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Signaling pathways of a structural analogue of apelin-12 involved in myocardial protection against ischemia/reperfusion injury

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ABSTRACT

Exogenously administered chemically modified apelin-12 (MA) has been shown to exhibit protective effects in myocardial ischemia/reperfusion (I/R) injury. They include reduction of ROS formation, cell death and cardiometabolic abnormalities. The aim of the present study was to explore the role of the underlying signaling mechanisms involved in cardioprotection afforded by MA. Isolated perfused working rat hearts subjected to global ischemia and anaesthetized rats in vivo exposed to LAD coronary artery occlusion were used. Myocardial infarct size, cell membrane damage, cardiac dysfunction and metabolic state of the heart were used as indices of I/R injury at the end of reperfusion. Administration of specific inhibitors of MEK1/2, PI3K, NO synthase (NOS) or the mitochondrial ATP-sensitive K^+ (mito K_{ATP}) channels (UO126, LY294002, L-NAME or 5-hydroxydecanoate, respectively) reduced protective efficacy of MA in both models of I/R injury. This was evidenced by abrogation of infarct size limitation, deterioration of cardiac function recovery, and attenuation of metabolic restoration and sarcolemmal integrity. An enhancement of functional and metabolic recovery in isolated reperfused hearts treated with MA was suppressed by U-73122, chelerythrine, amiloride or KB-R7943 (inhibitors of phospholipase C (PLC), protein kinase C (PKC), Na^+/H^+ or Na^+/Ca^{2+} exchange, respectively). Additionally, co-infusion of MA with amiloride or L-NAME reduced the integrity of cell membranes at early reperfusion compared with the effect of peptide alone. In conclusion, cardioprotection with MA is mediated by signaling via PLC and survival kinases, PKC, PI3K, and MEK1/2, with activation of downstream targets, NOS and mito K_{ATP} channels, and the sarcolemmal Na^+/H^+ and Na^+/Ca^{2+} exchangers.

1. Introduction

The adipokine apelin and its G protein-coupled receptor APJ are involved in signaling pathways implicated in numerous cardiac and vascular functions [1]. Apelin is produced as a 77 amino acid prepropeptide which is cleaved to shorter biologically active C-terminal fragments including apelin-36, -19, -17, -13 (or its pyroglutamate analogue [Pyr]^1-apelin-13) and -12 [2]. C-terminal apelin fragments and APJ receptor have been shown to play an important role in the maintenance of cardiovascular homeostasis and protection against ischemia/reperfusion (I/R) injury. Apelin-12, 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 [3–5]. Post-infarct treatment with [Pyr]^1-apelin-13 decreases infarct size and attenuates cardiac tissue injury in rats in vivo [6]. In cultured cardiomyocytes, apelin-13 suppresses apoptosis induced by glucose deprivation [7]. Involvement of apelin-13 in stabilizing cell membranes of the myocardium and reducing formation of reactive oxygen species (ROS) were demonstrated in I/R of rat heart in vivo [8]. This peptide maintains sarcoplasmic reticulum function and Ca^{2+} transient in cardiomyocytes during reperfusion of rat heart [9]. The beneficial effects of C-terminal apelin fragments are attributed to mobilization of the PI3K–Akt and MEK1/2–ERK1/2 salvage kinases, and inhibition of the mitochondrial permeability transition pore (mPTP) opening [3,10]. Phosphorylation and activation of endothelial nitric oxide synthase (eNOS) are also implicated in myocardial protection afforded by apelin [8,11]. Some studies imply possibility of activation of transmembrane Na^+/H^+ and Na^+/Ca^{2+} exchange by apelin-13 [12]. Therefore, apelin peptides

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Table 1
Structure of apelin-12 and its analogue MA.

H-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe-OH	Apelin-12
H-(**N$^{\alpha}$Me**) Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-**Nle**-Pro-Phe-OH	MA

The substitutions are shown in bold.

are potent endogenous signals for triggering multiple protective mechanisms in myocardial I/R stress and may represent a new class of potential therapeutic agents. However, development of pharmacological agonists of APJ receptor is expedient to eliminate the limitations related to the short circulating half-life of natural apelin peptides [13,14].

Earlier Hamada et al. synthesized cyclic apelin-12 analogues [15]. These compounds act as the effective APJ agonists and activate Akt and extracellular signal-regulated kinase (ERK1/2) in cell-based in vitro assays but not in the physiologically relevant models. The first non-peptidic agonist of APJ receptor, namely E339-3D6, is able to induce the vasorelaxation of rat aorta precontracted with norepinephrine and suppress systemic vasopressin release in mice when intracerebroventricularly injected [16]. More recent studies directly indicate the possibility of reducing myocardial I/R injury by modified apelin peptides. Thus, a 40 kDa polyethylene glycol N-terminal conjugated apelin-36 (PEG-apelin-36) was examined in rats with myocardial infarction (MI) [17]. The PEG-apelin-36 had a prolonged circulating life compared to apelin-36 and its infusion significantly increased cardiac ejection fraction in rats with MI. Two modified [Pyr]^{1}-apelin-13 analogues, more resistant to cleavage by angiotensin-converting enzyme 2 than natural [Pyr]^{1}-apelin-13, were synthesized by Wang et al., and their effects were tested in mice models of myocardial I/R injury [18]. One of these analogues improved postischemic functional recovery of perfused hearts and reduced MI size compared with control due to activation of survival pathways and promotion of angiogenesis.

Previously, we synthesized structural analogues of apelin-12 more resistant to degradation by proteolytic enzymes and having greater storage stability in comparison with the natural peptide apelin-12 [19]. These bioactive compounds were evaluated on various models of I/R injury, including cultured cardiomyocytes, isolated perfused rat hearts and anaesthetized rats in vivo. Their protective effects were comparable to the action of natural apelin-12 and apelin-13 and tightly related to upregulation of cardiac antioxidant defense systems and attenuation of lipid peroxidation and ROS production [20,21]. Nevertheless, the underlying signaling mechanisms of structural analogues of apelin-12 remain obscure. The objective of the present study was to characterize the role of phospholipase C (PLC), protein kinase C (PKC), the components of the reperfusion injury salvage kinase (RISK) pathway, PI3K/Akt and MEK1/2–ERK1/2, and NOS in reduction of I/R injury by the most efficient structural analog of apelin-12 (hereinafter MA, modified apelin-12, Table 1). In addition, we assessed the significance of the mitochondrial ATP-sensitive K^+ (mito K_{ATP}) channels, and Na^+/H^+ and Na^+/Ca^{2+} exchangers as the end-effectors of MA signaling involved in postischemic cardiac function recovery. Myocardial infarct size, cell membrane damage, cardiac dysfunction and metabolic state of the heart were used as indices of myocardial I/R injury.

2. Materials and methods

2.1. Chemicals

Peptide MA was synthesized by the automatic solid phase method using an Applied BioSystems 431A peptide synthesizer (Germany) and Fmoc technology (Table 1). It was purified by preparative HPLC and identified by ^{1}H NMR spectroscopy and mass spectrometry [19]. Enzymes and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO USA). Inhibitors were acquired from Selleckchem (Munich, Germany) and Abcam Biochemicals (Cambridge, UK). Solutions were prepared using deionized water (Millipore Corp. Bedford, MA, USA).

2.2. Animals

Male Wistar rats weighing 300–340 g were housed in cages in groups of three, maintained at 20–30 °C with a natural light-dark cycle. All animals had free access to standard pelleted diet (Aller Petfood, St. Petersburg, Russia) and tap water. The care and use of the animals were conducted in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (No. 123 of 18 March 1986).

2.3. Perfusion of isolated rat hearts

Rats were heparinized by intraperitoneal injection (1600 IU/kg body weight) and anaesthetized with urethane (1.3 g/kg body weight). Hearts were perfused with Krebs–Henseleit buffer (KHB) as previously described [20]. 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 contractile function intensity index was calculated as the LV developed pressure-heart rate product (LVDP × HR), where LVDP is the difference between LV systolic and LV end-diastolic pressure. Cardiac pump function was assessed by cardiac output (CO), the sum of aortic output and coronary flow.

2.4. Experimental protocol for perfused hearts

The steady state values of cardiac function were recorded after preliminary 20 min of 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. Isolated hearts were randomly assigned onto one of ten groups:

(1) Control (n = 12). After preliminary working perfusion, a 5-min infusion of KHB without additives was applied at a constant flow rate of 4 ml/min before global ischemia. Then the hearts were subjected to 35 min of normothermic global ischemia followed by 5 min of Langendorff perfusion at a flow rate of 4 ml/min with subsequent 25 min of working reperfusion
(2) MA (n = 12). After preliminary working perfusion, a 5-min infusion of 140 μM MA at a constant flow rate of 4 ml/min was used. Further procedure was the same as for the control group. Previously we have shown that such way of MA administration is optimal for cardiac function recovery in this protocol [19]. Peptide MA was dissolved in KHB immediately prior to the infusion.
(3) U + MA (n = 12). A 5-min infusion of 108 μM U-73122 hydrate, a phospholipase C (PLC) inhibitor, and 140 μM MA at a flow rate of 4 ml/min was used prior to global ischemia.
(4) Che + MA (n = 12). A 5-min infusion of 110 μM chelerythrine, the specific protein kinase C (PKC) inhibitor and 140 μM MA, at a flow rate of 4 ml/min was used prior to global ischemia.
(5) Am + MA (n = 12). A 5-min infusion of 150 μM amiloride hydrochloride, α selective inhibitor of Na^+/H^+ exchange, and 140 μM MA at a flow rate of 4 ml/min was used prior to global ischemia.
(6) KB + MA (n = 12). A 5-min infusion of 110 μM KB-R7943, an inhibitor of Na^+/Ca^{2+} exchange, and 140 μM MA at a flow rate of 4 ml/min was used prior to global ischemia.

(7) UO + MA (n = 12). A 5-min infusion of 150 μM UO126, a selective inhibitor of MAPK MEK1/2, and 140 μM MA at a flow rate of 4 ml/min was applied before global ischemia.
(8) LY + MA (n = 12). A 5-min infusion of 110 μM LY294002, a selective inhibitor of phosphatidylinositol 3-kinase (PI3K), and 140 μM MA at a flow rate of 4 ml/min was used prior to global ischemia.
(9) N + MA (n = 12). A 5-min infusion of 128 μM L-NAME, the non-selective NO synthase (NOS) inhibitor, and 140 μM MA at a flow rate of 4 ml/min was applied prior to global ischemia.
(10) HD+MA (n = 12). A 5-min infusion of 120 μM 5-hydroxydecanoate, the mitoK_{ATP} channels blocker, and 140 μM MA at a flow rate of 4 ml/min was used prior to global ischemia.

In groups 3–10, further protocol was the same as for the control group. Inhibitors U, Che, UO and LY were dissolved in dimethyl sulfoxide (DMSO). Effects of the inhibitors and the vehicle (0.5% DMSO) on cardiac function were studied in preliminary experiments. The used concentrations of the inhibitors and 0.5% DMSO did not affect recovery of cardiac function compared with control.

At the end of reperfusion, the hearts were freeze-clamped in liquid nitrogen for metabolite analysis. To determine the initial content of metabolites, the hearts were freeze-clamped in liquid nitrogen after the preliminary working perfusion (steady state). The myocardial effluent was collected in ice-cold tubes during both periods of Langendorff perfusion for immediate assessment of lactate dehydrogenase (LDH) activity.

2.5. Anesthetized rats in vivo

Rats were anesthetized with 20% urethane (1200 mg/kg body wt i.p.) and artificially ventilated with a KTR-5 animal respirator (Hugo Sacks Electronik) with a volume of 2–3 ml at a rate of 70–75 breaths/min. Further preparation of animals was performed as described in [22]. The chest was opened by a left thoracotomy in the fifth intercostal space, and the heart was exposed by removing the pericardium. After pericardiotomy, a 5-0 prolene ligature was placed under the left anterior descending (LAD) coronary artery where it emerges from beneath the left atrial appendage and the ends were threaded through a small plastic tube to form a snare for reversible LAD coronary artery occlusion. Complete LAD coronary artery occlusion was confirmed by observing cyanosis of the myocardium as well as the ST-segment elevation and immediate fall in MAP by 15–30 mm Hg. Arterial blood pressure was recorded with a pressure transducer (Statham p23Db, Oxnard, USA) using a polygraph Biograph-4 (St. Petersburg, Russia). The MAP, HR and standard lead II ECG were recorded on a computer using a LabVIEW 7.1 data acquisition system (National Instruments, USA).

2.6. Experimental design for anesthetized rats

Acute myocardial infarction was induced as described previously [22]. After 30-min stabilization of hemodynamic parameters (steady state), LAD coronary artery was occluded for 40 min to simulate regional ischemia; the duration of subsequent reperfusion was 1 h. The prepared animals were randomly assigned onto one of six groups:

(1) Control (n = 8). After the period of LAD coronary artery occlusion, 0.5 ml of saline was administrated by i.v. bolus injection at the onset of reperfusion.
(2) MA (n = 8). MA (0.35 μmol/kg) was administrated by i.v. bolus injection at the onset of reperfusion. Previously we have found that this dose is optimal for MA [22].
(3) UO + MA (n = 8). UO126 (22 mg/kg) and MA (0.35 μmol/kg) were administrated by i.v. bolus injection at the onset of reperfusion.
(4) LY + MA (n = 8). LY294002 (0.3 mg/kg) was injected intraperitoneally 10 min before reperfusion, MA (0.35 μmol/kg) was administrated by i.v. bolus injection at the onset of reperfusion.
(5) N + MA (n = 8). L-NAME (10 mg/kg) was injected i.v. 10 min before reperfusion, MA (0.35 μmol/kg) was administrated by i.v. bolus injection at the onset of reperfusion.
(6) HD + MA (n = 8). 5-Hydroxydecanoate (10 mg/kg) was injected i.v. 5 min before reperfusion, MA (0.35 μmol/kg) was administrated by i.v. bolus injection at the onset of reperfusion.

The peptide MA was dissolved in saline before administration. Effects of inhibitors UO, LY, N and HD on myocardial infarct size and activity of necrosis markers were assessed in separate series of experiments. At the end of the reperfusion, LAD coronary artery was reoccluded and 2 ml of 2% Evans Blue (Sigma, USA) solution was injected through the jugular vein to distinguish the myocardial non-ischemic area from area at risk (AAR). In separate series of experiments with N, the myocardial AAR was freeze-clamped in liquid nitrogen after steady state or at the end of reperfusion for metabolite analysis.

2.7. Determination of myocardial infarct size

After staining with Evans Blue, the heart was excised, and the LV was transversely cut into 1.5 mm thick slices which were incubated in 0.1 M sodium phosphate buffer pH 7.4, containing 1% 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma, USA) 10 min at 37 °C. The AAR was stained to deep red, infarct tissue grey white, and non-ischemic area blue. The slices were fixed in 10% formalin for 5 min. Then they were placed between two transparent glasses and captured using a scanner at 600 dpi resolution; the saved images were analyzed by computerized planimetry using Imagecal software. The slices were then weighed for determination of LV weight. The AAR was expressed as a percentage of LV weight, the infarct size (IS) was expressed as a percentage of the AAR in each group [22].

2.8. Determination of necrosis markers

At the end of the steady state and reperfusion, blood samples were collected for plasma separation. Plasma LDH activity was determined enzymatically with pyruvate as substrate by using standard kits from BioSystems S.A. (Barcelona, Spain). Plasma CK-MB activity was assessed by an immunoinhibition method using standard kits from BioSystems S.A. (Barcelona, Spain) from the rate of NADPH formation by means of the hexokinase and glucose-6-phosphate dehydrogenase coupled reactions.

2.9. Analysis of metabolites

Frozen isolated perfused hearts or AAR excised from the LV were quickly homogenized in cooled 6% $HClO_4$ (10 ml/g) using an Ultra-Turrax T-25 homogenizer (IKA-Labortechnik, Staufen, Germany), and the homogenates were centrifuged at 2800 × g for 10 min at 4 °C. The supernatants were then neutralized with 5 M K_2CO_3 to pH 7.40, and the 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), creatine (Cr) and lactate in neutralized tissue extracts were determined spectrophotometrically by enzymatic methods [23].

Table 2
Inhibition of PLC, the RISK pathway or Na^+/H^+ exchange reduces metabolic protection of reperfused rat heart induced by MA.

	ATP	ΣAN	EC	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*	16.90 ± 0.99*	0.58 ± 0.03*	12.69 ± 1.59*	6.93 ± 1.29*
MA	14.59 ± 1.03*,**	23.56 ± 0.95**	0.75 ± 0.02*,**	15.20 ± 1.41*,**	1.47 ± 0.24**
U + MA	7.27 ± 0.90*,***	17.88 ± 1.42*,***	0.54 ± 0.03*,***	8.56 ± 0.80*,**,***	8.89 ± 1.40*,***
Am + MA	8.74 ± 1.45*,***	17.08 ± 1.06*,***	0.64 ± 0.03*,***	12.65 ± 1.96*	6.81 ± 2.31*,***
UO + MA	7.38 ± 2.37*,***	16.79 ± 1.33*,***	0.62 ± 0.01*,***	9.82 ± 2.43*	6.57 ± 1.10*,***
LY + MA	8.03 ± 0.67*,***	16.68 ± 2.24*,***	0.63 ± 0.02*,***	10.07 ± 2.53*	7.27 ± 1.53*,***
N + MA	10.82 ± 0.25*,**,***	18.68 ± 1.34*,***	0.69 ± 0.03*	11.69 ± 2.03*	4.74 ± 0.30*,***

Values are the mean ± SEM of 8 experiments and expressed in μmol/g dry wt. for metabolites. ΣAN = ATP + ADP + AMP. The energy charge (EC) = (ATP + 0.5ADP)/ΣAN. St. state, steady state; MA, modified apelin-12; U, U-73122 hydrate, a PLC inhibitor; Am, amiloride hydrochloride, an inhibitor of Na^+/H^+ exchange; UO, UO126, an inhibitor of MAPK MEK1/2; LY, LY294002, a PI3K inhibitor; N, L-NAME, a NOS inhibitor.
* $p < 0.05$ vs. steady state.
** $p < 0.05$ vs. control.
*** $p < 0.05$ vs. MA.

2.10. 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. Comparisons between two groups involved use of the Student's unpaired *t*-test. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Functional recovery of isolated rat hearts after global ischemia

Cardiac function indices of isolated perfused rat heart at the end of reperfusion were considerably depressed in the control group. Thus recovery of LVDP × HR product and CO was 45, and 30% as compared with the steady state values, respectively; LV diastolic pressure (LV P_d) was significantly higher the initial value (10 ± 1 vs. −3 ± 1 mm Hg, respectively, Fig. 1). Preischemic MA infusion enhanced recovery of LVDP × HR product and CO by 27 and 37% compared with control, respectively. This effect was accompanied by a reduction of LV P_d up to 3 ± 1 mm Hg. The co-infusion of a PLC inhibitor, a PKC inhibitor and inhibitors of Na^+/H^+ or Na^+/Ca^{2+} exchange (U, Che, Am or KB, respectively) along with MA substantially reduced protective effects of MA on recovery of LVDP × HR product and CO and significantly raised LV P_d by the end of reperfusion. Similarly the co-infusion of MA with inhibitors of MEK1/2, PI3K, NOS or the mito K_{ATP} channels blocker (UO, LY, N or HD, respectively) decreased recovery of LVDP × HR product and CO during reperfusion (Fig. 2). In these groups, LV P_d markedly increased and did not differ significantly from the control. The infusion of the inhibitors alone did not aggravate recovery of cardiac function indices compared with the control.

3.2. The metabolic state of isolated rat hearts at reperfusion

Since the effects of the inhibitors on postischemic functional recovery of the hearts were similar, we selectively assessed action some of them on myocardial metabolic state and membrane integrity 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 aerobic metabolism at the end of reperfusion. A dramatic decrease in myocardial ATP content was accompanied by a reduction of the ΣAN pool and the energy charge (EC) (to 30, 65 and 64% of the steady state values, respectively). PCr content was 52% of the steady state value, while myocardial lactate was four times higher. Preischemic infusion of MA enhanced restoration of ATP two times, completely restored ΣAN content and significantly increased EC in reperfused hearts compared with control. These effects were combined with reduction of myocardial lactate content to the initial value. MA infusion slightly increased recovery of PCr compared with control. Inhibition of PLC, Na^+/H^+ exchanger, MEK1/2 or PI3K with U, Am, UO or LY, respectively, abrogated the beneficial effects of MA on restoration of myocardial ATP, ΣAN and EC at the end of reperfusion. In the U + MA group, PCr content was significantly lower than after administration of MA or the value in control. The co-infusions of MA with U, Am, UO or LY increased lactate content to the value in control. Coadministration of L-NAME and MA significantly impaired metabolic effects of the peptide but to a lesser extent compared with other inhibitors.

3.3. LDH leakage from isolated perfused rat hearts

Effects of inhibition of Na^+/H^+ exchange and NOS on LDH leakage were assessed in isolated rat hearts treated with MA before ischemia. LDH activity in the perfusate did not differ significantly between the groups before ischemia (Table 3). Therefore, infusion of MA alone or together with inhibitors Am or N 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 preischemic MA infusion compared with control, thus suggesting fewer membrane defects. However, the co-infusion of MA with Am or N increased LDH release after ischemia to the values that did not differ significantly from control.

3.4. Myocardial infarction in anesthetized rats in vivo

The AAR of the left ventricle (AAR/LV wt., %) was similar among all treated groups and did not differ significantly from the value

Table 3
Effects of infusion of MA and inhibitors of Na^+/H^+ exchange or NOS on LDH release in isolated rat heart before and after global ischemia.

	Before ischemia	After ischemia
Control	5.45 ± 0.78	11.79 ± 1.26**
MA	4.76 ± 0.82	6.53 ± 1.08*
Am + MA	5.65 ± 0.89	9.16 ± 1.12**
N + MA	6.15 ± 1.03	9.75 ± 1.33**

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. MA, modified apelin-12; Am, amiloride hydrochloride, an inhibitor of Na^+/H^+ exchange; N, L-NAME, a NOS inhibitor.
* $p < 0.05$ vs. control.
** $p < 0.05$ vs. the value before ischemia.

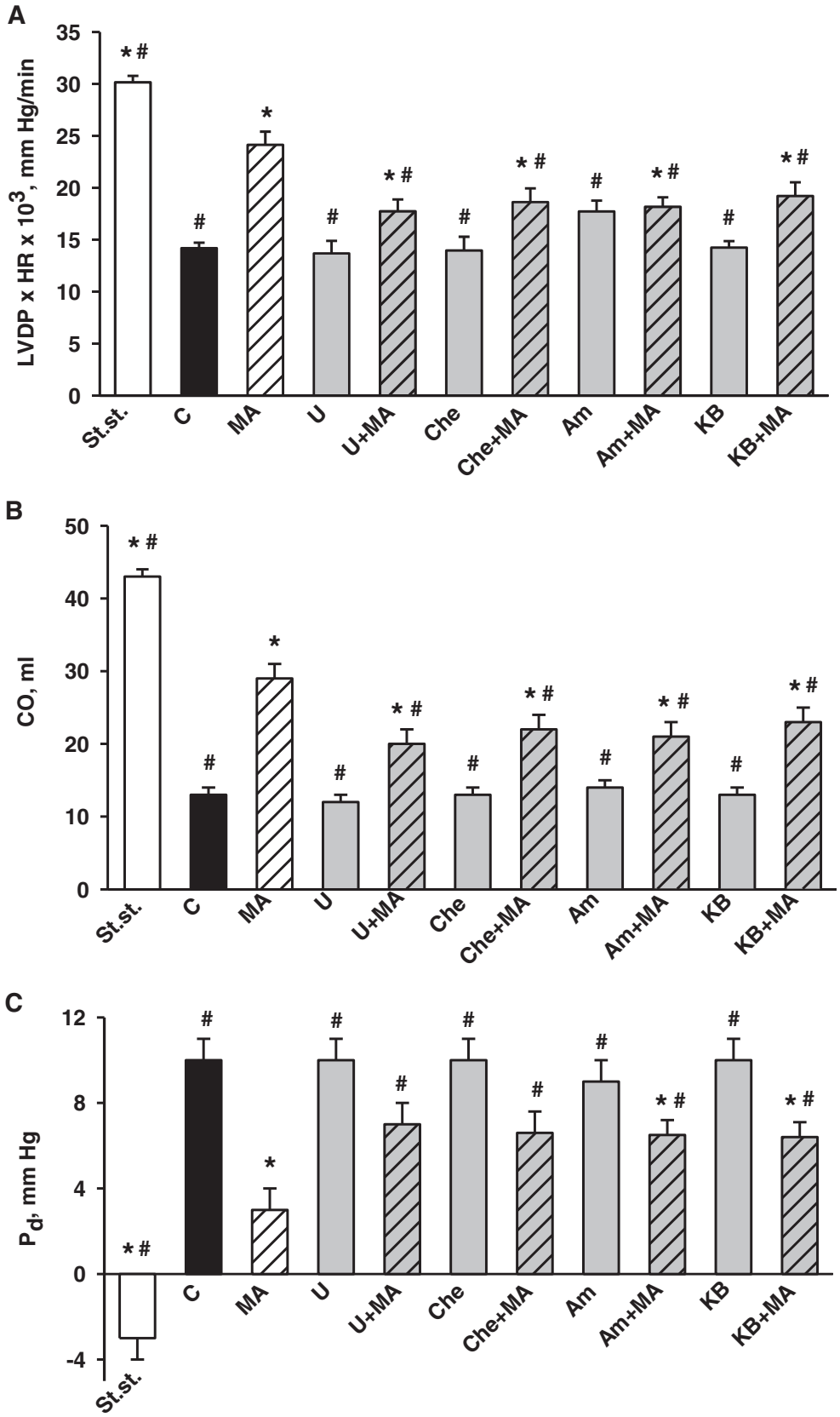

Fig. 1. Functional recovery of isolated rat heart during reperfusion induced by MA is suppressed in presence of signaling pathway inhibitors U and Che or ion transport blockers Am and KB. Left ventricular developed pressure-heart rate product, LVDP × HR (A), cardiac output, CO (B); left ventricular diastolic pressure, P_d (C). St.st., steady state; C, control; MA, modified apelin-12; U, U-73122 hydrate, a PLC inhibitor; Che, chelerythrine, a PKC inhibitor; Am, amiloride hydrochloride, ɑn inhibitor of Na^+/H^+ exchange; KB, KB-R7943, an inhibitor of Na^+/Ca^{2+} exchange. The values are expressed as means ± SEM for twelve experiments. $^*p < 0.05$ vs. control; $^\#p < 0.05$ vs. MA.

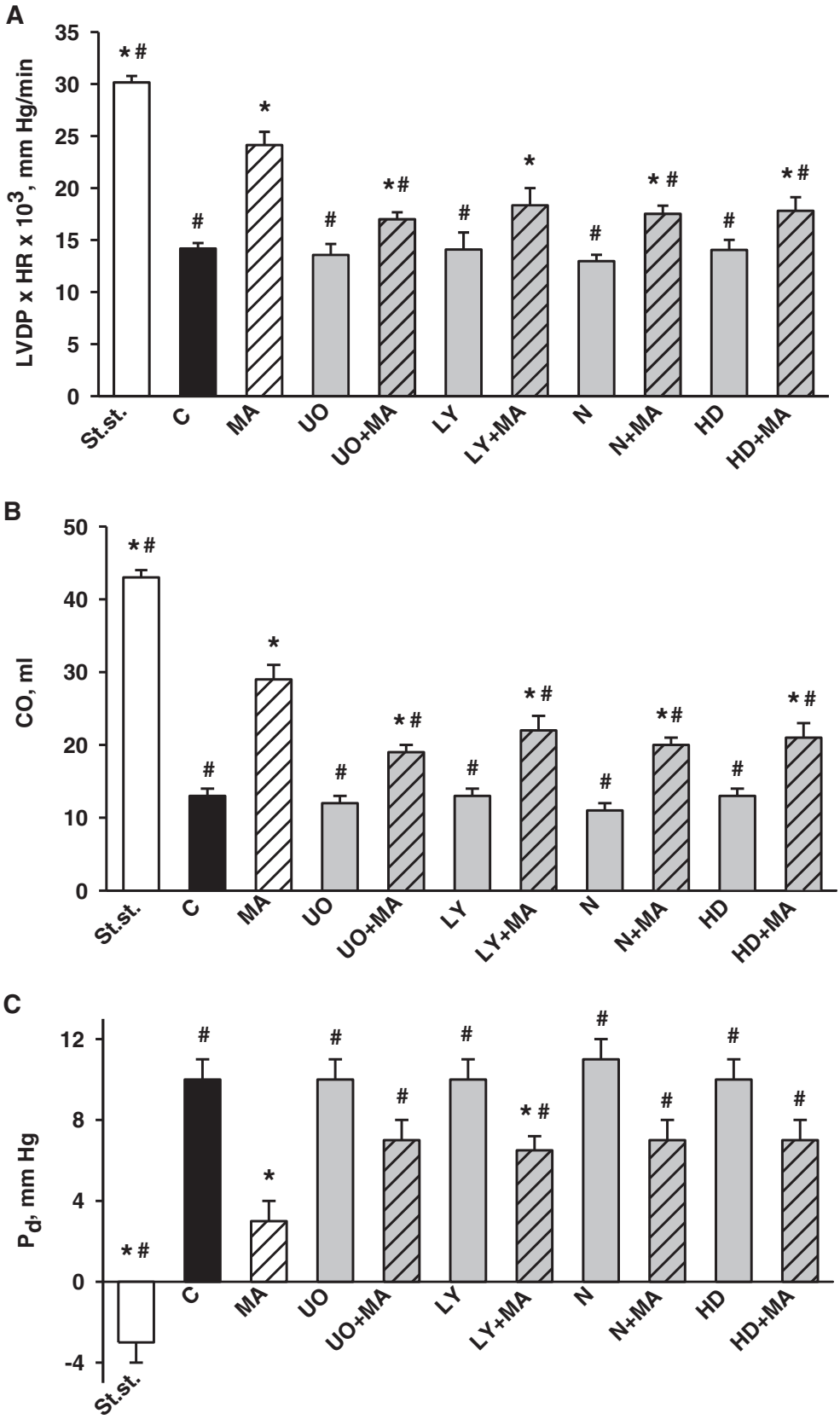

Fig. 2. Inhibitors of upstream (UO and LY) and downstream (N and HD) signals of the RISK pathway reduce protective effects of MA on functional recovery of isolated rat heart during reperfusion. Left ventricular developed pressure-heart rate product, LVDP × HR (A), cardiac output, CO (B); left ventricular diastolic pressure, P_d (C). St.st., steady state; C, control; MA, modified apelin-12; UO, UO126, an inhibitor of MAPK MEK1/2; LY, LY294002, a PI3K inhibitor; N, L-NAME, a NOS inhibitor; HD, 5-hydroxydecanoate, the mito K_{ATP} channels blocker. The values are expressed as means ± SEM for twelve experiments. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. MA.

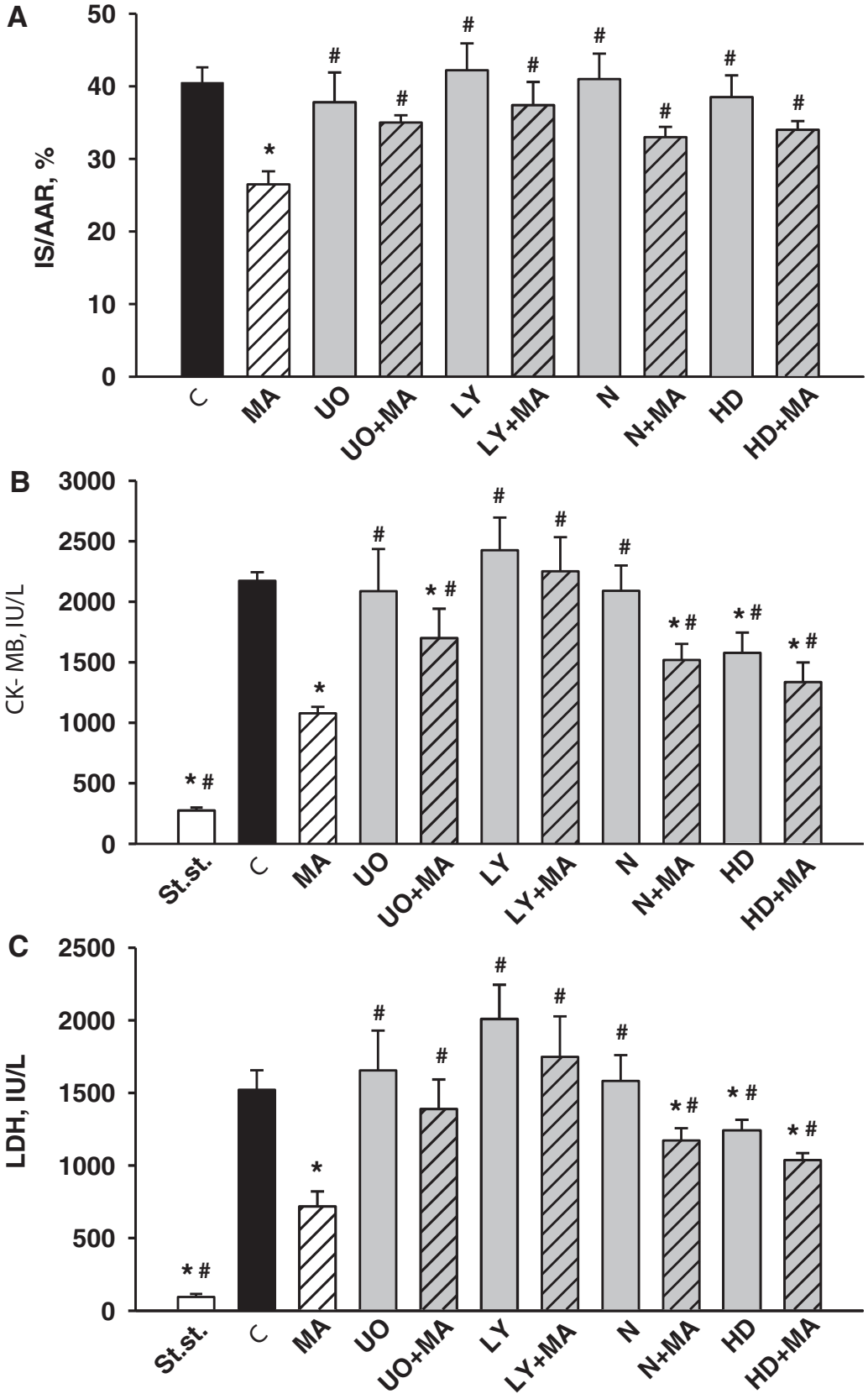

Fig. 3. Inhibitors of the RISK pathway reduce beneficial effects of MA on myocardial infarct size (IS/AAR,%, A) and plasma activity of necrosis markers CK-MB (B) and LDH (C) in rats in vivo. St.st., steady state; C, control; MA, modified apelin-12; UO, UO126, an inhibitor of MEK1/2; LY, LY294002, a PI3K inhibitor; N, L-NAME, a NOS inhibitor; HD, 5-hydroxydecanoate, the mito K_{ATP} channels blocker. The values are expressed as mean ± SEM of eight experiments. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. MA.

Table 4
Inhibition of NOS attenuates metabolic effects of MA on the AAR at the end of reperfusion in rats in vivo.

	Steady state	LAD reperfusion		
		Control	MA	N + MA
ATP	20.21 ± 1.13	5.84 ± 0.42*	9.41 ± 0.74*,**	6.28 ± 0.68*
ΣAN	27.18 ± 1.19	11.89 ± 1.02*	14.89 ± 0.96*	12.26 ± 0.43*,***
EC	0.85 ± 0.02	0.67 ± 0.01*	0.75 ± 0.01*,**	0.72 ± 0.01*
PCr	20.50 ± 1.77	6.72 ± 0.85*	18.92 ± 3.05**	12.02 ± 1.03*,**
ΣCr	66.62 ± 3.29	42.85 ± 1.31*	56.75 ± 2.89**	41.42 ± 1.26*,***
Lactate	0.67 ± 0.10	8.44 ± 0.73*	3.22 ± 0.34*,**	10.18 ± 1.14*,***

Metabolite contents are expressed as mean ± SEM in μmol/g dry wt. for 6 experiments. MA, modified apelin-12; N, L-NAME, a NOS inhibitor. ΣAN = ATP + ADP + AMP. The energy charge (EC) = (ATP + 0.5ADP)/ΣAN. Total creatine (ΣCr) = PCr + Cr.
* $p < 0.05$ vs. steady state.
** $p < 0.05$ vs. control.
*** $p < 0.05$ vs. MA.

in control (38.6 ± 1.60%). At the end of reperfusion, the percentage ratio of IS/AAR was 40.5 ± 2.1% in control (Fig. 3A). Treatment with MA reduced the percentage ratio of IS/AAR by 35% compared with control. Coadministration of inhibitors of upstream signals, such as UO or LY (inhibitors of MEK1/2 and PI3K, respectively) along with MA increased the percentage ratio of IS/AAR to the values close to control. Similarly, treatment with MA and antagonists of downstream signals N or HD (inhibitors of NOS or the mito K_{ATP} channels, respectively) significantly reduced infarct-limiting effect of the peptide. Administration of these inhibitors alone did not affect the percentage ratio of IS/AAR compared with control.

3.5. Plasma CK-MB and LDH activity

A plasma CK-MB activity was 275.0 ± 24.2 IU/l in the steady state (Fig. 3B). In the control animals, the CK-MB activity increased to 2173.4 ± 71.0 IU/l by the end of reperfusion. Intravenous administration of MA reduced the CK-MB activity twofold compared with control ($p < 0.05$). Coadministration of inhibitors of MEK1/2, PI3K, NOS or the mito K_{ATP} channels blocker (UO, LY, N or HD, respectively) and MA significantly increased the plasma CK-MB activity compared with MA treatment, although in the UO + MA, N + MA and HD + MA groups these values were lower than in control.

In the steady state, a plasma LDH activity of 94.4 ± 21.0 IU/l was observed (Fig. 3C). By the end of reperfusion, the plasma LDH activity increased by 16 times in the control animals. Administration of MA reduced the LDH activity by half compared with control. Treatment with MA in conjunction with inhibitors of MEK1/2, PI3K, NOS or the mito K_{ATP} channels blocker (UO, LY, N or HD, respectively) caused a significant increase in plasma LDH activity compared with MA administration. Moreover, in the case of coadministration of UO or LY and MA, the plasma LDH activity did not differ from the value in control.

3.6. The metabolic state of the area at risk

Metabolic effects of coadministration of downstream signals inhibitor N and MA are presented in Table 4. By the end of reperfusion, ATP and ΣAN pool in the AAR of the control animals were reduced by 4 and 2 times as compared with the steady state values, respectively. These alterations in the content of adenine nucleotides resulted in a reduction of EC by 20% compared with the preischemic value. The AAR of the control group exhibited poor recovery of PCr, dramatic loss of ΣCr and substantial increase in lactate content in comparison with the values before LAD coronary artery occlusion. Treatment with MA significantly increased ATP preservation and slightly enhanced ΣAN pool in the AAR compared with control. MA administration restored the contents of PCr and ΣCr almost to the steady state values and markedly reduced lactate accumulation in the AAR compared with control. Coadministration of N and MA attenuated protective action of MA on the energy state of the AAR. In the N + MA group, ATP, ΣAN pool and EC did not differ from the values in control; additionally ΣAN pool was preserved significantly worse than after treatment with MA. A clear trend to reduction of PCr content was combined with a profound decrease of ΣCr to the value close to the control. In the presence of N, lactate content increased in the AAR to the value in the control.

4. Discussion

The apelin/APJ system is involved in regulation of the cardiovascular system in coronary artery disease, and, therefore, represents an important therapy target [24]. The present study revealed, for the first time, intracellular signal transduction pathways of peptide MA, a structural analogue of apelin-12, that related to myocardial protection against I/R. Indeed, inhibitors of MEK1/2 and PI3K reduced protective efficacy of MA in both in vitro and in vivo models. This was evidenced by deterioration of cardiac function recovery, abrogation of infarct size limitation and attenuation of metabolic restoration when MA and LY or UO were administered together. PI3K and MEK1/2, the upstream kinases of the RISK pathway, are, therefore, the principal intracellular molecules involved in the effects of MA. This finding is consistent with previous studies, which demonstrated that cardioprotection with natural apelin-13 and apelin-36 is mediated via MEK1/2–ERK1/2 and PI3K/Akt signaling that triggers reducing both necrosis and apoptosis [3,8,25]. However, the protective effect of [Pyr]^1-apelin-13 in myocardial I/R injury is not abolished by wortmannin, the PI3K inhibitor, and thus may not depend on the cascade involving the activation of PI3K/Akt [4]. Alternatively other components of the RISK cascade, such as JNK, or prostacyclin may be activated by apelin and induce cardioprotection by preventing the opening of mPTP [26].

Phosphorylation of Akt and subsequent activation of mammalian target of rapamycin in cardiomyocytes leads to the activation of eNOS. In the present study, inhibition of NOS by N reduced the beneficial effects of MA in isolated rat hearts and in rats in vivo. Although the inhibitory effects of N on myocardial metabolism and release of LDH and CK-MB were poor than that of UO or LY, these data confirm MA signaling via NOS. Besides 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 [27]. These results indicate that apelin upregulates eNOS gene expression in vessels, but it is not excluded that MA could initiate a similar effect in the myocardium. In addition, NO formation, catalyzed by eNOS, requires L-arginine (Arg) transport into the myocardial cells [28], which was found to increase in aortic tissue in response to apelin [27]. Therefore, the role of the RISK pathway in NO-dependent mechanisms of MA-induced car-

Table 5
The similarity in the signaling pathways between natural apelin peptides and MA.

Cascade component, end effector	Inhibitor	Effect of the inhibitor		
		natural apelins		MA
PI3K-Akt	LY294002	+	[3,7,9]	+
	Wortmannin	+	[8]	
	Wortmannin	–	[4]	
MEK1/2-ERK1/2	UO126	+	[3,36]	+
	PD098059	+	[8]	
	MEK inhibitor 1	+	[3]	
PKC	Chelerythrine	+	[9]	+
	Staurosporine	+	[12]	
	Bis	+	[36]	
PKCϵ	ϵV1-2	+	[9]	
p70S6	Rapamycin	–	[4]	
NOS	L-NAME	+	[11]	+
	L-NNA	+	[5]	
Mito K_{ATP} channels	5-Hydroxydecanoate	+	[9]	+
PLC	U73122	+	[12]	+
Na^+/H^+ exchanger	Zoniporide	+	[12]	+ (Amiloride)
Na^+/Ca^{2+} exchanger	KB-R7943	+	[12]	+

Models: myocardial infarction [3–5]; cardiomyocyte apoptosis [7,8]; postischemic cardiac dysfunction [9,11]; cardiomyocyte shortening [9,12]; cardiac performance [12,36].

dioprotection may be supplemented by the enhanced membrane Arg transport activity and the increased eNOS protein expression.

Production of NO is responsible for the opening of mito K_{ATP} channels via guanylyl cyclase-cGMP cascade and the activation of protein kinases G and Cϵ [29]. Mito K_{ATP} channel opening prevents the opening of mPTP and provides positive feedback by altering upstream components, such as ROS or PKC, being a trigger and an effector of cardioprotection [30]. Our study clearly demonstrated that the activation of the mito K_{ATP} channels was tightly related to protective action of MA in both models of I/R injury. This is confirmed by the fact that coadministration of MA and HD, a blocker of the mito K_{ATP} channels, abolished beneficial effects of MA on myocardial infarct size, activity of necrosis markers and functional recovery of isolated rat heart at reperfusion. It is noteworthy, the opening of mito K_{ATP} channels, which blocks the opening of mPTP, occurs due to the activation of various survival kinases, such as, p38-mitogen activated protein kinase, c-Jun N-terminal kinase, PKC, Akt, and tyrosine kinase [3,31]. Taking into account the similarity of the inhibitory effects of LY, N and HD in this study, we may assume that reduction of I/R injury by MA might be mediated by the PI3K-PKCϵ–mito K_{ATP} channels pathway.

In this study, U as well as Che blocked an improvement of functional recovery and a decrease in LV P_d in isolated reperfused hearts treated with MA. Additionally, coadministration MA and U abrogated enhancement of metabolic recovery of postischemic hearts induced by MA. These findings imply that MA binding to receptor APJ stimulates PLC signaling, including the hydrolysis of phosphatidylinositol 4,5-biphosphate to inositol triphosphate (IP3) and diacylglycerol (DAG), and subsequent activation of PKC by DAG. As it is known, PKC activation is a crucial mechanism of protection against contractile dysfunction by preserving diastolic function in postischemic hearts [32]. On the other hand, elevation of IP3 in the cytosol triggers release of Ca^{2+} via the ryanodine receptor and IP3 receptor from the sarcoplasmic reticulum [33]. Thus, MA may influence cardiac contractile dysfunction, which is attributed to impaired calcium handling of cardiomyocytes during I/R.

We did not assess which of PKC isoforms were involved in cardioprotection in the present study. However, it has been recently demonstrated that a specific PKCϵ inhibitor, ϵV1-2, abolished effect of apelin-13 on recovery of contractile function and increased LDH release during reperfusion on a rat model of myocardial I/R injury in vitro [9]. These data indirectly support our assumption on involvement of PKCϵ in cardioprotective effects of MA, although they do not exclude participation of other PKC isoforms. Activated PKC can phosphorylate a wide spectrum of cellular proteins including the sarcolemmal Na^+/H^+ exchanger [34]. In our experiments, Am, a selective inhibitor of Na^+/H^+ exchange, reduced protective effects of MA on recovery of cardiac contractile and pump function, myocardial metabolic state and membrane integrity, suggesting that activation of Na^+/H^+ exchange contributes to the impact of the peptide. 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^+ mediated by activation of Na^+/H^+ exchange can promote a rise in intracellular Ca^{2+} via the reverse mode of operation of the Na^+/Ca^{2+} exchanger [35]. In the present study, KB-R7943, a selective inhibitor of the reverse mode Na^+/Ca^{2+} exchange markedly suppressed the MA-induced improvement of cardiac function recovery. Taken together, the results obtained in perfused rat hearts suggest that enhanced functional and metabolic recovery induced by MA involves signaling via PLC and PKC, and the activation of sarcolemmal Na^+/H^+ and Na^+/Ca^{2+} exchange. This mechanism may also play a pivotal role in reducing abnormalities in intracellular Ca^{2+} transients in I/R injury.

Apelin peptides, the endogenous ligands for the G-protein-coupled APJ receptor, exert cardioprotective effects in myocardial I/R injury as well as the bioactive analogue of apelin-12 MA. In addition to their role in cardioprotection, contribution of PKCϵ and extracellular signal-regulated kinase 1/2 (ERK1/2) to the positive inotropic effect of these compounds was demonstrated in various cell types [36,37]. Modest activation of PKCϵ and ERK1/2 signaling improves cardiac contractile function thus enhancing significance of these pathways for the treatment of ischemic heart. The similarity in signal mechanisms between natural C-terminal fragments of apelin and MA is shown in Table 5. These facts strongly suggest that analogue MA interacts with the APJ receptor as a pharmacological ligand and triggers signal cascades comparable to those produced by natural apelin peptides.

In conclusion, a structural analogue of apelin-12, MA, reduces irreversible cardiomyocyte damage, improves cardiac dysfunction,

and enhances metabolic restoration and membrane integrity in experimental models of myocardial I/R injury. This cardioprotection is mediated by signaling via PLC and survival kinases, PKC, PI3K, and MEK1/2, with activation of downstream targets, NO synthase and mito K_{ATP} channels, and sarcolemmal Na^+/H^+ and Na^+/Ca^{2+} exchangers. We believe that MA may be a promising tool for treating cardiovascular diseases. In view of this feature, further studies of the MA-APJ receptor signaling pathways are warranted to discover the optimal therapy with this peptide.

Conflict of interest

The authors declare that they have no conflict of interest.

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