Apelin-13 protects the heart against ischemia-reperfusion injury through the RISK-GSK-3β-mPTP pathway

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Abstract

Introduction: Apelin plays an important role in the protection against myocardial ischemia-reperfusion (I/R) injury, while the mechanism still remains unclear. In the current study, we aimed to evaluate the protective effect of apelin-13, and the main mechanism.

Material and methods: The in vivo I/R injury model (Sprague-Dawley rat) was established, then infarct size, expression levels of phospho-protein kinase B (p-Akt), phospho-extracellular signal-regulated kinase (p-ERK) and phospho-glycogen synthase kinase-3β (p-GSK-3β) were measured. The fluorescence intensity of tetramethylrhodamine ethyl ester perchlorate (TMRE) of the isolated myocardial cells was determined to evaluate the opening of the mitochondrial permeability transition pore (mPTP) caused by oxidant stress and hypoxia/reoxygenation.

Results: For the established I/R injury model, apelin-13 and SB216763 (GSK-3β inhibitor) significantly reduced the infarct size (p < 0.05), which could be abolished by LY294002 (PI3K inhibitor), PD98059 (MEK inhibitor) and atracyloside (mPTP accelerator). The enhanced expression levels of p-Akt, p-ERK and p-GSK-3β caused by apelin-13 (p < 0.05) could be counteracted by LY294002 and PD98059. The reduced fluorescence intensity of TMRE in the H₂O₂/apelin-13 and H₂O₂/SB216763 treated groups was significantly lower (p < 0.05), indicating that apelin-13 and SB216763 could reduce the decline in mitochondrial membrane potential caused by oxidant stress, and the fluorescence intensity in the hypoxia/reoxygenation + apelin-13 group was significantly lower (p < 0.05), which suggested that apelin-13 could inhibit the mitochondrial membrane potential changes induced by hypoxia/reoxygenation.

Conclusions: The protective mechanism of apelin-13 might be that inactivation of GSK-3β could inhibit the opening of mPTP by activating PI3K/Akt and ERK1/2 involved in the reperfusion injury salvage kinase (RISK) pathway.

Key words: myocardial ischemia-reperfusion injury, apelin-13, reperfusion injury salvage kinase, glycogen synthase kinase-3β, mitochondrial permeability transition pore.

Introduction

Myocardial ischemia-reperfusion (I/R) injury is the most important factor in the therapy of acute myocardial infarction. It has been confirmed
that early myocardial injury is regularly associated with acute liver failure [1]. In recent years, protection against myocardial ischemia-reperfusion injury has focused on the ischemic pre- and post-conditioning. It has been shown that production of transient remote ischemia preconditioning in the lower extremities reduces damage in the spinal cord secondary to ischemia, probably by the increase of heat shock protein [2]. Also, levels of transforming growth factor-β1, fibrotic and apoptotic activities were reduced in the I/R model treated with decorin [3]. What is more, administration of L-arginine in the pre-ischemic and in the initial phase of reperfusion stimulates increase in nitric oxide release, which is positively correlated with the increase of coronary flow [4].

One of the major protective mechanisms involves activation of the reperfusion injury salvage kinase (RISK) pathway [5], which incorporates phosphatidylinositol 3-OH kinase (PI3K)-cellular Akt/protein kinase B (Akt) and p44/42 mitogen-activated protein kinase (MAPK) extracellular regulated protein kinases (ERK1/2) [6–10]. The other important mechanism involves inhibition of the opening of the mitochondrial permeability transition pore (mPTP), which could evaluate the degree of reperfusion injury [11, 12]. Also, mPTP is a non-specific aperture located on the mitochondrial inner membrane which is related to cell apoptosis. Several studies have shown that opening of the mPTP is correlated with activation of PI3K, which is regulated by PI3K-Akt and ERK1/2 [13, 14].

Apelin, appeared to act as an endogenous ligand for the previously orphaned G-protein-coupled APJ receptor [15], has several biological actions including depressurization, administration on water-electrolyte metabolism and biologic rhythm. Among apelin fragments which have been isolated and classified according to the number of amino acids, apelin-13 is considered to have the most potent effect on myocardial protection and have significant therapeutic potential for end-stage heart failure [16–18]. Apelin-13 could stimulate vascular smooth muscle cell proliferation by promoting the G1-S phase transition mediated in part by an apelin-pERK1/2-cyclin D1 signal cascade [19]. Rastaldo et al. have found that apelin-13 could limit infarct size and improve cardiac post-ischemic mechanical recovery only if given after ischemia [20]. Furthermore, Tao et al. reported that apelin-13 protects the heart against ischemia-reperfusion injury through inhibition of ER (endoplasmic reticulum)-dependent apoptotic pathways [21]. Studies have also shown that apelin acutely protects the heart against I/R injury via the activation of PI3K/Akt/endothelial nitric oxide synthase (eNOS) and ERK signaling pathways [16, 22]. The apelin–API system may protect the myocardium from ischemia reperfusion injury by its actions on the RISK pathway [23]. However, the specific mechanism of apelin-13 in ischemia-reperfusion injury still remains unclear.

Therefore, the aims of the present investigation are to study the effects of apelin-13 on the RISK pathway, GSK-3β (glycogen synthase kinase-3β) and mitochondrial membrane potential, and to discuss the relationships among them.

**Material and methods**

**Materials**

Adult male Sprague-Dawley (SD) rats, weighing between 250 and 280 g, were purchased from the experimental animal center of Fudan University in China. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee. Apelin-13 came from Bachem corporation, LY294002 (PI3K inhibitor) and PD98059 (MEK inhibitor) were from Sigma Chemical Co, SB216763 (GSK-3β inhibitor) was purchased from Tocris Bioscience and atractyloside (mPTP accelerator) was from CALBIOCHEM. Primary and secondary antibodies against total Akt/protein kinase B (Akt) and phosphorylated Akt, ERK1/2 and GSK-3β were from Cell Signaling Technology.

**Establishment of ischemia-reperfusion injury model**

The ischemia-reperfusion injury model was established as described previously [24, 25]. Briefly, the SD rats were ventilated with room air at 80–100 breaths/min with the tidal volume set to 0.8–1.5 ml/100 g body weight with body temperature maintained at 37°C after anesthesia with pentobarbital sodium (40 mg/kg i.p.) and endotracheal intubation. Using sterile surgical procedures, the right carotid artery was cannulated with an angiocatheter for drug infusion. A left thoracotomy was performed through the third or fifth intercostal space, and the ribs were gently retracted to expose the heart. After a pericardiotomy, the left coronary artery (LCA) was encircled by a 6-0 prolene suture just distal to its first branch, and its ends were threaded through polyethylene-50 tubing to form a snare for reversible coronary artery occlusion. Cardiac ischemia was confirmed by an initially pale and later cyanotic area below the suture and ST-T elevation shown in ECG (electrocardiograph), whereas reperfusion was characterized by the rapid disappearance of cyanosis followed by vascular blush. The duration of ischemia was 30 min, and then rats were injected with drugs before reperfusion. Ischemia-reperfusion (I/R) injury was assessed 120 min after reperfusion.

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Experimental groups

For both infarct size assessment and measurement of phosphorylation by western blot analysis, the SD rats were randomly divided into 7 groups: (1) SO: sham-operated group; (2) C: control (normal saline); (3) A: apelin-13 (0.1 mg/kg); (4) AL: apelin-13 (0.1 mg/kg) and LY294002 (0.3 mg/kg); (5) AP: apelin-13 (0.1 mg/kg) and PD98059 (0.3 mg/kg); (6) AA: apelin-13 (0.1 mg/kg) + atractyloside (5 mg/kg); (7) SB: SB216763 (0.6 mg/kg). Six rats in each group were used for the measurement of infarct size 120 min after reperfusion, and 3 rats in each group were used for the detection of western blot 10 min after reperfusion.

Measurement of area at risk and infarction size

At the end of the reperfusion for 120 min, the left anterior descending (LAD) coronary artery was ligated, and 1% Evans blue dye (1 ml) was injected to determine the area at risk (blue: non-ischemic area; uncolored: area at risk). The heart was removed and cut into five to six slices followed by incubation for 3–5 min at 37°C in 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution to determine the infarcted myocardium. The extent of the areas of left ventricle (LV), area at risk (AAR) and infarction size (IS) were quantified by the Leica QWin V3 computer image analysis system. Then, the ratio of AAR to LV (AAR/LV) and the ratio of IS to LV (IS/LV) were calculated.

Western blot analysis

Myocardium was homogenized and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) to gain protein followed by bicinchoninic acid (BCA) protein quantification. Then the proteins were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membrane. After blocking with 5% bovine serum albumin (BSA) for 2 h, the membranes were immunoblotted with antibodies of total and phosphorylated Akt, ERK1/2, GSK-3β (1 : 3000) overnight at 4°C, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 10 000) for 1 h at room temperature. Immunoreactive bands were detected by the method of enhanced chemiluminescence using the Bio-Rad imaging system and analyzed by Leica image analysis software.

Isolation and culture of myocardial cells

The myocardial cells were isolated from the neonatal rat by the digestion method. The heart was washed with D-Hank’s solution three times and cut into small pieces. After digestion with 0.125% trypsinogen at 37°C for 10 min, the digest was mixed with DMEM culture medium, and centrifuged at 4°C, 1200 rpm for 5 min. The precipitation was digested several times until the color became white in DMEM medium supplemented with 10% fetal bovine serum (FBS). The enchytema was collected and cultured in a culture dish under a 5% CO2 humidified atmosphere at 37°C in DMEM medium supplemented with 10% FBS.

Observation of mitochondrial membrane potential by laser confocal scanning microscopy

The myocardial cells were washed with PBS three times and stained with tetramethylrhodamine ethyl ester perchlorate (TMRE, 100 nM) for 20 min at 37°C away from light. Then, the cells were washed with PBS three times to remove residue.

In order to compare the changes of mitochondrial membrane potential caused by oxidant stress, the cells were divided into three groups as follows: the control group treated with H2O2 (200 μM) for 20 min; the apelin-13 group first treated with apelin-13 (1000 nM) for 5 min and then H2O2 (200 μM) for 20 min; the SB216763 group first treated with SB216763 (3 μM) for 5 min and then H2O2 (200 μM) for 20 min.

Furthermore, the changes of mitochondrial membrane potential caused by hypoxia/reoxygenation were compared according to the following two groups. One was the hypoxia/reoxygenation group (control) and the other one was the hypoxia/reoxygenation + apelin-13 group. In the hypoxia/reoxygenation group, cells were stimulated with the ischemia reperfusion injury in vitro to evaluate the mitochondrial membrane potential changes. Briefly, the isolated myocardial cells were placed in hypotonic balanced salt solution (1.3 mM CaCl2, 5 mM KCl, 0.3 mM KH2PO4, 0.5 mM MgCl2, 0.4 mM MgSO4, 69 mM NaCl, 4 mM NaHCO3, and 0.3 mM Na2HPO4) without glucose or serum and made hypoxic for 40 min at 37°C. Hypoxia was achieved by using an air-tight jar with hypoxia agent. Then, the plates were removed from the chamber and placed in sterile air for reoxygenation (20 min).

For the hypoxia/reoxygenation + apelin-13 group, apelin-13 (1000 nM) was added into the cell solution in the process of reoxygenation (20 min).

The fluorescence intensity of TMRE in each group was measured using an LSM-510 laser confocal scanning microscope (Carl Zeiss, Jena, Germany) with the 543 nm excitation wavelength and 560 nm emitting wavelength. Then, all images were analyzed by LSM-510 V2.3 software.
Statistical analysis

Experiments were performed three times and the difference was analyzed by T-test using SPSS10.0 statistical software. The data were expressed as means ± SD of three independent experiments and $p < 0.05$ was considered statistically significant.

Results

Area at risk and left ventricle

Compared with group C, the AAR/LV in groups A, AL, AP, AA and SB did not change significantly ($p > 0.05$, Figure 1 A). The IS/AAR and AAR/LV in groups A and SB were lower than those in group C. The IS/AAR in group A (34.73 ±7.20%) and group SB (36.22 ±6.22%) significantly decreased compared with that in group C (53.03 ±8.90%, $p < 0.05$, Figure 1 B), which indicated that apelin-13 and SB216763 (GSK-3β inhibitor) could reduce the infarct size. However, the IS/AAR in group AL (46.60 ±4.23%), group AP (50.84 ±8.05%) and group AA (52.5 ±7.8%) showed no significant difference compared with group C ($p > 0.05$, Figure 1 B), which suggested that LY294002 (PI3K inhibitor), PD98059 (MEK inhibitor) and atractyloside (mPTP accelerator) could inhibit the effects of apelin-13 on reducing the infarct size.

Expression levels of total and phosphorylated Akt, ERK1/2, GSK-3β

Western blot was used to study the effect of apelin-13 on the RISK pathway and GSK-3β, which are involved in the underlying mechanism of heart protection mediated by apelin-13 infusion. The results showed that the expression levels of total Akt, ERK and GSK-3β were almost unchanged among the four groups, while the expression levels of phospho-Akt (p-Akt), phospho-ERK (p-ERK) and phospho-GSK-3β (p-GSK-3β) were increased significantly in group A compared with group C ($p < 0.05$, Figure 2), which indicated that apelin-13 could enhance the expression levels of p-Akt, p-ERK and p-GSK-3β. However, the expression of p-GSK-3β did not change significantly in groups AL and AP compared with group C ($p > 0.05$, Figure 2 C). Therefore, LY294002 and PD98059 counteracted the effect of apelin-13.

Mitochondrial membrane potential changes

The fluorescence intensity of TMRE was determined using a laser confocal scanning microscope to evaluate the opening of the mitochondrial permeability transition pore. The reduced fluorescence intensity of TMRE in the H2O2/apelin-13 and H2O2/SB216763 treated groups was significantly lower than that in the H2O2 treated group ($p < 0.05$, Figure 3). This result indicated that the roles of apelin-13 and SB216763 might be to reduce the decline in mitochondrial membrane potential caused by oxidant stress. Meanwhile, the reduced fluorescence intensity of TMRE in the hypoxia/reoxygenation + apelin-13 group was significantly less than that in the hypoxia/reoxygenation group ($p < 0.05$, Figure 4), which suggested that apelin-13 could also inhibit the mitochondrial...
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Discussion

It has been reported that apelin plays important roles in the protection against ischemia-reperfusion injury and maintenance of cardiovascular homeostasis, while the knowledge of molecular and signaling mechanisms of apelin in response to ischemia-reperfusion injury is still incomplete [26]. In our study, we concluded that the protective mechanism of apelin-13 might be that inactivation of GSK-3β could inhibit the opening of mPTP by activating PI3K/Akt and ERK1/2 involved in the reperfusion injury salvage kinase (RISK) pathway.

The RISK pathway represents the PI3K/Akt (phosphatidylinositol 3-OH kinase/protein kinase B) and ERK 1/2, which are involved in heart protection against cell apoptosis and death during the phase of reperfusion [27]. Some reports have shown that ischemic pre- and post-conditioning could decrease cell apoptosis and myocardial injury, which could activate the PI3K/Akt and ERK1/2 signal, phosphorylate eNOS, p70S6K, GSK-3β, GLUT4 and anti-apoptosis proteins such as Bcl2, Bad, Bax, Caspase, then delivery of Cyt-C is inhibited and cell apoptosis is blocked [7–10, 28]. However, these protective effects are weakened when the phosphorylation of PI3K/Akt and ERK1/2 is inhibited by a specific repressor of the signaling pathway [29]. It has been reported that Akt and p44/42 phosphorylation were enhanced by apelin at all reperfusion time points [23]. Simpkin et al. found that the protective effect of apelin-13 was abolished by LY294002 and UO126, and the delayed effect on mPTP opening caused by apelin-13 was inhibited by LY294002 and MEK inhibitor 1 [16]. Amadou et al. found that administration of apelin not only completely abolished the activation of endoplasmic reticulum stress-induced apoptosis membrane potential changes induced by hypoxia/reoxygenation.

**Figure 2.** Expression levels of p-Akt (A), p-ERK 1/2 (B) and p-GSK-3β (C) in cardiac muscle tissue of SD rats detected by western blot

SO – Sham-operated group, C – control group, A – apelin-13 group, AL – apelin-13 + LY294002 group, AP – apelin-13 + PD98059 group, AA – apelin-13 + atractyloside group, SB – SB216763 group. *P < 0.05 vs. control group.
signaling pathways at 2 h of reperfusion but also significantly attenuated time-related changes at 24 h of reperfusion, and PI3K/Akt, AMPK, and ERK activation were involved in the protection against I/R injury via inhibition of endoplasmic reticulum stress-dependent apoptosis activation using pharmacological inhibition [30]. In the present study, we also found that the protective effect of apelin-13 could be suppressed by LY294002 (PI3K inhibitor) and PD98059 (MEK inhibitor), which was associated with mPTP. Therefore, the protective effect of apelin-13 was related to activation of the RISK pathway. The expression levels of p-Akt and p-ERK1/2 were increased significantly, which was consistent with previous studies indicating that the cardioprotective actions of apelin involve the PI3K-Akt pathway [16, 21].

Several reports have shown that the PI3K/Akt cell signal could be activated following the inhibition of GSK-3β activity in the process of ischemic pre-conditioning [31–33]. However, the molecular mechanism for the effects of apelin-13 on GSK-3β is still unknown. In our study, the expression level of p-GSK-3β was increased in the apelin-13 treated group, whereas the effectiveness was inhibited on condition that a PI3K inhibitor or MEK inhibitor was present. Meanwhile, SB216763 (GSK-3β inhibitor) could reduce the decline in mitochondrial membrane potential caused by opening of mPTP. Therefore, GSK-3β might participate in the inhibition of opening of mPTP. Our study proved that apelin-13 could decrease the infarction size through activation of the PI3K-Akt and ERK1/2 pathway followed by the increased expression of
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p-GSK-3β, which was inactivated. Therefore, GSK-3β might be the downstream target of the RISK pathway in which apelin-13 exhibited anti-ischemia-reperfusion injury activity.

Bopassa et al. reported that activation of PI3K resulted in inhibition of mPTP opening in an isolated reperfusion injury model, and Juhaszova et al. stated that the inactivation of GSK-3β could prevent the mPTP opening in the process of ischemic post-conditioning [34–36]. In our study, the change of mitochondrial membrane potential, which indicated the opening of the mitochondrial permeability transition pore, was more obvious in apelin-13 and SB216763 treated groups compared with the H2O2 model group. Similar results were also obtained in the model of hypoxia/ventilation conditions. Our results indicated that apelin-13 could inhibit the opening of mPTP. Meanwhile, atractyloside, which promotes mPTP opening, could attenuate the protective effects that apelin-13 induced. It is worth noting that SB216763 (GSK-3β inhibitor) could also decrease the opening of mPTP.

In conclusion, our results indicated that mPTP was the downstream target of GSK-3β, and the major protective mechanism of apelin-13 was that it could activate the RISK pathway followed by the inactivation of GSK-3β, and then inhibited the opening of mPTP. However, there are some limitations in our study. Expression levels of phosphorylated Akt, ERK1/2 and GSK-3β would be better assessed at several reperfusion time points. What is more, the protective mechanisms of apelin-13 in ischemia-reperfusion injury might...
be more scientifically and exactly investigated by delivering activation genes of GSK-3β into myocardi- 24. Shuansuo Yang, Hui Li, Lei Tang, Guanghao Ge, Jiangwei Ma, Zengyong Qiao, Huajin Liu, Weiyi Fang 25. Acknowledgments 26. The authors declare no conflict of interest. 27. References 28. 1. Papoutsidakis N, Arkadopoulos N, Smyrniotis V, et al. Early myocardial injury is an integral component of experimental acute liver failure — a study in two porcine models. Arch Med Sci 2011; 7: 217-223. 2. Selimoglu O, Ugurlucan M, Basaran M, et al. Efficacy of remote ischaemic preconditioning for spinal cord protection against ischemic injury: association with heat shock protein expression. Folia Neuropathol 2008; 46: 204-12. 3. Alan C, Kocoglu H, Altintas R, Alici B, Resit Ersay A. Protective effect of decorin on acute ischemia-reperfusion injury in the rat kidney. Arch Med Sci 2011; 7: 211-6. 4. Okonski P, Banach M, Rysz J, et al. 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