor agonists synthesized in the myocardium to forming resistance to ischemic and reperfusional injury in "nonadapted" cardiomyocytes. Experiments were performed using an analogous scheme with the same blockers but without prior reproduction of sessions of ischemic postconditioning.

Inhibition of cardiac opioid receptors in these conditions had virtually no effect on the level of cardiac injury in ischemia-reperfusion (Fig. 2).

Discussion

As noted above, the cardioprotective effect of ischemic postconditioning has been reproduced both in in vivo experiments and in vitro. Thus, formation of this protective phenomenon may be due to processes occurring in myocardial cells themselves rather than humoral factors or neural regulation. Published data have provided grounds for suggesting that endogenous opioids and opioid receptors play an important role in forming postconditioning [14]. The results obtained from our experiments showed that naloxone, which blocks all types of opioid receptor, completely eliminated the cytoprotective effect of the protective phenomenon studied here, which is entirely consistent with published data. At the same time, there is significant controversy regarding the question of which myocardial opioid receptor subtypes are involved in postconditioning. The opioid peptides leu- and met-enkephalin, which are endogenously synthesized in the myocardium, have affinity for δ_1 and δ_2 receptors, and also for μ receptors, so the protective effects of these compounds may be mediated via any of these receptor subtypes. Studies in 2005 observed that the phenomenon of postconditioning can also be imitated by exogenously administered opioids [7]. Thus, i.v. injection of morphine, an agonist of all opioid receptor types, at a dose of 0.3 mg/kg, before reperfusion, decreased infarct size [7]. These data were supported in 2007 by Gross et al. [11, 12]. The infarct-limiting effect of morphine persisted on the background of exposure to the δ receptor antagonist naltrindole but did not develop in conditions of blockade of κ opioid receptors by norbinaltorphimine [8]. These observations may provide evidence for the involvement of κ receptors in postconditioning. However, along with morphine, this phenomenon was also reproduced using the selective δ agonist BW373U86 [12]. Our results from studies of the receptor specificity of the involvement of the opioid system in the protective effect of postconditioning support the view that cardiac δ_1 receptors play a defining role in the mechanism of cardiac tolerance to the harmful action of reperfusion after ischemic postconditioning. The mechanism mediating this adaptive phenomenon may in our view be as follows. Increased production of reactive oxygen species is known to be one of the key elements in the pathogenesis of ischemic and reperfusion injury to cardiomyocyte membranes [4, 6]. Inhibition of lipoperoxide reactions may, conversely, promote decreases in the number of irreversibly damaged cardiomyocytes in anoxia-reoxygenation [4]. Data obtained by American and Japanese investigators [20, 21] indicate that one of the mechanisms of the protective action of postconditioning is a decrease in the reoxygenation-related gen-

eration of reactive oxygen species in cardiomyocytes. Another group showed that postconditioning prevented the increase in the plasma malondialdehyde level in response to ischemia-reperfusion of the heart [15]. These authors took the view that this effect might be directly related to limitation of the size of the necrotic focus. Of the other hand, stimulation of δ opioid receptors in the myocardium is known to be accompanied by decreases in the levels of peptide peroxidation products in the myocardium in conditions of oxidative stress, due to recovery of superoxide dismutase and catalase levels [5]. Considering these points, we suggest that endogenous agonists of δ_1 receptors play an important role in the mechanism of the protective effect of postconditioning, mediating the cardioprotective action by reducing the intensity of peroxidation reactions during reperfusion.

In summary, we should also note the results obtained from our experiments using opioid receptor blockers without modeling postconditioning of the heart. In these conditions, inhibition of opioid receptors with the corresponding antagonists had no effect on the level of hart damage induced by reperfusion. This means that peptide agonists of opioid receptors, endogenously synthesized in the myocardium [17], did not affect the resistance of the "nonadapted" heart to the actions of ischemia-reperfusion or that their basal levels are not high enough. It can be suggested that ischemic postconditioning is accompanied by a nonspecific increase in the level of endogenous opioids in the heart and, perhaps, changes in the receptor apparatus of cardiomyocytes which are specific to this adapting treatment.

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