HYPOTHALAMIC PROLINE-RICH PEPTIDE-1 PROTECTS AGAINST MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

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Keywords: Hypothalamic proline-rich peptide-1; cardiac output; ischemia-reperfusion; myeloperoxidase; myocardial infarction; neutrophils

Hypothalamic proline-rich peptide-1 (PRP-1), a neurosecretory cytokine appeared to be involved in the multiple mechanisms of cardioprotection. Dose-dependent effects of PRP-1 (4 and 8 μg) were studied in an open-chest model of myocardial ischemia-reperfusion injury (IRI). Adult male Sprague-Dawley rats underwent 40-minute left anterior descending coronary artery occlusion, under isoflurane anesthesia followed by 2 and/or 24 h of reperfusion. Groups treated with PRP-1 were compared to control. It has been revealed that the efficient dose of PRP-1 can restore in a time-dependent manner the contractile activity of the myocardium and suppress the both inflammation and necrosis via amelioration of oxidative stress in the cardiac tissues therethrough contributing to the *in vivo* reduction of myocardial infarct volume and an improvement the cardiac hemodynamics and coronary circulation. New studies are needed to ascertain a beneficial effect of PRP-1 in humans and its future clinic use for the myocardial IRI treatment

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INTRODUCTION

Early reperfusion, the process of restoring blood flow to the ischemic myocardium is the most efficient way of prevention the deleterious impact of pathogenic factors associated with myocardial ischemia, to reduce the size of a myocardial infarct and improve the clinical outcome.[1] Although the beneficial effects of myocardial reperfusion are reduced because of the development of ischemia-reperfusion injury (IRI) that leads to arrhythmia, myocardial stunning (the reversible reduction of function of heart contraction after reperfusion), overproduction of reactive oxygen species (ROS). It is accompanied by necrosis apoptosis, and autophagy is contributing to the death of cardiomyocytes viable before myocardial reperfusion.[2,3] Mechanisms underlying IRI are complex and not well understood why there is still no effective, proven therapy against IRI.[4]

We have recently reported that hypothalamic proline-rich peptide-1 (PRP-1) discovered in H. Buniatyan Institute of Biochemistry NAS RA is involved in the mechanisms of cardioprotection by the maintenance of the calcium binding properties of the cardiomyocytes membrane proteins interfering with the standard molecular mechanisms of myocardial damage caused by pancreatic necrosis and/or muscle compression injury.[5,6] At the same time there is evidence that PRP-1 could protect heart tissues on the one hand via inhibition of the phospholipase A2 and the processes of ROS generation and lipid peroxidation, on the other via stimulation the activity of catalase and the energy metabolism, exhibiting membrane-stabilizing effects.[7] Notably, the increased lipoprotein-associated phospholipase A2 levels detected early after myocardial infarction are strongly and independently associated with mortality.[8] This study was performed to investigate whether administration of PRP-1 after induction of ischemia had any effect on the hemodynamic parameters, infarct size and inflammation following ischemic - reperfusion injury.

EXPERIMENTAL

Materials and Methods

The experiments were performed in accordance with the European Communities Council Directives (86/609/EC) on care and use of animals for experimental procedures. These directives are approved by the Animal Care and Ethics Committee of the Center for Vascular Research of Lowe Cancer Research Center of New South Wales University (Australia) and H. Buniatian Institute of Biochemistry (Republic of Armenia). Bovine serum albumin was purchased from Carl Roth (GmbH, Karlsruhe). Solid-phase synthesis of proline-rich peptide-1 (PRP-1) was performed at Moscow laboratory of academic A. A. Galoyan. The PRP-1 preparations were dissolved in saline and filtered (0.22 μm) before use. All other reagents were purchased from Sigma-Aldrich (USA). The work is done in the Center for Vascular Research of Lowe Cancer Research Center of New South Wales University during of June 2010 – August 2010.

Animals and study design

Adult male Sprague-Dawley rats weighing 200-240 g were randomly divided into groups (n=15/group). Control – intact rats; experimental – rats subjected to myocardial ischemia subdivided to untreated, treated with intraperitoneal (*ip*) injections of saline or PRP-1 dissolved in saline (4 or 8 μg) and studied following 2 and/or 24 h reperfusion.

Table 1. Dose-, and time-dependent effects of PRP-1 on cardiac output following myocardial ischemia/reperfusion.

Conditions	**Control**	**Ischemia**		**Ischemia/saline**		**Ischemia/4 μg PRP-1**		**Ischemia/8μg PRP-1**	
Time after releasing the ligature		2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
Cardiac output (%)	98	54-57	62-68	54-56	62-67	64-68	96-97	56-58	68-70

A rat model of myocardial ischemia-reperfusion.

Prior to surgery rats were anesthetized with a mixture of ketamine and xylazine (25 and 75 μg kg^{-1} body weight *ip*, respectively). The PRP-1 impact on the heart was studied in an open-chest model of myocardial ischemia-reperfusion injury (IRI), and inhalational anesthesia with isoflurane was performed throughout the experiment.[9,10] Myocardial ischemia was induced by occlusion of the left anterior descending coronary artery (LAD) using a silk suture with a section of silica gel tubing and confirmed by regional cyanosis and ST-segment elevation. After 40 min of ischemia, reperfusion was initiated via releasing the ligature and removing the silica gel tubing. Reperfusion was confirmed by a rapid color change on the heart surface. In the drug treatment group 2 min after artery ligation and the beginning of surgical procedures, rats were *ip* injected with 0.2 ml PRP-1 diluted with normal saline to 4 or 8 μg. An equal volume of saline was used as a vehicle control. Indices of oxidative stress and inflammation were assayed and the myocardial IRI measured at the end of reperfusion.

Determination of myocardial IRI was performed following 2 and/or 24 h reperfusion. LAD was retied, and 2.0 % Evans blue (Sigma-Aldrich, St. Louis, MO, USA) was injected into the left ventricular cavity to delineate the area at risk of myocardial infarction retrospectively.[11] The heart was removed, washed in phosphate buffered saline, and then stained with 1.5 % 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich) via incubation for 15 min at 37°C, to discriminate between the viable non-ischemic area (blue) and the zone at risk (white and red).

Echocardiography was performed prior to ligation and as 40 min ischemia followed by 2 and/or 24 h reperfusion using the Vevo 770 micro-ultrasound system High-Resolution In Vivo Micro-Imaging System (FujiFilm VisualSonics Inc.).Indices of oxidative stress referring to lipid peroxidation processes were established by measuring malondialdehyde (MDA) using thiobarbituric acid.[12] Briefly: Samples were deproteinized with 10 % TCA, and the precipitates were removed by centrifugation at 15000 rpm for 3 min, and supernatants were mixed with 0.72 % TBA and 0.6 N HCl, heated for 15 min in boiling water bath. After that, the absorbance of samples was measured at 535 nm against reagent blank containing all the reagents minus the sample.

The superoxide radical content was measured using cellular ROS/Superoxide Detection Assay Kit (ab139476). Quantification of neutrophil accumulation in the myocardial tissues using hematoxylin and eosin (H&E) staining.[13] Formalin-fixed, paraffin-embedded sections of myocardial tissues were stained with H&E and examined under a light microscope (magnification, ×400).

Myeloperoxidase assay in heart tissues was based on the cytochemical determination of oxybenzidine formed from the benzidine oxidized in the presence of myeloperoxidase.[14] Fresh smears were fixed by 4 % formalin-alcohol solution for 30 seconds, washed, dried, treated with peroxidase reagent for 5 min, washed again, dried and stained with Romanowsky-Giemsa dye. Myeloperoxidase is detected in the cytoplasm of cells as brown granules.

Results and discussion

Inflammation plays a pivotal role in the myocardial IRI pathophysiology, and anti-inflammatory compounds may attenuate detrimental consequences of infection-reperfusion [15, 16]. New hypothalamic cytokine, PRP-1 (primary structure, AGAPEPAEPAQPGVY and apparent molecular mass of 1475.26 Da) represented the C-terminal 25-39 fragment of neurophysin-vasopressin-associated glycoprotein, produced in hypothalamus nuclei (n. paraventricularis and n. supraopticus) and might be involved in the regulation of homeostasis via multiple mechanisms including anti-inflammatory/antioxidant effects.[7] Dose-dependent effects of PRP-1 (4 or 8 μg) were studied in an open-chest model of myocardial ischemia-reperfusion injury (IRI), which is the most acceptable and appropriate model for the reproduction of human myocardial infarction and screening of pharmacological activity of various compounds.[9,10] The different doses of PRP-1 were administered 2 min after artery ligation in accordance with recommendations to enhance the cardioprotective effects and reduce infarct size by introducing drugs before or during ischemia.[17] Quantification of cardiac output using ultrasound was performed prior to occlusion, and following 2 and/or 24 h reperfusion. Data on a left ventricular ejection fraction (LVEF) (the percentage, of blood that is pumped (or ejected) out of the ventricles with each contraction) measured by echocardiography are presented in Table 1.

We revealed that 2h after the release of ligature cardiac output in experimental animals decreased to 54-57 %, compared to control (98 %). Both saline and 8 μg PRP-1 could not improve the low cardiac output that remained approximately on the same level, while 4 μg PRP-1 appeared to be slightly increased LVEF up to 64-68 %. The more pronounced dose-dependent effect of PRP-1 on coronary outflow was observed following 24 h reperfusion, namely: almost restored normal cardiac output (96-97 %) detected in the groups that received 4 μg PRP-1, while no significant changes in the LVEF were seen in the case of the double dose of PRP-1. Time-dependent effects of 4 μg PRP-1 on cardiac output in experimental ischemia-reperfusion are presented in Figs 1 and 2. At this moment, the mentioned dose of PRP-1 appeared to be improved the cardiac hemodynamics and coronary circulation during 24 h reperfusion period.

The cellular and molecular mechanisms regulating the inflammatory response following myocardial ischemia and reperfusion, myocardial necrosis induces complement activation and free radical generation, triggering a cytokine cascade recruiting neutrophils in the ischemic and reperfused myocardium.[18, 19] Treatment with the only efficient dose of PRP-1 (4 µg, *ip*) could decrease the oxidative stress processes in cardiac muscle tissues at early reperfusion period causing the reduction in the reactive oxygen species, particularly superoxide anion ($O_2^{\cdot -}$) and the MDA levels up to 40-45 %, and 20-25 % compared respectively to the rest of groups.

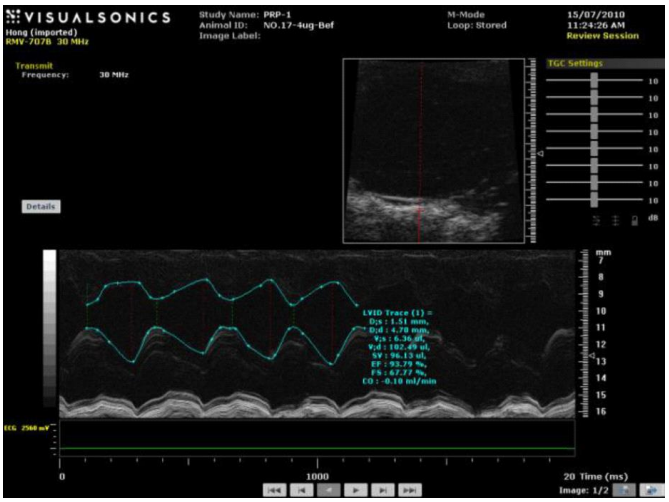


Figure 1. The PRP-1 (4 µg, *ip*) impact on cardiac output (left ventricle) following 2 h reperfusion after coronary artery occlusion in anesthetized rats. Results are shown as mean ± SEM of n = 15/group. $P < 0.05$ compared to control.

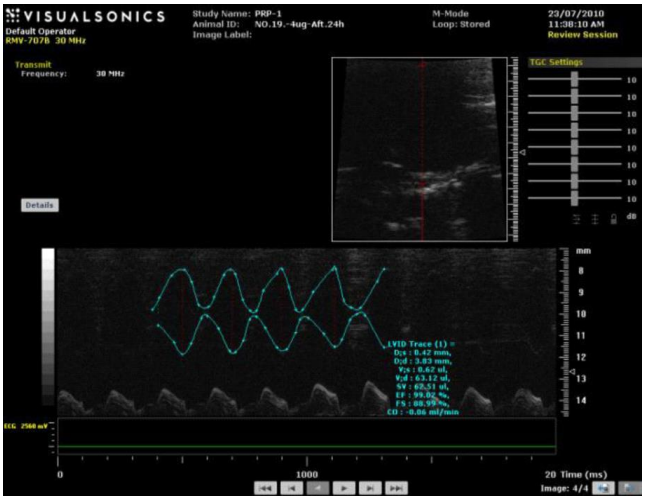
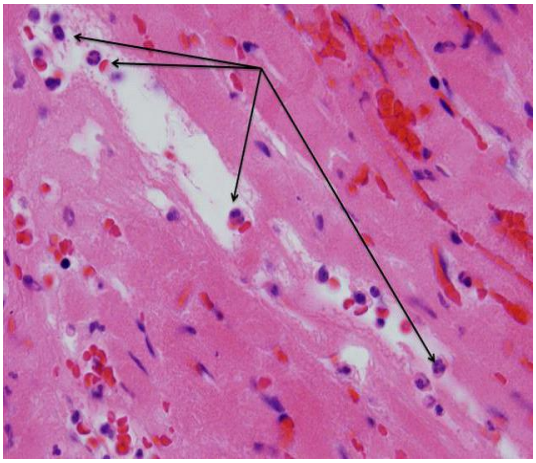


Fig. 2. The PRP-1 (4 µg, *ip*) impact on cardiac output (left ventricle) following 24 h reperfusion after coronary artery 40 min occlusion in anesthetized rats. Results are shown as mean ± SEM of n = 15/group. $P < 0.05$ compared to control.

Neutrophils are mainly contributed to a significant amount of myocardial injury induced by coronary artery occlusion followed by reperfusion.[20] Quantification of neutrophil accumulation in myocardial tissues as a marker of tissue damage showed that the effective dose of PRP-1 (4 µg µg, *ip*) caused a decrease in their number up to 7-8 in the field of view, compared to control following 24 h reperfusion (Fig. 3).

A



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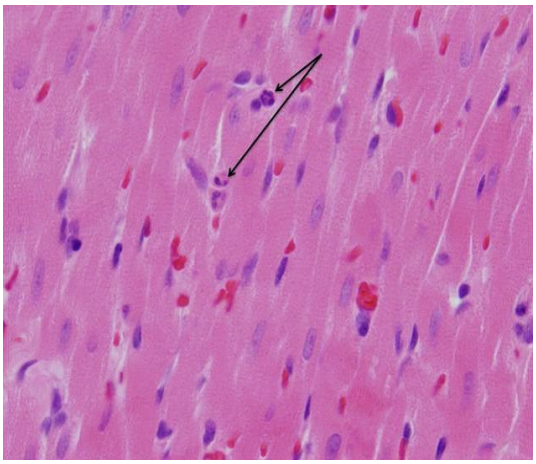

Figure 3. Neutrophil infiltration into cardiac tissues following 24 h reperfusion after coronary artery 40 min occlusion in anesthetized rats. (*A*) Saline-Treated rats (the number of neutrophils 40.3 ± 3.6 per field of view); (*B*) PRP-1-treated rats (4 µg, *ip*; the number of neutrophils per field of view – 7.9 ± 1.2). Hematoxylin and eosin staining (magnification, ×400). Results are shown as mean ± SEM of n = 15/group. $P < 0.05$ compared to control.

The results were confirmed by determination of the myeloperoxidase (MPO) level in the cardiac tissues. MPO is released by activated neutrophils from azurophilic granules and is recognized as a key regulator of neutrophil oxidant production, and an indicator for neutrophil infiltration in tissues; MPO may cause marked damage to cells, extracellular matrix, biological fluids and has been detected at sites of inflammation.[21] Targets and actions of the oxidants generated by MPO and mechanisms of its biological damage are reviewed elsewhere.[22] We found that only the effective dose of PRP-1 (4 µg) could decrease the MPO content in the myocardium during IRI in a time-dependent manner. Despite the administration of PRP-1, there are plenty of brown granules of oxybenzidine pointing to an unusual activity of MPO and indirectly confirm the infiltration of neutrophils following 2 h reperfusion, whereas they virtually disappear following 24 h reperfusion (Fig. 4).

The double dose of PRP-1 exhibited no effect on the MPO level in heart tissues under the same conditions, as well as saline-control.

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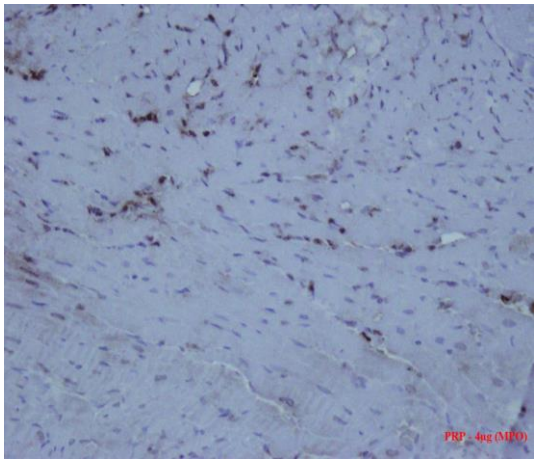

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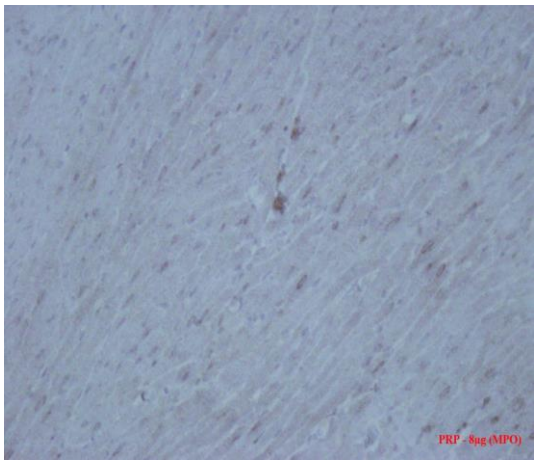

Figure 4. The impact of the effective dose of PRP-1 (4 μg) on the level of myeloperoxidase in cardiac tissues A. Following 2 hours reperfusion after coronary artery 40 min occlusion in anesthetized rats.; B. Following 24 h reperfusion after coronary artery 40 min occlusion in anesthetized rats.; (Hematoxylin staining, magnification, ×40). Results are shown as mean ± SEM of n = 15/group. $P < 0.05$ compared to control.

It should be noted that elevated levels of leukocyte- and blood-MPO are associated with the coronary artery disease.[23] High MPO activity is a risk factor for long-term mortality and adds prognostic value to LVEF measurements in patients with acute myocardial infarction.[24] On induction of IRI, MPO inhibition decreased postischemic apoptosis of cardiomyocytes and reduced cardiac infarct size.[25] Also, the infarct size was smaller in the PRP-1 (4 μg) groups compared to the rest of groups (Fig. 5).

Presumably, the antioxidant activity of the revealed dose of PRP-1 may contribute to the higher safety of cardiomyocytes and reduction of infarct zone. Our findings confirm the antioxidant potential of PRP-1 that could be used in myocardial IRI. However, be aware that not always it is possible to translate findings of animal experiments into clinical therapy.[1] Further research should be done to reproduce the PRP-1 ameliorating effects on myocardial IRI in the clinic.

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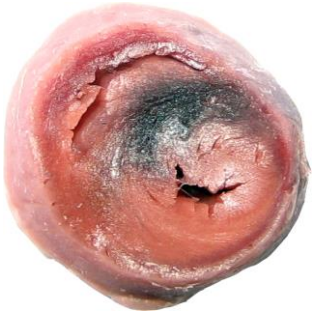

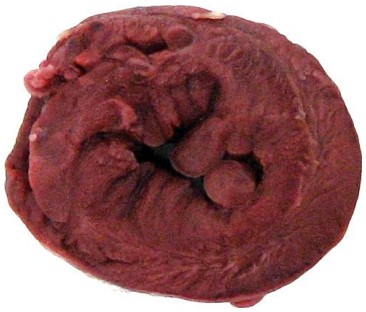

Figure 5. Myocardial ischemia-reperfusion injury (IRI) following 24 h reperfusion after transient occlusion of the left ventricle coronary artery in anesthetized rats. A. Myocardial infarction (the viable non-ischemic area (blue); B. an alleviation of myocardial IRI via ip injection of 4 μg PRP-1.

CONCLUSION

In conclusion, this study demonstrates that the hypothalamic cytokine PRP-1 could substantially reduce a reperfusion injury caused by regional ischemia-reperfusion in the rats in a dose and time-dependent manner. The effective dose of PRP-1 could restore the contractile activity of the myocardium and suppress the both inflammation and necrosis due to ameliorating of oxidative stress in the cardiac tissues and triggering the adaptation processes contributed to the improvement of heart structure and function. The data from this study clearly demonstrate that treatment with appropriate doses of PRP-1 may reduce the cardiomyocyte death, improve the cardiac hemodynamics and coronary circulation. In vivo, post-ischemic administration of PRP-1 significantly reduced myocardial infarct volume caused by transient occlusion of the coronary artery in rats, suggesting that this cytokine might be useful for the treatment of myocardial IRI and this possibility should be further investigated for introduction into clinical practice.

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