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This paper should be cited as follows:

Pisarenko, O.I. and Studneva, I. M. (2017). **"A Structural Analogue of Peptide Apelin-12 in Cardioprotection against Ischemia/Reperfusion Injury",** Athens: ATINER'S Conference Paper Series, No: **PHA2017-2192.**

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URL Conference Papers Series: www.atiner.gr/papers.htm
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ISSN: 2241-2891
10/05/2017

A Structural Analogue of Peptide Apelin-12 in Cardioprotection against Ischemia/Reperfusion Injury

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Abstract

This study was designed to explore the effects of chemically modified peptide apelin-12 [MeArg1,NLe10]-A12 (AI) on myocardial energy state, antioxidant enzyme activities and reactive oxygen species (ROS) formation in the ex vivo model of myocardial ischemia/reperfusion (I/R) injury. Peptide AI was synthesized by the automatic solid phase method using Fmoc technology and identified by 1H-NMR spectroscopy and mass spectrometry. Isolated perfused working rat hearts subjected to global ischemia and reperfusion were used. Preischemic infusion of AI improved the recovery of cardiac function and myocardial energy state, and enhanced the activity of Cu,Zn superoxide dismutase (Cu,Zn SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in reperfused hearts. These effects were combined with a reduction in ROS and malondialdehyde (MDA) formation during reperfusion. Administration of specific inhibitors of phosphoinositide 3-kinase (PI3K), mitogen activated extracellular regulated kinase 1/2 (MEK1/2), phospholipase C (PLC), NO synthase (NOS) or the sarcolemmal Na+/Ca2+ exchanger reduced protective efficacy of AI. This was evidenced by the deterioration of cardiac function recovery, attenuation of metabolic restoration and sarcolemmal integrity. Thus, cardioprotection with peptide AI is mediated by signaling via PLC, the upstream kinases of the reperfusion injury salvage kinase (RISK) pathway, PI3K and MEK1/2, and the activation of NOS and the sarcolemmal Na+/Ca2+ exchange. Mechanisms of AI action include the upregulation of cardiac antioxidant defense system and attenuation of ROS formation and lipid peroxidation.

Keywords: apelin, ischemia and reperfusion, myocardial energy metabolism, membrane integrity, antioxidant effects, signaling

Acknowledgments: This work was supported by the Russian Foundation for Basic Research (grants 14-04-00012a and 15-04-00359a). The authors are grateful to Dr. M.V. Sidorova for the synthesis of peptide AI and the discussion of the results. The authors are very grateful to Dr. A.A. Timoshin and Dr. V.Z. Lankin for the discussion of the results of this work.

Introduction

Myocardial ischemia/reperfusion (I/R) injury involves complex pathophysiologic events that contribute to cardiac dysfunction and cardiomyocyte death. Several therapeutic approaches such as the induction of pre- or postconditioning and administration of pharmacological agents have been examined to prevent or limit I/R injury. However, the suitable treatment modalities have not been fully proved yet (Ferdinandy et al. 2007). A promising approach to solving this issue is the application of natural biomolecules or their synthetic analogues that may trigger mechanisms of endogenous cardioprotection. One of these compounds is an adipocytokine apelin, the endogenous ligand for the G-protein-coupled APJ receptor (Kleinz and Davenport 2005). Apelin is produced as a 77 amino acid prepropeptide, which is cleaved to shorter biologically active C-terminal fragments (Tatemoto et al. 1998). Positive inotropic and hypotensive effects of exogenous apelin-36, apelin-13 and its pyroglutamated form, [Pyr^1]apelin-13, in normal and failing myocardium are well documented in experimental and clinical studies (Berry et al. 2004, Chen et al. 2003, Japp et al. 2010). Apelin-12 (A12), [Pyr^1]apelin-13, apelin-13 and, to a lesser extent, apelin-36 are capable to reduce infarct size and to augment contractile function recovery in the heart of rodents after regional or global ischemia (Simpkin et al. 2007, Kleinz and Baxter 2008, Zeng et al. 2009, Pisarenko et al. 2010, Pisarenko et al. 2013). In cultured cardiomyocytes, apelin-13 suppressed apoptosis and delayed the opening of the mitochondrial permeability transition pore (mPTP) (Simpkin et al. 2007, Zeng et al. 2009, Zhang et al. 2009). The beneficial effects of some of these peptides are attributed to recruiting the PI3K–Akt, MEK 1/2-ERK 1/2 signaling cascades (Zeng et al. 2009, Masri et al. 2005, Smith et al. 2007, Rastaldo et al. 2010). These facts suggest that administration of these peptides may be promising in the treatment of coronary heart disease. However, apelin peptides rapidly cleared from the circulation with a half-life of several minutes (Japp et al. 2010). It is believed that this is due to their rapid hydrolysis by various peptidases including angiotensin-converting enzyme 2 (Vickers et al. 2002).

Previously, we have synthesized a modified analog of the C-terminal fragment of apelin-12 (H-(N$^\alpha$Me)Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Nle-Pro-Phe-OH, AI) more resistant to degradation by proteolytic enzymes and having greater storage stability in comparison with the natural peptide apelin-12 (Sidorova et al. 2012). The protective efficacy of AI is comparable with the action of natural apelin-12 and -13 in various models of myocardial I/R injury (Pisarenko et al. 2013, Sidorova et al. 2012, Pisarenko et al. 2014). The objective of the present paper was to summarize data on metabolic and antioxidant activity of AI in ex vivo model of I/R injury of rat heart. We also evaluated the role of PLC and survival kinases, PI3K, and MEK1/2, NOS and the sarcolemmal Na^+/Ca^{2+} exchanger in mechanisms of AI action using the specific inhibitors. The presented results demonstrate a tight link between the AI ability to reduce functional disturbances in postischemic heart on the one

hand, and metabolic protection and membrane integrity afforded by the peptide, on the other hand.

Materials and Methodology

Chemicals

Peptides A12 and its structural analogue AI (Table 1) were synthesized by the automatic solid phase method using Fmoc technology. They were purified by preparative HPLC and identified by ^{1}H-NMR spectroscopy and mass spectrometry (Sidorova et al. 2012). Enzymes and chemicals were purchased from Sigma Chemical Co. (St Louis, MO USA).

Table 1. *Structure of Apelin-12 and its Analogue AI*

Peptide	Structure	Mw, g/mol
A12	H-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe-OH	1422,7
AI	H-(**N$^\alpha$Me**)Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-**Nle**-Pro-Phe-OH	1418,7

Note: The substitutions are shown in bold.

Isolated Perfused Rat Hearts

Male Wistar rats (300-340 g) were heparinized by intraperitoneal injection (1600 IU/kg body weight) and anaesthetized with urethane (i.e., 1.3 g/kg body weight). Their hearts were perfused with Krebs-Henseleit buffer (KHB) supplied with 11 mM glucose. A needle was inserted into the left ventricular (LV) cavity to register LV pressure via a Gould Statham P50 transducer, SP 1405 monitor and a Gould Brush SP 2010 recorder (Gould, Oxnard, Ca, USA). The steady state values of the cardiac function were recorded after preliminary 20-min perfusion in working mode according to a modified method of Neely under constant left atrium pressure and aortic pressure of 20 and 100 cm H_2O, respectively. Details of this method were described previously (Pisarenko et al. 2010).

In the control group, a 5-min infusion of KHB was applied according to Langendorff mode after preliminary working perfusion before global ischemia. Then the hearts were subjected to 35 min of normothermic global ischemia followed by a 5-min Langendorff perfusion with subsequent 25-min working reperfusion. In the experimental groups, preischemic infusion was performed with KHB containing 140 μM AI or A12. The infusion of AI with specific inhibitors of the intracellular signaling or the sarcolemmal Na^+/Ca^{2+} exchange was performed before ischemia in a separate series of experiments. Further the protocol was the same as for the control group. The used inhibitors were: 108 μM U-73122 hydrate (U), a PLC inhibitor; 150 μM UO126 (UO), a selective inhibitor of MEK1/2; 110 μM LY294002 (LY) , a selective inhibitor of PI3K;

128 μM L-NAME (N), a non-selective NOS inhibitor; 110 μM KB-R7943 (KB), an inhibitor of Na^{+}/Ca^{2+} exchange.

After preliminary perfusion and at the end of reperfusion, the hearts were freeze-clamped in liquid nitrogen for biochemical studies. The myocardial effluent was collected in ice-cold tubes during both periods of Langendorff perfusion for assessment of lactate dehydrogenase (LDH) activity and spin-trap measurements.

Analysis of Metabolites

A portion of frozen LV of isolated perfused hearts was quickly homogenized in cooled 6% $HClO_4$ (10 ml/g); the homogenates were centrifuged and then neutralized with 5M K_2CO_3 to pH 7.40. The tissue extracts were centrifuged after cooling to remove $KClO_4$ precipitate. Tissue dry weights were determined by weighing a portion of the pellets after extraction with 6% $HClO_4$ and drying overnight at 110°C. Concentrations of ATP, ADP, AMP, phosphocreatine (PCr) and lactate in neutralized tissue extracts were determined by enzymatic methods (Bergmeyer 1974).

Determination of Antioxidant Enzymes Activity and MDA

The remaining part of the frozen tissue samples was homogenized in 50 mM Na-phosphate buffer, pH 7.4 (1:10 wt/vol). The homogenates were centrifuged at 1000 g for 10 min at 4^{0}C. The supernatants were used for assessment of activities of Cu, Zn SOD, CAT and GSH-Px as described elsewhere (Pisarenko et al. 2014). The MDA content was estimated by the standard method in the reaction with 2-thiobarbituric acid (Lankin et al. 2012). The protein in the supernatants was estimated by the method of Lowry et al. (Lowry et al. 1951).

Spin-trap Measurements in Perfusate

100 mM 5,5-dimethyl-1pyrroline-N-oxide (DMPO) was added to aliquots of the coronary effluents collected at the end of the steady state and at early reperfusion. The effluent samples were stored in liquid nitrogen until the electron paramagnetic resonance (EPR) measurements. EPR signals of DMPO spin adducts were recorded in a glass capillary tube at room temperature using a Varian E-109 E X-band electron spin resonance spectrometer (Pisarenko et al. 2014).

Statistical Analysis

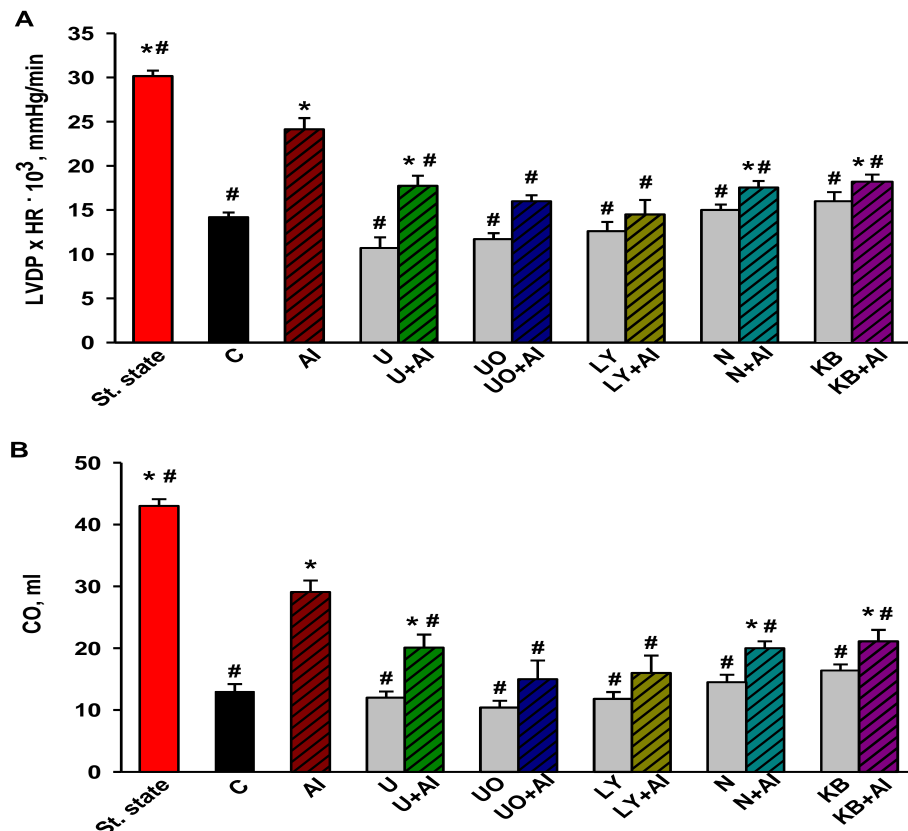
All data are presented as means ± SEM. Results were analyzed by one-way ANOVA followed by Bonferroni multiple range test post-hoc analysis for calculation differences between more than two groups. The comparisons

between two groups involved the use of the Student's unpaired t-test. A P<0.05 was considered statistically significant.

Results

Functional Recovery of Isolated Rat Hearts after Global Ischemia

Figure 1. *Enhanced Recovery of LVDP×HR Product (A) and Cardiac Output (B) Induced by Preischemic AI Infusion is Suppressed by the Inhibitors of PLC, MEK1/2, PI3K, NOS and Na^+/Ca^{2+} Exchange*



Note: St.st., steady state; C, control; CO, cardiac output. The values are expressed as means ± SEM for 10 experiments. *P<0.05 vs. control; #P<0.05 vs. AI.

Cardiac function indices of isolated perfused rat heart at the end of reperfusion were considerably depressed in the control group (Figure 1). The recovery of contractile function intensity index was calculated as the LV developed pressure-heart rate product (LVDP×HR) and cardiac output (CO) was 45 and 30% of the steady state values, respectively. The LV diastolic pressure (P_d) was significantly higher than the initial value (10±1 vs. -3±1 mm Hg, respectively). Preischemic AI infusion enhanced the recovery of LVDP×HR and CO by 27 and 37% compared with control, respectively. This effect was accompanied by a reduction of LV P_d to 3±1 mm Hg. The co-

administration of AI and the inhibitors of the RISK pathway, PLC, NOS or Na^+/Ca^{2+} exchange blocker (UO, LY, U, N or KB, respectively) substantially reduced or abolished the protective effects of AI on recovery of cardiac function during reperfusion. Preischemic administration of the inhibitors alone did not affect recovery of cardiac function during reperfusion compared with the control.

The Metabolic State of Isolated Rat Hearts at Reperfusion

Changes in the myocardial contents of metabolites at the end of reperfusion in the studied groups are compared with the steady state values in Table 2. The control group exhibited poor recovery of the aerobic metabolism at the end of reperfusion. A dramatic decrease in myocardial ATP was accompanied by a reduction of the adenine nucleotide pool (ΣAN) and adenylate energy charge (AEC) as compared to the steady state values. Phosphocreatine (PCr) content was half as much value in the steady state, while myocardial lactate was four times higher. Preischemic infusion of AI enhanced restoration of ATP two times, almost completely restored ΣAN and significantly increased AEC in reperfused hearts compared with control. These effects were combined with the reduction of myocardial lactate content to the initial value. AI infusion slightly increased the recovery of myocardial PCr compared with these indices in control.

Inhibition of PLC, PI3K, MEK1/2 or Na^+/Ca^{2+} exchanger with U, LY, UO or KB, respectively, abrogated the beneficial effects of AI on restoration of myocardial ATP, ΣAN and AEC at the end of reperfusion. In the U+AI group, the PCr content was significantly lower than after administration of AI or the value in control. The co-infusions of AI with U, LY, UO or KB increased lactate content to the value in control. Coadministration of N and AI significantly impaired the metabolic effects of the peptide but to a lesser extent compared with other inhibitors. Thus, all studied inhibitors of intracellular signaling and Na^+/Ca^{2+} exchange reduced or completely suppressed AI ability to improve the metabolic state of reperfused heart.

Table 2. *Inhibition of PLC, the RISK Pathway or Na^+/Ca^{2+} Exchange Reduces Metabolic Protection of Reperfused Rat Heart Induced by AI*

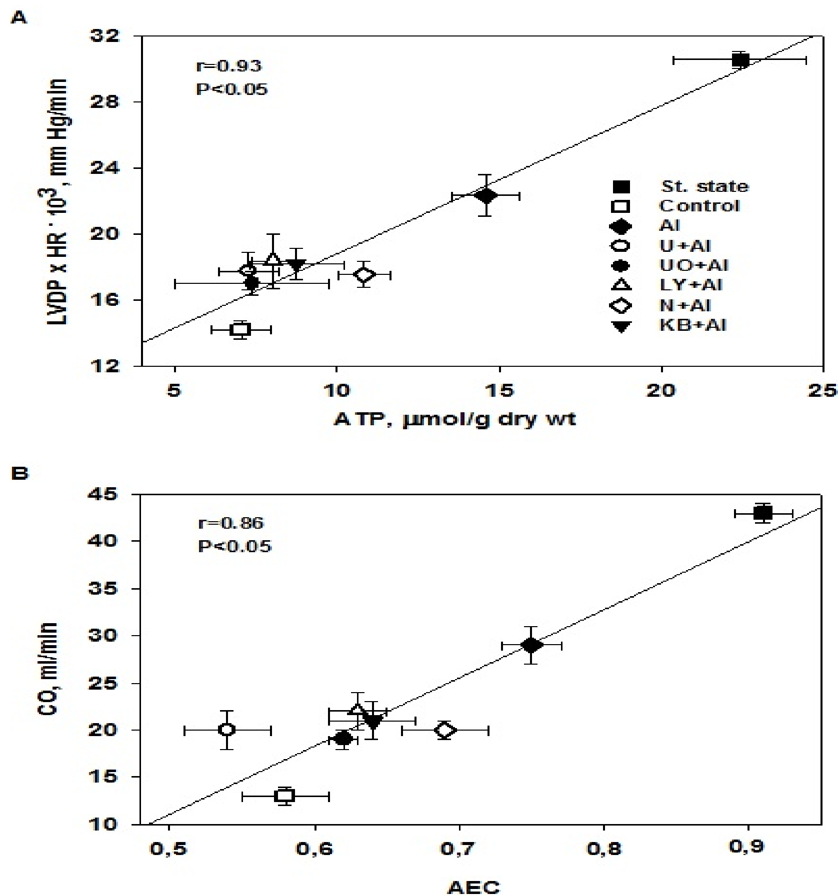
	ATP	**ΣAN**	**AEC**	**PCr**	**Lactate**
St. state	22.42±2.06	25.93±1.45	0.91±0.02	24.30±2.30	1.72±0.19
Control	7.04±0.92[a]	16.90±0.99[a]	0.58±0.03[a]	12.69±1.59[a]	6.93±1.29[a]
AI	14.59±1.03[ab]	23.56±0.95[b]	0.75±0.02[ab]	15.20±1.41[a]	1.47±0.24[b]
U+AI	7.27±0.90[ac]	17.88±1.42[ac]	0.54±0.03[ac]	8.56±0.80[abc]	8.89±1.40[ac]
KB+AI	8.74±1.45[ac]	17.08±1.06[ac]	0.64±0.03[ac]	12.65±1.96[a]	6.81±2.31[ac]
UO+AI	7.38±2.37[ac]	16.79±1.33[ac]	0.62±0.01[ac]	9.82±2.43[a]	6.57±1.10[ac]
LY+AI	8.03±0.67[ac]	16.68±2.24[ac]	0.63±0.02[ac]	10.07±2.53[a]	7.27±1.53[ac]
N+AI	10.82±0.25[abc]	18.68±1.34[ac]	0.69±0.03[a]	11.69±2.03[a]	4.74±0.30[ac]

Note: Values are the mean ± SEM of 8 experiments and expressed in μmol/g dry wt. for metabolites. ΣAN=ATP+ADP+AMP. The adenylate energy charge (AEC) = (ATP+0.5ADP)/ ΣAN. St. state, steady state; [a] $P<0.05$ vs. steady state; [b] $P<0.05$ vs. control; [c] $P<0.05$ vs. AI.

Correlations between Functional and Metabolic Recovery of Reperfused Hearts

The correlation analysis revealed tight positive correlations between the mean values of cardiac function indices (LVDP×HR product and CO) and the mean values of myocardial content of ATP, ΣAN, PCr and AEC in the studied groups (r=0.84-0.93, P<0.05). Correlations between the mean values of LV P_d and the mean values of the myocardial energy state (ATP, ΣAN, AEC and PCr) were negative (r=-0.75-0.90). The average myocardial lactate contents negatively correlated with the LVDP×HR product and CO (r = -0.58-0.59) and positively with LV P_d (r= 0.58, P<0.05). The correlations between the LVDP×HR product and myocardial ATP and between CO and AEC in the studied groups are shown as an example in Figure 2 A,B. It is seen that the reduction of functional recovery of reperfused hearts when peptide AI was administered with the inhibitors were associated with a decrease or complete abolition of improving myocardial metabolic state.

Figure 2. *The Relationships between the Intensity of Contractile Function and Myocardial ATP (A) and Cardiac Output and Adenylate Energy Charge (B) in the Studied Groups*



Note: Values are the mean ± SEM of 8-10 experiments. LVDP×HR, contractile function intensity index; CO, cardiac output; AEC, adenylate energy charge.

Damage to Cell Membranes at Early Reperfusion

LDH activity in the myocardial effluent did not differ significantly between the groups before ischemia (Table 3). Therefore, infusion of AI alone or together with the inhibitors did not cause damage to the sarcolemma of nonischemic cardiomyocytes. In control, the release of LDH at early reperfusion increased by more than two-fold compared with the value before ischemia, thus indicating I/R membrane damage. The postischemic LDH leakage significantly decreased after the preischemic AI infusion compared with control, thus suggesting fewer membrane defects. The coadministration of AI and the inhibitors of MEK1/2 and PI3K, (UO and LY, respectively) increased LDH release after ischemia to the values that did not differ significantly from control. The ability of AI to reduce LDH leakage from postischemic cardiomyocytes decreased to a lesser extent in the presence of the inhibitors of NOS and Na^+/Ca^{2+} exchange (N and KB, respectively).

Table 3. *Effects of Infusion of AI and Inhibitors of RISK pathway, NOS and Na^+/Ca^{2+} Exchange on LDH Release in the Perfusate Before and After Global Ischemia*

	Before ischemia	After ischemia
Control	**5.45±0.78**	**11.79±1.26 [b]**
AI	4.76±0.82	6.53±1.08 [a]
UO+AI	4.98±0.86	10.14±1.54 [b]
LY+AI	5.05±0.72	9.93±0.97 [b]
KB+AI	5.65±0.89	7.56±1.12 [a]
N+MA	5.45±0.83	8.55±1.20 [b]

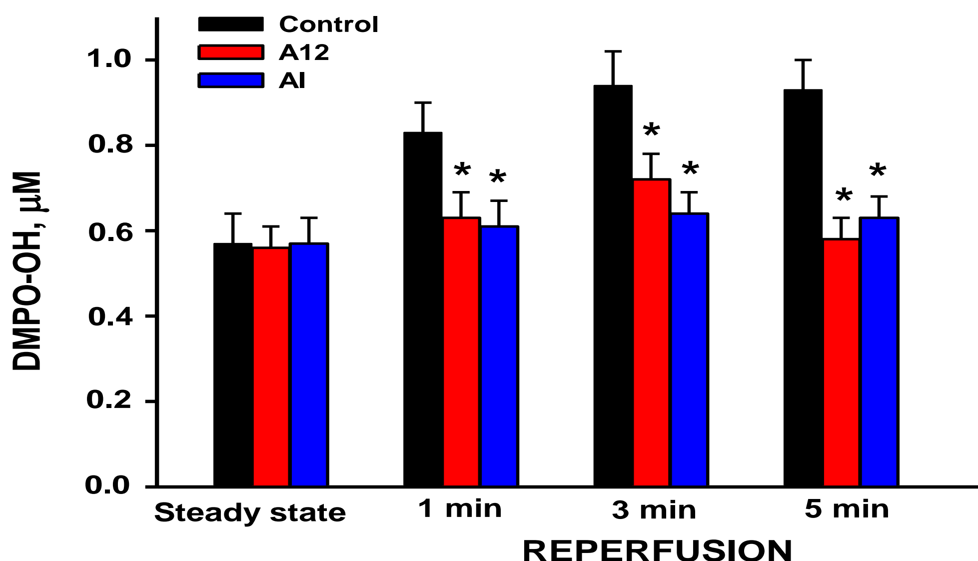
Note: Values are the means ± SEM for 8 experiments and are expressed in IU/g dry wt. for 5-min Langendorff perfusion before or after global ischemia. [a] $P<0.05$ vs. control; [b] $P<0.05$ vs. the value before ischemia.

DMPO-OH Adduct Formation in Myocardial Effluent

To elucidate the possible mechanisms of reducing damage to cell membranes induced by peptide AI, we evaluated its effect on the formation of reactive oxygen species (ROS) using the spin trap DMPO. The natural peptide A12 was used for comparison in a separate series of experiments. The effluent DMPO-OH concentrations did not differ significantly between the groups in the steady state (Figure 3). A significant increase in DMPO-OH concentration was observed at early reperfusion in the control group as compared to the steady state value. Preischemic administration of A12 or AI substantially decreased DMPO-OH formation during reperfusion compared with control. In this case DMPO-OH concentrations did not differ from the steady state values. The observed increase in DMPO-OH concentration could be related to a decomposition of superoxide radical adducts DMPO-OOH and a trapping of $OH^{\cdot}$ Radicals generated in the Haber-Weiss and the Fenton reactions (Kappusamy and Zwier 1989). Since the detection of DMPO-OH adduct in myocardial effluent does not directly reflect ROS formation in the heart, this

data demonstrates that both peptides prevented the release of ROS-generating systems and H_2O_2 from myocardial tissue.

Figure 3. *Effects of A12 or AI Infusion before Ischemia on DMPO-OH Adduct Concentrations in Myocardial Effluent of Isolated Perfused Rat Heart*

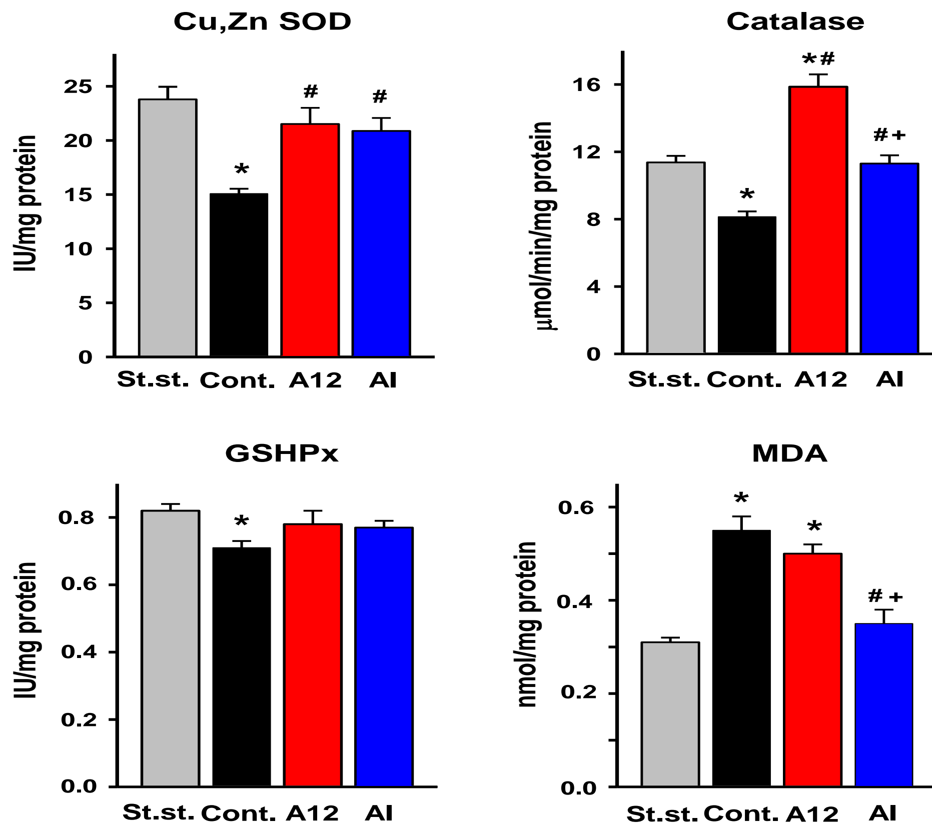


Note: The values are expressed as means ± SEM of six experiments. *P<0.05 vs control.

Activities of Antioxidant Enzymes and MDA Formation

A significant decrease in activities of the key myocardial antioxidant enzymes, Cu,Zn SOD, CAT and GSH-Px, was observed at the end of reperfusion in the control group as compared to the steady state values (Figure 4). The reduction in the efficiency of the antioxidant defense was accompanied by a twofold increase in myocardial content of MDA, the product of lipid peroxidation. A complete recovery of Cu,Zn SOD activity and an increase in CAT activity was observed in the A12 treated group as compared to the control group. Preischemic A12 infusion also restored myocardial GSH-Px activity and slightly reduced MDA level in myocardial tissue as compared to the control group. Preischemic treatment with peptide AI recovered the activities of Cu,Zn SOD, CAT and GSH-Px in reperfused hearts almost to the steady state values. AI infusion prevented lipid peroxidation, as evidenced by the reduction of MDA content to the initial value.

Figure 4. *Effects of A12 or AI Infusion before Ischemia on Antioxidant Enzyme Activities and MDA Content in Isolated Perfused Rat Heart*



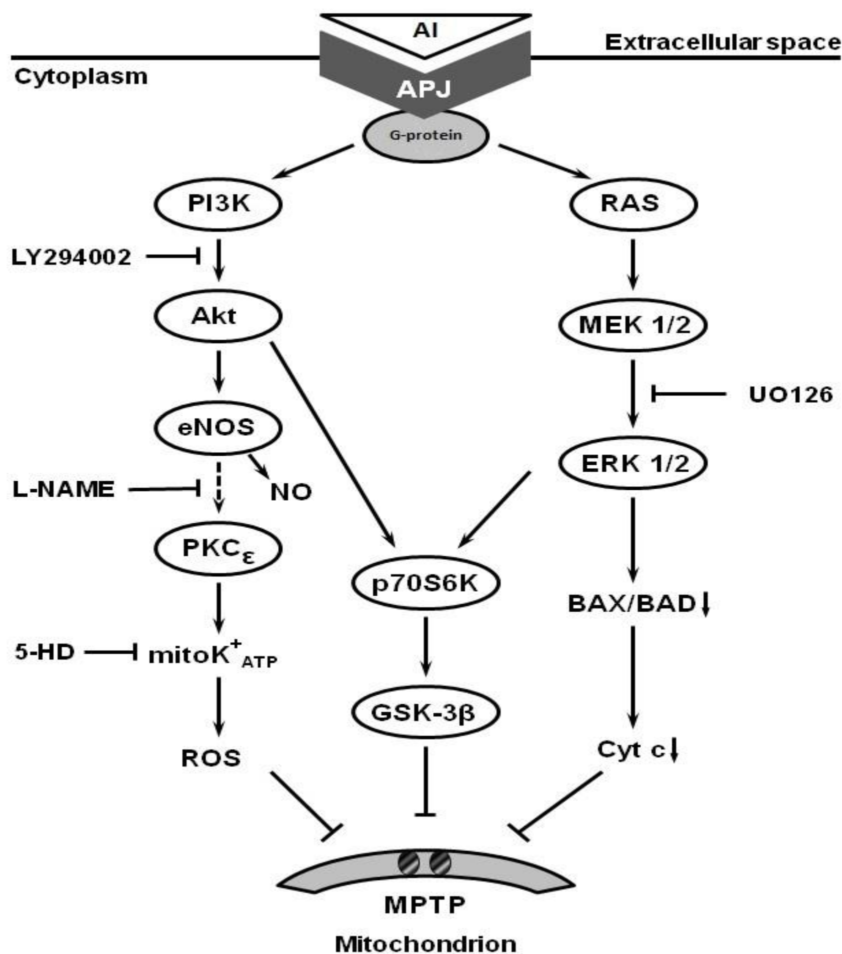
Note: The values are expressed as means ± SEM of eight experiments. St. st., steady state; Cont., control; A12, infusion of 140 μM A12; AI, infusion of 140 μM AI. *P<0.05 vs steady state; $^{\#}$P<0.05 vs control; $^{+}$P<0.05 vs A12.

Discussion

Myocardial I/R injury may lead to severe impairment of cardiac function, the deterioration of energy state of the heart, cardiomyocyte membrane damage and ultimately cell death (Turer and Hill 2010). The therapeutic strategies to attenuate these pathophysiologic events include the controlled reperfusion and administration of various interventions (Ferdinandy et al. 2007). The present study demonstrates that preischemic infusion of biologically active peptide AI, a structural analogue of natural A12, reduces functional and metabolic responses of isolated perfused rat heart to global ischemia and reperfusion. These beneficial effects are accompanied by less formation of hydroxyl radical adduct DMPO-OH and decreased LDH leakage in the myocardial effluent during the early reperfusion. Moreover, intracellular signaling triggered by AI is closely linked with the regulation of metabolic state and membrane integrity of a reperfused heart. Indeed, the inhibitors of MEK1/2 and PI3K (UO126 and LY294002) suppressed the protective efficacy of AI impairing functional and metabolic recovery of the heart and increasing membrane damage. This finding

is consistent with previous studies, which demonstrated that natural apelin-13 and apelin-36 is reduce both necrosis and apoptosis via MEK1/2–ERK1/2 and PI3K/Akt signaling (Simpkin et al. 2007, Zeng et al. 2009, Tao et al. 2011). Therefore, PI3K and MEK1/2, the upstream kinases of the RISK pathway, are the principal intracellular molecules involved in the protective effects of AI (Figure 5).

Figure 5. *The Binding of Peptide AI with APJ Receptor Triggers the RISK Pathway Preventing the opening of Mitochondrial Permeability Transition Pores (MPTP) in Cardiomyocytes during Reperfusion*



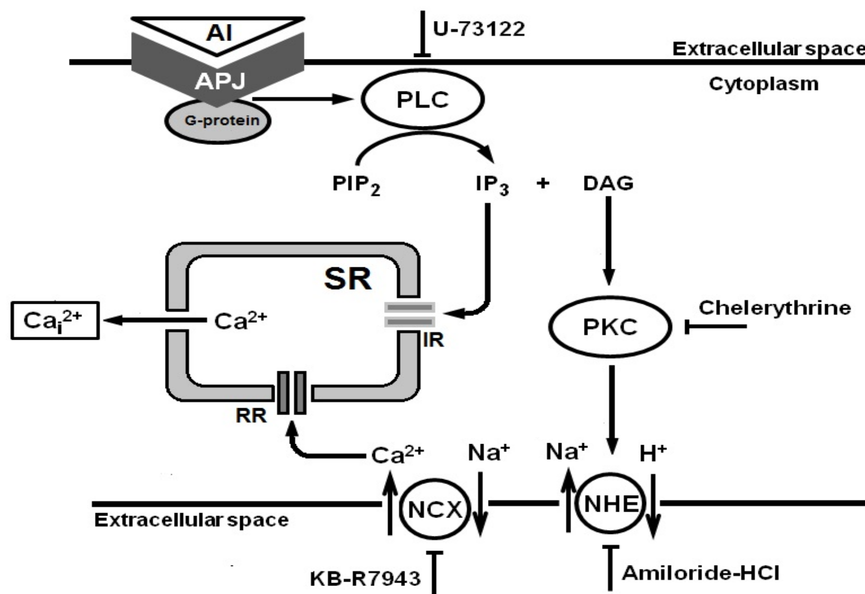
Note: Cardioprotective effects of AI is blocked in the presence of the inhibitors of intracellular signaling LY294002, L-NAME, 5-HD (5-hydroxydecanoate) and UO126. PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; eNOS, endothelial NO synthase; PKCε, ε isoforms of PKC; mito$K^{+}_{ATP,}$ mitochondrial ATP-dependent K^{+}-channels; ROS, reactive oxygen species; P70S6K, ribosomal protein S6 kinase; GSK-3β, glycogen synthase kinase 3β; RAS, a superfamily of signaling proteins; MEK1/2, mitogen activated extracellular regulated kinase 1/2; ERK1/2, extracellular signal regulated kinase 1/2; BAX/BAD, proapoptotic proteins; Cyt c, cytochrome c.

Our work demonstrates an important role of PLC in the mechanisms of AI action, since U-73122, a specific inhibitor of PLC, blocked cardioprotection. We believe that AI binding to the APJ receptor stimulates PLC, leading to the hydrolysis of phosphatidylinositol 4,5-biphosphate to inositol triphosphate (IP_3) and diacylglycerol (DAG), followed by the activation of protein kinase C (PKC) by DAG. As it is known, PKC activation is a crucial mechanism of protection against contractile dysfunction by preserving the diastolic function in postischemic hearts (Cave and Apstein 1996). On the other hand, elevation of IP_3 in the cytosol triggers the release of Ca^{2+} via the ryanodine receptor and the IP3 receptor from the sarcoplasmic reticulum, thus improving an impaired calcium handling in cardiomyocytes during I/R injury (Berridge 2009). It has been recently demonstrated that a specific PKCε inhibitor, 1V1-2, abolished the effect of apelin-13 on recovery of contractile function and increased LDH release during reperfusion on a rat model of myocardial I/R injury ex vivo (Wang et al. 2013). These data support our pervious findings on involvement of PKC in cardioprotective effects of AI obtained with chelerythrine, a selective inhibitor of group A and B *PKC isoforms* (Pisarenko et al. 2015). Activated PKC can phosphorylate a wide spectrum of cellular proteins including the sarcolemmal Na^+/H^+ exchanger (Karmazyn et al. 1999). Stimulation of Na^+/H^+ exchange may result in an increase in intracellular pH and sensitization of cardiac myofilaments to intracellular Ca^{2+}. Additionally, accumulation of intracellular Na^+ can promote a rise in intracellular Ca^{2+} via the reverse mode of the *operation* of the Na^+/Ca^{2+} *exchanger (Pisarenko et al. 2015, Kentish 1999).* In our experiments, KB-R7943, a selective inhibitor of the reverse mode Na^+/Ca^{2+} *exchange* markedly suppressed the AI-induced improvement of cardiac function, metabolic recovery and membrane integrity (Figure 6). This fact implies that the activation of Na^+/Ca^{2+} exchange contributes to the impact of the peptide. Therefore, the results obtained by us and other authors in perfused rat hearts indicate that enhanced functional and metabolic recovery induced by apelin peptides involves signaling via PLC and PKC, and the activation of sarcolemmal Na^+/H^+ and Na^+/Ca^{2+} exchange. This mechanism may play a pivotal role in reducing abnormalities in intracellular Ca^{2+} transients in I/R injury.

The results of the present study confirm the involvement of NO in cardioprotective effects of AI although the inhibitory effects of L-NAME on myocardial metabolism and release of LDH were poor than that of UO126 or LY294002. These data are consistent with earlier studies, which have shown that NO is implicated in endothelium-dependent vasorelaxation triggered by natural apelin peptides (Lee et al. 2000, Cheng et al. 2003). This effect is blocked by L-NAME, indicating that the peptides exert vasodilation via the activation of eNOS pathway (Tatemoto et al. 2001). Besides the enhancement of NO production by the activation of eNOS in the RISK pathway, the C-terminal fragments of natural apelin are capable to increase vascular eNOS mRNA level with remarkable increase of eNOS protein (Jia et al. 2007). This finding indicates that apelin peptides upregulate eNOS gene expression in vessels, probably the analogue AI may initiate a similar effect in the

myocardium. In the context of our work, it is important that apelin-induced increase in NO bioavailability was accompanied by reduction in ROS generation in studies employing models of diabetes and atherosclerosis (Zhong et al. 2007, Chun et al. 2008). Since NO prevents mitochondrial oxygen damage and lipid peroxidation (Shultz et al. 2004), we can assume that the antioxidant properties of peptide AI in our model of I/R injury could be associated with increased NO formation. Formation of NO promotes the opening of mitochondrial ATP- dependent K^+-channels (mitoK_{ATP}) via guanylyl cyclase-cGMP cascade and the activation of protein kinases G and Cε (Oldenburg et al. 2002). The mitoK_{ATP} channel opening prevents the opening of mPTP being a trigger and an effector of cardioprotection. Effects of 5-hydroxydecanoate (5HD), the mitoK_{ATP} blocker, and peptide AI were explored by us recently in an ex vivo and in vivo models of myocardial I/R injury (Pisarenko et al. 2017). 5HD administration abolished the beneficial effects of MA on cardiac function recovery, limitation of infarct size and the sarcolemma integrity reducing restoration of myocardial energy state during reperfusion. Thus, mitoK_{ATP} are an important component of the cardioprotective mechanisms of AI. Taken together, these experimental facts convincingly demonstrate the importance of NOS-dependent mechanisms in AI-induced attenuation of myocardial I/R injury.

Figure 6. *Activation of Phospholipase C (PLC) by peptide AI via G-Protein Causes an Increase in Cytosolic Ca^{2+} Concentration Enhancing Myocardial Inotropy*



Note: Inotropic action of AI is reduced in the presence of the inhibitors of PLC, PKC and the sarcolemmal Na^+/H^+ and *Na^+/Ca^{2+} exchange (U-73122, chelerythrine, amiloride-HCl and KB-R7943, respectively). PiP_2,* phosphatidylinositol 4,5-bisphosphate*; IP_3,* inositol 1,4,5-trisphosphate*; DAG,* diacylglycerol*; SR,* sarcoplasmic reticulum; *IR,* IP_3 receptor*; RR,* ryanodine channel receptor*; PKC, protein kinase C; NCX,* Na^+/Ca^{2+} exchanger*; NHE,* Na^+/H^+ exchanger.

It is well recognized that the mechanisms of I/R injury include an overproduction of ROS (Murphy and Steenbergen 2008). The interaction of ROS with cell membrane lipids and essential proteins contribute to myocardial cell damage, leading to irreversible tissue injury with the concomitant depletion of endogenous antioxidant enzymes (Dhalla et al. 2000). The present study demonstrates that exogenous peptide AI enhances antioxidant defense against myocardial I/R injury. This is manifested by an increase in the enzymatic activity of myocardial Cu,Zn SOD, CAT and GSH-Px and the inhibition of lipid peroxidation. In addition, the peptide is capable to decrease the generation of short-lived ROS as proved by the attenuation of the DMPO-OH adduct formation in myocardial effluent of the isolated rat heart. The obtained results are principally consistent with the previously described antioxidant properties of unmodified C-terminal fragments of apelin. Apelin-13 treatment ameliorated the inhibited SOD activity and reduced ROS formation in cultured cardiomyocytes subjected to hypoxia/reoxygenation (Zeng et al. 2009). In isoproterenol treated rats, the administration of exogenous apelin-13 decreased the LDH activity in plasma and myocardial MDA content (Jia et al. 2006). Activation of CAT by [Pyr^1]apelin-13 prevented oxidative stress-linked hypertrophy in cultured rat cardiomyocytes (Foussal et al. 2010). Reasons for the increase in antioxidant enzyme activities under the influence of apelin peptides as well as their direct antioxidant action remain unexplored. To our knowledge, only the fact that [Pyr^1]apelin-13 induced CAT mRNA expression in cultured rat cardiomyocytes (Foussal et al. 2010). Exploration of the nature of antioxidant properties of these compounds undoubtedly deserves further expanded research is mentioned.

The fact that the modification of the natural peptide A12 does not impair its antioxidant capacity seems important. Both peptides enhance the antioxidant enzymes activity and attenuate hydroxyl radical adducts formation during reperfusion almost equally. However, the advantages of analogue AI may be more apparent in situations with a prolonged exposure to peptides due to its higher proteolytic stability. The half-life of AI in human blood plasma is ten times more than that of the natural peptide (unpublished data). Additionally, this peptide reduces the mean arterial pressure and HR to a significantly lesser degree than A12 when administered intravenously (Pisarenko et al. 2013) and has greater storage stability.

In conclusion, the results of this study provide the evidence that the structural apelin analogue AI attenuates excessive ROS production and preserves myocardial metabolic status and cell membrane integrity in a rat model of myocardial I/R injury. These beneficial properties are combined with the improvement of cardiac function recovery during reperfusion. We propose that the use of analogue AI may be promising in cardiac surgery (as an additive to cardioplegic and reperfusion solutions) and treatment of ischemic heart disease.

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