Apelin protects heart against ischemia/reperfusion injury in rat

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ARTICLE INFO

Article history:
Received 23 January 2009
Received in revised form 11 February 2009
Accepted 11 February 2009
Available online 24 February 2009

Keywords:
Apelin
APJ
Ischemia/reperfusion injury
Apoptosis

ABSTRACT

Apelin, the endogenous ligand of the G protein-coupled APJ receptor, is a peptide mediator with emerging regulatory actions in the heart. We aimed to determine whether the endogenous apelin/APJ system is an intrinsic protective pathway in ischemic/reperfusion injury. A Langendorff model of perfused isolated rat hearts and primary cultured myocardial cells from neonatal rats were used. Cardiac function was monitored and apelin/APJ expression was determined by real-time PCR and Western blot analysis. In rats under I/R, cardiac function was significantly decreased as compared with controls, and APJ was overexpressed at both the mRNA and protein levels (by 7-fold and 35%, respectively, both p < 0.01). However, pre-administration of apelin (30 pmol/L) greatly ameliorated the reduced heart function. To gain mechanistic insight into the cardio-protective effects of apelin/APJ, cultured cardiomyocytes were treated with apelin (30 pmol/L), and those under hypoxia/re-oxygenation showed H/R-induced apoptosis and upregulated apelin/APJ mRNA expression by 6-fold and 7-fold, respectively (both p < 0.01). And lactate dehydrogenase leakage was greatly increased as well. Meanwhile, apoptosis, the generation of reactive oxygen species and malonaldehyde content as well as lactate dehydrogenase leakage were inhibited by apelin. Furthermore, apelin enhanced superoxide dismutase activity and phosphorylation of extracellular signal-regulated kinase 1/2 and Akt after hypoxia/re-oxygenation. In conclusion, apelin/APJ has protective effects in ischemic heart disease and might constitute an important therapy target.

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1. Introduction

Severe myocardial dysfunction and tissue damage resulting from ischemia/reperfusion (I/R) is a common clinical scenario in patients with some heart diseases and therapies such as thrombolysis, percutaneous coronary intervention, coronary artery bypass grafting, and cardiac transplantation. The phenomenon that reperfusion causes damage in addition to that caused by the ischemia insult is referred to as I/R injury [36]. Growing evidences suggested that the myocardium adapts to I/R by synthesizing and responding to various stress-induced growth factors and cytokines and that identification of these endogenous homeostatic mechanisms may open new avenues to limit I/R injury [15,22]. It is becoming increasingly clear that intrinsic factors – adipokines such as leptin and adiponectin, cytokines such as vascular endothelial growth factor, and vaso-active peptides such as adrenomedullin and ghrelin [3,13,25,31] – likely mediate the protective effects in I/R injury.

The peptide apelin, described as an adipokine because of the production and secretion by isolated mature adipocytes [2], has been newly identified as the endogenous ligand of the human orphan G-protein-coupled receptor APJ (putative receptor protein related to the angiotensin receptor AT1) by reverse pharmacology [34]. Variously produced in different tissues and especially highly expressed in the cardiovascular system [20,26], apelin/APJ shows significant hypotension and positive inotropic action [1,5,14,35]. These facts suggest its important regulatory role in cardiovascular homeostasis.

Our previous studies revealed that apelin administration attenuated myocardial injury induced by an overdose of isoproterenol in rats [17]. Recent articles even showed that exogenously administrated apelin protected the ischemic myocardium against I/R injury [19,29,30,40]. However, it is disputed how apelin/APJ reduces I/R injury. In fact, no study has ever evaluated the actions of endogenous apelin/APJ in I/R injury in the heart.

This study was performed in rat heart and neonatal cardiomyocytes to investigate apelin/APJ expression after I/R or hypoxia/re-oxygenation (H/R) and to answer whether endogenous apelin/APJ could ameliorated myocardial I/R injury by its anti-apoptosis actions.

2. Materials and methods

2.1. Materials

Male Wistar rats (220–250 g) and neonatal Wistar rats less than 48 h old were acquired from the Animal Department, Capital
and reagents were of analytical grade. Lysis buffer used in Western sample loading control. All the above sequences of oligonucleotide sequences of oligonucleotide primers were APJ-F, 5' - CACCTGGCT-GAGACTCTCTACA-3'; pelin-F, 5' - GGCTAGAAAGGGCAACATGC-3'; and pelin-R, 5' - CCGCTGTCCGAAATTTCCCT-3'; GAPDH-F, 5'- CCTGGAGAACCCTGCCAAGT-3'; and GAPDH-R, 5'- TACCCAGGTGCCTTTCAG-3' for sample loading control. All the above sequences of oligonucleotide primers were synthesized by Sangon (Beijing, China). The chemicals and reagents were of analytical grade. Lysis buffer used in Western blot were composed with 1% NP-40, 20 mmol/L Tris/HCl (pH 8.0), 137.5 mmol/L NaCl, 1 mmol/L Na2VO4, 1 mmol/L PMSF, and 10 µg/ml aproninin. TBST solution were composed with 25 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.05% (vol/vol) Tween 20.

2.2. Langendorff model of myocardial I/R injury

Adult male rats were given 100 U of heparin by intraperitoneal injection before cervical dislocation. Hearts were then excised and perfused retrogradely via the aorta at a constant flow of 8 ml with oxygenated Krebs–Henseleit (K–H) buffer, pH 7.4 at 37°C [7,31]. Myocardial temperature (thermal probe) and heart function (ventricular balloon) were monitored throughout the perfusion periods. Isolated hearts underwent 20-min stabilization, then 40-min global ischemia (achieved by total perfusion arrest) and 30-min reperfusion. Necessary K–H buffer containing 30 pmol/L apelin-13 was substituted for normal buffer throughout the experiment; these hearts corresponding to the cardio-protectant treated group. Following the appropriate reperfusion period, the perfusion solution was collected for assaying LDH release, and hearts were snap-frozen and stored at –80°C until analysis.

2.3. Cell culture and H/R treatment

To further demonstrate the involvement of apelin/APJ in myocardial I/R injury, primary neonatal ventricular cardiomyocytes were isolated from neonatal (less than 48-h-old) Wistar rats and maintained as described [41]. Briefly, single cells were dissociated from minced hearts of rats with use of a 0.25% solution of crude trypsin and collagenase and then cultured as monolayers at 5 × 10⁴ cells/cm² in Dulbecco’s Modified Eagles Medium (DMEM) at 37°C. The medium contained 10% calf serum and 2 µmol/L fluoroodeoxyuridine, the latter was used to prevent proliferation of non-cardiomyocytes. Three days after the cells were seeded; the cultured cardiomyocytes were randomly divided into the following groups for treatment. Control group: cardiomyocytes were continuously cultured for 5 h with or without wortmanin (1.0 µmol/L) and PD098059 (50 nmol/L) in normal culture medium. H/R group: cells were subjected to 3 h simulated hypoxia (approximately 2% O₂/5% CO₂), then 2 h of normoxia (approximately 20% O₂/5% CO₂) as described [12]. Apelin groups: 30 pmol/L apelin with or without wortmanin (1.0 µmol/L) and PD098059 (50 nmol/L) added to the culture medium 30 min before hypoxia. Drugs were dissolved in pre-warmed medium and added directly to the culture. For controls, equivalent volumes of medium were added. Only cultures that consisted of >95% rhythmically beating cells, determined by counting 300 cells in 3 different fields, were used in the analysis.

2.4. Cell viability assay

Cell viability was determined by 3-(4,5)-dimethylthiahiazolo(3,4-yl)-3,5-di-phenyterazoliumromide (MTT) assay. Cells were cultured in 96-well plates, MTT was added to each well under sterile conditions (with a final concentration of 0.5 mg/mL) immediately after 2 h of re-oxygenation, and the plates were incubated for 5 h at 37°C. The supernatant was removed, and dimethylsulfoxide (DMSO) (150 µL/well) was added. The plates were then agitated on a plate shaker. The absorbance of each well was measured at 490 nm with a Wellscan MK 3 automated EIA Analyzer (Labsystems Dragon, Taiwan). The viability of control cells was taken as 100%, and the others were expressed as percentages of control.

2.5. TUNEL staining

Cell apoptosis was detected by the terminal transferase-mediated dUTP nick end-labeling (TUNEL) method with the DeadEnd™ Fluorometric TUNEL System (G3250, Promega, San Luis Obispo, CA, USA). In accordance with the manufacturer’s protocol, cells were fixed with 4% paraformaldehyde for 30 min at 4°C. After three washes in PBS, they were permeabilized with 0.1% Triton X-100 for 5 min at room temperature, then incubated with 50 µL TUNEL reaction mixture for 60 min at 37°C. Diaminobenzidine was used to generate an insoluble colored substrate at the site of DNA fragmentation. Finally, the cells were counterstained with methyl green for morphological evaluation and characterization of normal and dead cells. All cells were analyzed under a microscope. The percentage of dead cells was calculated as the ratio of the number of TUNEL-positive cells to the total number of cells, which were counted in three different random fields.

2.6. Assay of LDH activity

The LDH activity in the medium and perfusion solution was measured by an automatic biochemistry analyzer (Roche, 04744934001, CH-4070 Basel, Switzerland) according to the manufacturer’s instructions; namely, 50 µL medium and 50 µL mix of reagent A and B were co-incubated for 30 min and then the absorbance was detected at 492 nm with use of a spectrophotometer (Labsystems Dragon, Taiwan). The results were demonstrated with zero in control group and 100% in I/R or H/R group.

2.7. Assessment of oxidative stress

To evaluate oxidative damage, MDA content in heart tissue and primary neonatal myocardium was measured by use of a commercial kit (Jian-Cheng Biochemical Engineering). The MDA content was measured by the thiobarbituric acid assay on a spectrophotometer at 532 nm [10]; 1,1,3,3-tetramethoxypropane was used as an external standard, and the level of MDA was expressed as nanomoles per milligram protein for myocardium. Protein concentrations were determined by a protein assay kit.
Activity of the anti-oxidative enzyme SOD in heart tissue and primary neonatal myocardial cells was determined by its inhibition of pyrogallol auto-oxidation as previously described [24]. One unit of the SOD activity was defined as the amount of enzyme that inhibited the rate of pyrogallol auto-oxidation by 50% in a minute in this assay.

Intracellular reactive oxygen species (ROS) generated in primary neonatal myocardial cells were measured by DHE assay, as previously described [39]. In brief, DHE was prepared in DMSO. The stock solutions were then further diluted in cell-culture medium to the desired concentrations. After experimental treatments, the cells were incubated with 5 μmol/L DHE and 1:10,000 Hoechst in culture medium for 5 min at 37 °C in the dark, DHE will react with ROS in the cell and displayed red fluorescence in the nucleus, and then fluorescence images were taken to count the proportion of DHE-positive nuclear cells, which exhibited red fluorescence, to total cells. Three independent experiments were performed.

2.8. Real-time PCR of mRNA levels of apelin/APJ

To evaluate the apelin/APJ mRNA level, total RNA was extracted, and hearts or cells were lysed with Trizol reagent. One microgram of total RNA was reverse-transcribed into single-strand cDNA with use of M-MulV reverse transcriptase and random primers. From sequences reported in GenBank (AF179679/AB033170), primers and probes were designed for apelin/APJ (101 bp/111 bp) and glyceraldehyde-3-phosphatedehydrogenase (GAPDH, 83 bp) with use of the Primer Express Software. Real-time PCR involved a 25-μL reaction mixture prepared with SYBR GREEN PCR Master Mix (Warrington, UK) containing an appropriately diluted cDNA solution, 0.2 μmol/L each of primer, at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 45 s. Real-time PCR reactions were analyzed by use of the ABI 7700 Prism Sequence Detection System (PE-ABI). Each tissue sample of rats was run in triplicate. Rat housekeeping gene GAPDH mRNA from samples was measured as an internal control. ΔΔCt was calculated for every sample, and the expression were indicated with 2^{-ΔΔCt}.

Fig. 1. I/R or H/R stimulates APJ synthesis in myocardium on RT-PCR. (A) The primer for rat GAPDH and rat APJ with the appropriate molecular weight products. (B and C) APJ mRNA levels (n = 6) normalized to GAPDH expression relative to control (CTL) hearts or cells (1.0). ΔΔCt = [CτAPJ - CτGAPDH]I/R or H/R - [CτAPJ - CτGAPDH]CTL was calculated for every sample, and the expression were indicated with 2^{-ΔΔCt}. (D) Ventricular (heart, left two rows) and primary cultured myocardial (myocardium, right two rows) homogenates in control and I/R or H/R groups were subjected to SDS–PAGE and probed for APJ expression at 42 kDa (upper panel) and α-tubulin at 56 kDa (lower panel). (E) APJ expression levels normalized to α-tubulin relative to CTL (n = 3). Results are the means ± SE of at least three independent experiments. *p < 0.05 compared with CTL hearts or cells.
2.9. Western blotting analysis [21]

Primary neonatal myocardial cells were quickly scraped into PBS, centrifuged at 1000 rpm for 5 min, re-suspended and homogenized in lysis buffer and then centrifuged. Protein samples (80 or 60 μg) were separated on SDS-polyacrylamide denaturing gels and trans-blotted to nitrocellulose filter (Hybond ECL, Amersham). The membranes were blocked overnight at 4 °C with 1% Blot-Qualified BSA in TBST solution, then incubated for 2 h at room temperature under gentle agitation with the primary antibody (rabbit anti-APJ [1:500], rabbit polyclonal antibody) at room temperature under gentle agitation with the primary antibody (rabbit anti-APJ [1:500], rabbit polyclonal antibody against rat eNOS [1:500], ERK1/2 [1:500] and goat polyclonal antibody against rat Akt [1:500], and α-tubulin [1:700]). After three washings with TBST, the membranes were incubated with AP-labeled secondary antibody in TBST solution for 1 h at room temperature, then, washed as above. The positive protein bands were developed with the BCIP/NBT solution and quantified on imageJ. Results were expressed as the ratio of the optical density of the protein band of interest to that of α-tubulin.

2.10. Statistical analysis

All data were expressed as mean ± SE. Statistical differences between groups were evaluated by one-way ANOVA and then Least-Significant-Difference test; comparisons between 2 groups involved use of the Student’s t test. "p < 0.05 was considered statistically significant.

3. Results

3.1. Up-regulation of apelin/APJ expression in the myocardium or cardiomyocytes under cardiac I/R or cardiomyocyte H/R injury

Compared with the control group (n = 6), hearts under I/R (40/30 min) showed an increase in APJ mRNA by about 8-fold (1.0 vs. 8.43 ± 1.08, n = 6, p < 0.01, Fig. 1B).

The cultured neonatal myocardium under H/R (3/2 h) showed increased expression of apelin and APJ mRNA, by 6- and 7-fold, respectively (6.25 ± 0.75 for apelin, n = 6, p < 0.01, Fig. 2; 7.35 ± 1.15 for APJ, n = 6, p < 0.01, Fig. 1C), as compared with the control group (as 1.0).

As well, APJ protein expression was up-regulated in I/R or H/R-treated groups by about 41% and 35% (1.0 vs. 1.41 ± 0.21; 1.0 vs. 1.35 ± 0.14, n = 3, both p < 0.01, Fig. 1E).

3.2. Perfusion with apelin ameliorated I/R injury in isolated rat heart

Before ischemia, the parameters of cardiac function and coronary perfusion pressure were not statistically different between the groups (all p > 0.05, data not shown). During the experimental period, the cardiac function in the control groups (perfusion with K–H solution alone for 90 min or perfusion with K–H plus 30 pmol/L apelin for 90 min) was steady (all p > 0.05, some data shown in Table 1). However, in I/R group, cardiac function was severely inhibited during reperfusion. At the time of reperfusion for 30 min, the values of left-ventricular end-diastolic and end-systolic pressure (LVEDP; LVESP) were higher by 60.8%, and lower by 21.5%, than those of controls respectively. Values for maximal left-ventricular developed pressure (+LVdp/dtmax; +LVdp/dtmax) were lower by 56.5% and 46.2%, than those of controls respectively (all n = 6, p < 0.01, Table 1). Meanwhile, coronary perfusion pressure was markedly increased after 30-min reperfusion (171.1% of basal value, n = 6, p < 0.01, Table 1). Perfusion of the hearts with apelin (30 pmol/L in K–H solution) significantly ameliorated cardiac dysfunction induced by I/R. Compared with the I/R-alone group, with apelin perfusion, LVESP, +LVdp/dtmax and –LVdp/dtmax were augmented (119.3%, 209.9% and 173.0% of the I/R group after 30-min reperfusion, n = 6, p < 0.01, Table 1). Apelin-treated hearts showed decreased coronary perfusion pressure and LVEDP as compared with the I/R-alone group (61.3% and 78.8%, respectively, of the values in I/R group, n = 6, all p < 0.01, Table 1). In addition, apelin treatment diminished myocardial LDH leakage induced by I/R (25% ± 3.2% of the values for the I/R-alone group, n = 6, p < 0.01, Fig. 3A).

3.3. Apelin attenuated cell death induced by H/R in rat primary neonatal cardiomyocytes

Treatment with H/R (3/2 h) reduced the number of viable cells, and apelin treatment increased cell viability as determined by the

### Table 1

Cardiac function of normal and I/R-treated rats (reperfusion for 30 min, R 30 min) treated with or without apelin (30 pmol/L) in perfusion solution (mean ± SE, n = 6).

<table>
<thead>
<tr>
<th></th>
<th>Control (R 30 min)</th>
<th>I/R (R 30 min)</th>
<th>Apelin + I/R (R 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+LVdp/dtmax (mmHg/s)</td>
<td>2605 ± 220</td>
<td>1132 ± 231*</td>
<td>2376 ± 153</td>
</tr>
<tr>
<td>−LVdp/dtmax (mmHg/s)</td>
<td>1352 ± 109</td>
<td>727 ± 108*</td>
<td>1259 ± 48</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>9.7 ± 0.5</td>
<td>15.6 ± 0.8*</td>
<td>12.3 ± 0.6</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>110.7 ± 4.9</td>
<td>86.9 ± 4.8*</td>
<td>103.7 ± 5.2</td>
</tr>
<tr>
<td>Perfusion pressure (mmHg)</td>
<td>39.4 ± 1.3</td>
<td>67.4 ± 1.7*</td>
<td>41.3 ± 1.6</td>
</tr>
</tbody>
</table>

LVEDP: left-ventricular end-diastolic pressure; LVESP: left-ventricular end-systolic pressure; perfusion pressure: pressure of the aorta which reflex the tension of coronary artery; +LVdp/dtmax: maximal left-ventricular developed pressure/diastolic time.

* p < 0.01 vs. I/R group.

* p < 0.01 vs. control group.
MTT method. Cell viability in the H/R group was 56.7% that of the control group and apelin incubation increased the viability to 89.7% (n = 6, p < 0.01, Figs. 3C and 4A). Presence of the inhibitors of phosphatidylinositol 3-kinase (PI3K) or extracellular signal-regulated kinase (ERK), wortmanin or PD098059, in culture medium blocked the protective effect of apelin on H/R-induced cell death (63.2% or 64.4% of the control group value in wortmanin or PD098059 treated H/R group, vs. 54.0% of the control value in the H/R-alone group, n = 6, all p > 0.05; Figs. 3C and 4A), whereas incubation with wortmanin or PD098059 without apelin treatment had no effect on cell viability (105% or 97.8% of the control group value in the cells without H/R, and 58.3% or 56.6% of the control group value in H/R-treated cells, n = 6, all p > 0.05; Figs. 3C and 4A).

Apelin treatment greatly reduced the amount of cardiomyocyte LDH leakage induced by H/R (27.4% LDH leakage compared with 100% in H/R-alone treatment, n = 6, p < 0.01; Fig. 3B). This effect was blocked by wortmanin or PD098059: in the wortmanin+ or PD098059 + apelin group, LDH leakage was 86.3% or 84.1%, respectively, that of the H/R-alone group value (n = 6 for each group, all p < 0.01; Figs. 3B and 4B), whereas incubation with wortmanin or PD098059 without apelin treatment had no effects on LDH leakage (n = 6 for each group, all p > 0.05; Figs. 3B and 4B).

H/R increased the number of TUNEL-positive cells, from 4.3% at baseline (n = 3, control group) to 47.4% (n = 3, p < 0.01; Fig. 5). Apelin incubation reduced this number to 12.3% (n = 3, p < 0.01 vs. H/R-alone group; Fig. 5). Wortmanin blocked the protective effects of apelin on TUNEL-positive cells to 41.8% (n = 3, p > 0.05 vs. H/R-alone group, Fig. 5), whereas wortmanin itself did not induce TUNEL-positive cells (n = 3, p > 0.05 vs. control group; Fig. 5) in the normoxia condition.

3.4. Apelin ameliorated myocardial oxidative stress caused by I/R or H/R

The proportion of DHE fluorescence-positive cells was significantly increased in cells under H/R (3/2 h) (8.4% in control group and 56.1% in the H/R group, n = 6, p < 0.01, Fig. 6A). Cells treated with apelin and stimulated by H/R showed lower DHE fluorescence than those treated with H/R alone (23.6% in apelin-treated group, p < 0.01 vs. H/R-alone group, Fig. 5). Wortmanin blocked the protective effects of apelin on TUNEL-positive cells to 41.8% (n = 3, p > 0.05 vs. H/R-alone group, Fig. 5), whereas wortmanin itself did not induce TUNEL-positive cells (n = 3, p > 0.05 vs. control group; Fig. 5) in the normoxia condition.

Fig. 3. Effect of apelin on LDH leakage normalized as 100% in the I/R group and 0% in the control group, and cell viability normalized as 100% in the control group. (A) LDH release from the heart after I/R treatment. Treatment for the three groups: control group (CTL) perfused with K–H solution for 90 min; ischemia and reperfusion group (I/R), KH solution stopped for 40 min and reperfusion for 30 min; apelin-treated I/R group (apelin + I/R) perfused with 30 pmol/L apelin in K–H solution and treated with I/R. (B) LDH content in cell-culture medium normalized as 100% in H/R group and 0% in control group. (C) Cell viability normalized as 100% in control group. Treatment for the cells in (B) and (C), hypoxia and re-oxygenation group (H/R): cells were subjected to 1% O2–5% CO2 for 3 h hypoxia, then 2 h 95% air–5% CO2. Apelin and wortmanin treatment: concentration of apelin and/or wortmanin added into medium before H/R treatment. Values are means ± SE (n = 6 for each group). *p < 0.05 compared with I/R or H/R group.

Fig. 4. Effects of ERK1/2 inhibitor PD098059 on LDH leakage and cell viability. (A) Cell viability normalized as 100% in control group. (B) LDH content in cell-culture medium normalized as 100% in H/R group and 0% in control group. Values are means ± SE (n = 6 for each group). *p < 0.05 compared with I/R or H/R group.
Apelin treatment ameliorated the inhibited myocardial SOD activity induced by I/R injury (398/6.5 vs. 790/6.37 U/mg protein in the I/R-alone and the apelin + I/R group, respectively, n = 6, p < 0.01, Fig. 6D). Similarly, SOD activity was significantly lower in H/R-treated cardiomyocytes than in the control group (370/6.62 vs. 588/6.70 U/mg protein, n = 6, p < 0.01, Fig. 6E), but apelin treatment increased the SOD activity to 522/6.44 U/mg protein (n = 6, p < 0.01 vs. H/R alone, Fig. 6E).

3.5. Apelin modulated I/R- or H/R-induced phosphorylation of ERK1/2, Akt and eNOS expression

Western blot analysis of the phosphorylation of ERK1/2 and Akt, and expression of eNOS in primary cultured cardiomyocytes revealed that H/R decreased the phosphorylation of ERK1/2, and apelin treatment reversed the H/R-induced decreased activation of ERK1/2 (n = 3, p < 0.05, Fig. 7A and B). H/R increased the phosphorylation of Akt, by 30% (n = 3, p > 0.05, Fig. 7C and D), which was further enhanced by apelin (increased by 34% compared with H/R-alone group, n = 3, p < 0.01 vs. H/R group, Fig. 7C and D). H/R injury significantly decreased the expression of eNOS, but apelin treatment enhanced the eNOS expression by 40% as compared with the H/R-alone group (n = 3, p < 0.01, Fig. 8A and B).

4. Discussion

Recent researches have suggested that the adipocytokine apelin may be a new and potentially important cardio-protective autacoid, which is up-regulated rapidly after myocardial ischemia, activating the reperfusion injury salvage kinase pathway and delaying the mitochondrial permeability transition pore opening [19,29,30]. Apelin has been described as an adipokine because of its production and secretion by isolated mature adipocytes [2]. In rodents, the up-regulated mRNA expression of both apelin and APJ has been observed in several tissues including the heart and vessels [18,23,26], and the cardiovascular system appears to be a primary target of apelin because blood pressure decreases...
and cardiac function is potentiated [5] after administration of apelin in rats. Moreover, changes in apelin and APJ expression have been found in the human heart under cardiac dysfunction [4,8].

The present study identified apelin/APJ as a cytokine and the receptor system strongly induced in the myocardium after I/R injury, which provided a protective effect on cardiac I/R injury, possibly via resisting cardiomyocyte apoptosis. Our study is the first to demonstrate that apelin/APJ is an endogenous protective system against myocardial I/R injury and the first to illustrate the anti-apoptotic effects of apelin/APJ in protecting against I/R injury in the heart.

The Langendorff model of cardiac I/R injury was used in our study to simulate distinct clinical scenarios in patients with acute myocardial infarction receiving reperfusion therapy. Global ischemia followed by reperfusion decreased myocardial contractile function.

Fig. 6. Detection of the anti-oxidant function of apelin in primary cultured myocardial cells after I/R or H/R injury. (A) Cells in each groups were incubated with DHE for 5 min, ROS-positive cells will display red fluorescence in the nucleus. Then ROS-positive cells and total cells and were counted. Pictures show the red fluorescence in every group and bars show the averaged percentage of ROS-positive cells to total cells. (B and C) MDA contents in rat heart or cultured neonatal myocardium. (D and E) Supernatant SOD activities in rat heart or primary cultured neonatal myocardial cells. Values are means ± SE (n = 6 for each group). *p < 0.01 vs. I/R or H/R group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
n = 3; p < 0.05 vs. H/R group). (C) Primary cultured neonatal myocardium treated with H/R with or without apelin (30 pmol/L). Then the cells were harvested and subjected to Western blot analysis to detect eNOS expression (upper panel) and the same blots were also blotted for α-tubulin (lower panel) as a loading control. (D) Densitometry of eNOS levels normalized to α-tubulin in each treatment condition in (A) (mean ± SE; n = 3; p < 0.01 vs. H/R treatment).

function, as revealed by decreased values of ±LVdp/dtmax and LVESP and elevated coronary perfusion pressure and LVEDP, for evidence of severe I/R injury, as has been described [3].

To gain mechanistic insight into the cardio-protective effects of apelin/APJ, we used a cultured neonatal cardiomyocyte model of H/R. Consistent with previous investigations [11], H/R led to accelerated apoptosis in our cell-culture model, as shown by TUNEL staining. Use of two complementary techniques to assess cell death after H/R revealed reduced cell viability and induced LDH leakage.

We detected a robust induction of APJ levels in the rat heart after I/R, which emphasizes the potential role of APJ in I/R injury. Supporting the conclusion that cardiomyocytes are a major source of apelin/APJ expression after I/R injury, we revealed that apelin/APJ expression was up-regulated in cardiomyocytes after H/R. The hypoxia-inducible factor (HIF) appeared to be involved in the up-regulation of apelin in cardiomyocytes after I/R, because previous studies have shown apelin gene expression and secretion upregulated with HIF [28].

To demonstrate the apelin/APJ up-regulation is an endogenous protective pathway, we administrated apelin to I/R hearts and found that it significantly ameliorated I/R-induced decrease in myocardial contractile function, as shown by elevated values of ±LVdp/dtmax and LVESP as well as decreased perfusion pressure and LVEDP. As well, myocardial LDH leakage was markedly alleviated with apelin treatment. These data were supported by other reports [19]. Our results also showed that apelin, when added before hypoxia, suppressed cellular LDH leakage and increased cell viability in cardiomyocytes treated with H/R.

Oxygen free radicals are a key factor involved in I/R injury. Our study revealed that apelin decreased ROS generation and MDA content and increased SOD activity in cultured cardiomyocytes under H/R treatment. We also detected the same phenomenon in I/R hearts. These findings suggest that apelin/APJ may protect the heart by alleviating oxidative injury during I/R. Nitric oxide (NO), known to be a potent vasodilator and antioxidant, abolishes mitochondrial oxidant damage in adult rat cardiomyocytes [38]. NO is capable of inhibiting neutrophil superoxide anion production via a direct action on the membrane components of NADPH oxidase and the assembly of NADH/NADPH oxidase subunits [6,9]. Our present study showed that apelin promoted eNOS expression, decreased perfusion pressure and suppressed superoxide production in cardiomyocytes, as we have reported earlier [16]. These results indicate that apelin could improve I/R-induced cardiac dysfunction through increased NO formation.

Apoptosis plays an important role in I/R injury pathogenesis. Our study showed that apelin blocked H/R-induced apoptosis in cultured neonatal cardiomyocytes. Apelin was used at 30 pmol/L during these cell-culture studies, a dose that is probably pathophysiologically relevant: normal human left ventricles contain ~0.967 ng/mL apelin; however, up to 3-fold higher apelin levels have been reported in specific situations [4].

Apelin suppresses apoptosis in many kinds of cells through the PI3K-Akt and ERK pathway [27,33,37]. Our data indicate that phosphorylation of Akt and ERK might be a target of apelin/APJ in...
protecting cardiomyocytes against H/R injury. More importantly, from a mechanistic standpoint, the protective effects of apelin were abolished by PI3K and ERK inhibitors, which points to a critical involvement of the PI3K-Akt and ERK1/2 pathways in the cytoprotective effects of apelin, as was found in other results [29,30], which suggests that apelin/APJ is an endogenous defending pathway in myocardial I/R injury.

Our data showed that apelin, through APJ receptor activation, enhanced by I/R, improves cardiac dysfunction after myocardial I/R injury by suppressing myocardial apoptosis and resisting oxidation effects. The mechanisms mediating these protective effects of apelin may be through up-regulating eNOS and phosphorylating PI3K-Akt and ERK1/2 pathways. This study raises the intriguing possibility of the potential value of apelin/APJ in protecting ischemic heart disease, and the pathway might constitute an important therapy target.

Acknowledgements

This work was supported by the State Major Basic Research Development Program of the People's Republic of China (grant no. 2006CB503807), the Beijing Natural Science Foundation of the People's Republic of China (grant no. 7052041), and the National Natural Science Foundation of the People's Republic of China (grant no. 30600232).

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