In Vivo Reduction of Reperfusion Injury to the Heart with Apelin-12 Peptide in Rats

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Apelin-12 (A-12) peptide was synthesized by automated solid phase method and purified by reverse phase HPLC. Its homogeneity and structure were confirmed by HPLC, ^{1}H-NMR spectroscopy, and mass spectroscopy. Acute myocardial infarction was induced by 40-min occlusion of the left coronary artery with subsequent 60-min reperfusion in narcotized Wistar rats. Peptide A-12 was injected (intravenous bolus, 0.07 or 0.35 μmol/kg) to experimental animals simultaneously with the beginning of reperfusion. Injections of A-12 in these doses led to reduction of systolic BP to 67 and 85% of the initial level, respectively, which was virtually restored completely by the end of reperfusion, and to a significant reduction of the infarction focus in the myocardium (by 21 and 34% in comparison with the control, respectively). Injection of A-12 in a dose of 0.35 μmol/kg led to reduction of plasma concentrations of necrosis markers in comparison with the control by the end of reperfusion: MB-creatine kinase by 56%, lactate dehydrogenase by 30%. The results attest to vasodilatory effects of A-12 under conditions of heart reperfusion *in vivo*; the peptide injected after local ischemia limits the myocardial infarction size and reduces damage to cardiomyocyte membrane.

Key Words: *apelin-12; blood pressure; myocardial infarction; creatine kinase MB fraction; lactate dehydrogenase*

Early reperfusion of the myocardium is one of the main destructive factors of ischemic and reperfusion stress. Ca^{2+} overload and generation of reactive oxygen species under conditions of reduced oxidative phosphorylation promote irreversible damage to cellular and mitochondrial membranes leading to postischemic dysfunction of the heart and cardiomyocyte death [2]. Hence, the search for means reducing reperfusion injuries of the myocardium is a pressing problem of modern experimental cardiology.

Apelin adipokine isoforms with cardioprotective activity attracted much recent attention. It was shown that apelin-36 and apelin-13 peptides reduce the size of myocardial infarction foci and improved the contractile function of isolated perfused hearts from mice and rats after ischemia [5,7,11]. This was paralleled by an increase in SOD activity in the myocardium, less intensive production of MDA (LPO product), and less pronounced injuries to ischemic cardiomyocyte sarcolemma [11]. Apelin-13 stimulated the expression of NO synthase and formation of NO and inhibited the production of superoxide anion in a culture of cardiomyocytes subjected to ischemia and reoxygenation [11]. Apelin-13 inhibited opening of the mitochondrial pore and apoptosis in cardiomyocytes of rats under conditions of oxygenation stress [7,11]. Apelin-12 (A-12), completely identical to apelin C-terminal fragment in various animal species and in humans,

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was recently synthesized. Experiments on isolated rat heart showed that A-12 infusion after global ischemia provided effective recovery of the coronary blood flow and cardiac function during reperfusion [1]. These data suggested a possibility of reducing the reperfusion injury to the heart under the effect of A-12 *in vivo*.

We evaluated the capacity of A-12 to reduce irreversible changes in the myocardium caused by coronary artery occlusion and subsequent reperfusion in narcotized rats. The effects of intravenous injection of A-12 after a period of local ischemia on the size of myocardial infarction focus and cardiomyocyte membrane damage were studied.

MATERIALS AND METHODS

Peptide A-12 (H-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe-OH, M=1442.7) was obtained by automated solid phase synthesis on a peptide synthesizer (Applied Biosystems 431A) using Fmoc technology. The final product was purified by HPLC to 98% purity and characterized by ^{1}H-NMR spectroscopy and MALDI mass spectrometry (charge mass 1423.5; estimated molecular weight 1422.7). Amino acid derivatives, reagents, and solvents from Bachem and Fluka were used.

Artificial ventilation of the lungs with ambient air was carried out by means of KTR-5 device (Hugo Sacks Electronic) in male Wistar rats narcotized by 20% urethane (120 mg/kg intraperitoneally) under conditions of tracheotomy. The jugular vein was catheterized for heart staining with 1% Evans Blue solution at the end of experiment, the carotid artery was catheterized for BP registration. Systolic BP (SBP) and heart rate (HR) were recorded on a Biograph-4 polygraph (St. Petersburg University of Aerospace Engineering) after connection of the arterial catheter to a tensometric pickup. The data were recorded in a PC through an USB 6210 digital transformer (National Instruments) and Data Acquisition software.

After the animal was prepared, the hemodynamic parameters were allowed to stabilize over 30 min (initial status), after which 40-min occlusion of the left coronary artery (LCA) followed by 60-min reperfusion were modeled. Experimental animals were injected with A-12 in doses of 0.07 or 0.35 μmol/kg (intravenous bolus) after the period of local ischemia simultaneously with the beginning of reperfusion. Controls were injected with the same volume (0.5 ml) of saline. In order to identify the risk zone and intact regions of the myocardium, the LCA was re-occluded at the end of experiment and a bolus of 1% Evans Blue (2 ml) was injected into the jugular vain. The heart was then removed and the left ventricle (LV) was isolated for subsequent evaluation of myocardial infarction size.

Frozen LV was cut perpendicularly to the long axis of the heart into 4-5 slices about 1.5-2 mm thick; the slices were incubated for 10 min with 1% 2,3,5-triphenyltetrazoleum chloride in 0.1 M potassium phosphate buffer (pH 7.4 at 37°C). The resultant samples were scanned, the areas of myocardial infarction and risk zone were evaluated by computer planimetry using Imagecal software. The sections were then weighed for measurement of the LV weight. The risk zone/LV weight (RZ/LV) and myocardial infarction/risk zone (MI/RZ) ratios [10] were estimated for each group.

Cardiomyocyte membrane damage was evaluated by increment of lactate dehydrogenase (LDH) and MB-creatine kinase (MB-CK) activities in the plasma. About 0.5 ml blood was collected into heparin-treated tubes from the venous catheter initially and after 1 h of reperfusion. Plasma enzyme activities were measured on a Yanako UO-2000 spectrophotometer at λ=340 nm using BioSystems kits.

The differences between the groups were evaluated by Student's *t* test and were considered significant at $p<0.05$. The values were expressed as $M\pm m$.

RESULTS

Initially the mean SBP was virtually the same in all groups: 102±5 mm Hg; HR was 367±5 min^{-1}. Injection of saline (control) did not lead to changes in the mean SBP and HR during subsequent local ischemia and reperfusion. Injection of A-12 in a dose of 0.07 μmol/kg after local ischemia reduced the mean SBP to 67±3% of the initial level during the 3rd min of reperfusion (Fig. 1), while by the end of reperfusion it was restored to the initial level. Injection of A-12 in a dose of 0.35 μmol/kg led to an increase of the mean SBP by 25% during the 1st min of reperfusion, after which it decreased to 85±2% of the initial level by the 7th min of reperfusion. By the 30th min of reperfusion, the mean SBP returned to normal (93±5%) and virtually did not change afterwards.

Histochemical analysis of LV sections after reperfusion showed no appreciable differences in the risk

TABLE 1. Effects of Intravenous A-12 Injected at the Beginning of Reperfusion after Local Myocardial Ischemia on Reduction of Infarction Area in Narcotized Rats ($M\pm m$)

Experiment conditions	RZ/LV, %	MI/RZ, %
Control (n=12)	38.3±2.2	38.1±2.5
A-12, 0.07 μmol/kg (n=10)	34.6±2.3	30.1±2.2*
A-12, 0.05 μmol/kg	39.1±3.1	25.1±2.3**

Note. *$p<0.02$, **$p<0.001$ in comparison with the control.

zones in the groups (Table 1). Injection of A-12 in doses of 0.07 and 0.35 μmol/kg reduced significantly the size of infarction area – by 21 and 34% *vs.* control, respectively. Changes in the activities of necrosis markers (LDH and MB-CK) in the plasma were studied after injection of A-12 in the most effective dose (0.35 μmol/kg). The development of myocardial infarction in the control was associated with a significant increase in activities of both enzymes at the end of reperfusion in comparison with their initial levels (before LCA occlusion; Table 2). Injection of A-12 led to a significant reduction of MB-CK activity (by 56%; $p<0.02$) in comparison with the control. Activity of LDH in A-12 group decreased by 30% ($p=0.07$) by the end of reperfusion. Hence, vasodilatation effects of A-12 peptide under conditions of heart reperfusion and the capacity of this substance injected after local ischemia to reduce the size of myocardial infarction were demonstrated *in vivo* in rats. Reduction of cardiomyocyte membrane damage in the risk zone under the effect of the peptide was confirmed by lower activity of MB-CK (specific marker of necrosis) in the plasma.

Importantly, the results are in good correlation with reduction of the myocardial infarction zone during perfusion of the isolated rat heart with apelin-13 or its pyroglutamate analog [pyrGlu]-apelin-13 in local ischemia/reperfusion [5,7] and after intravenous injection of apelin-13 to narcotized rats with coronary artery occlusion at the beginning of reperfusion [7]. It was hypothesized that apelin-13 initiated the mechanisms of cell survival triggered by reperfusion kinase cascades [7,8]. This hypothesis was partially confirmed by finding that phosphatidyl-inositol-3-kinase, Akt kinase (Pl3K-Akt), and mitogen-activated MEK-Erk1/2 kinase inhibitors abolish the effect of apelin-13 on the mitochondrial pore opening and cardiomyocyte death [7,8,11]. Importantly that one of the targets of reperfusion kinases was endothelial NO synthase (eNOS), its expression increasing under the effect of apelin-13 [4,11]. Presumably, the protective effect of A-12 was also mediated by triggering of these mechanisms.

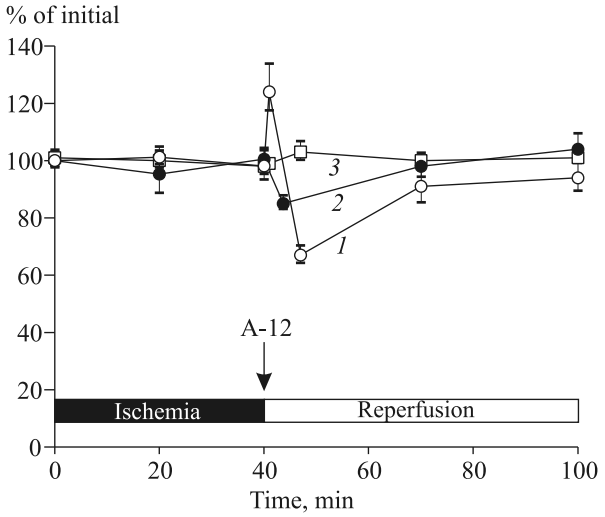

Fig. 1. Effects of intravenous A-12 infusion at the beginning of reperfusion of the myocardium on the time course of the mean SBP in narcotized rats. The means for series of 8-10 experiments are presented. *1*) A-12 in a dose of 0.35 μmol/kg; *2*) A-12 in a dose of 0.07 μmol/kg; *3*) saline in the same volume (control).

In our experiments, the decrease in the mean SBP (Fig. 1) determined by the vasodilatory effect of NO confirmed this possibility. One more evidence supporting this hypothesis was the hypotensive effect of A-12 in conscious and narcotized rats, which was paralleled by an increase of plasma levels of nitrites and nitrates and was canceled in the presence of NO synthase inhibitor , L-NAME methyl ester [3,9]. It is known that NO is not only a vasodilatory agent, but also a scavenger of active oxygen species reducing the reperfusion damage to the heart [6]. Less intensive generation of active oxygen species and together with lower MDA content in the isolated rat heart and cardiomyocyte culture in a model of ischemic and reperfusion stress [11] directly attested to the antioxidant effects of exogenous apelin-13. These facts prompt further studies of NO role in the mechanisms of myocardial infarction zone reduction under the effect of A-12.

One more prospective trend, continuing the present study, can be the synthesis of modified A-12 ana-

TABLE 2. Effects of Intravenous A-12 in a Dose of 0.35 μmol/kg after Local Myocardial Ischemia in Narcotized Rats on Plasma Activities of MB-CK and LDH at the End of Reperfusion (*M*±*m*)

Necrosis markers	Initial status	End of reperfusion	
		control	A-12, 0.35 μmol/kg
MB-CK, U/liter	274.9±42.4	1221.1±152.4	678.5±109.0*
LDH, U/liter	74.8±14.1	795.5±57.3	549.1±111.8

Note. The means for series of 6 experiments are presented. *$p<0.02$ in comparison with the control.

logs resistant to aminopeptidases, which can lead to creation of pharmacological APJ receptor agonists. This approach seems to be important for target regulation of the apelin/APJ receptor system in patients with acute coronary syndrome and heart failure.

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