Apelin-13 protects the heart against ischemia-reperfusion injury through inhibition of ER-dependent apoptotic pathways in a time-dependent fashion

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Tao J, Zhu W, Li Y, Xin P, Li J, Liu M, Li J, Redington AN, Wei M. Apelin-13 protects the heart against ischemia-reperfusion injury through inhibition of ER-dependent apoptotic pathways in a time-dependent fashion. Am J Physiol Heart Circ Physiol 301: H1471–H1486, 2011. First published July 29, 2011; doi:10.1152/ajpheart.00097.2011.—Endoplasmic reticulum (ER) stress is activated during and contributes to ischemia-reperfusion (I/R) injury. Attenuation of ER stress-induced apoptosis protects the heart against I/R injury. Using apelin, a ligand used to activate the apelin APJ receptor, which is known to be cardioprotective, this study was designed to investigate 1) the time course of changes in I/R injury after ER stress; 2) whether apelin infusion protects the heart against I/R injury via modulation of ER stress-dependent apoptosis signaling pathways; and 3) how phosphatidylinositol 3-kinase (PI3K)/Akt, endothelial nitric oxide synthase (eNOS), AMP-activated protein kinase (AMPK), and ERK activation are involved in the protection offered by apelin treatment. The results showed that, using an in vivo rat I/R model induced by 30 min of ischemia followed by reperfusion, infarct size (IS) increased from 2 h of reperfusion (34.85 ± 2.14%) to 12 h of reperfusion (48.98 ± 3.35, P < 0.05), which was associated with an abrupt increase in ER stress-dependent apoptosis activation, as evidenced by increased CCAAT/enhancer-binding protein homologous protein (CHOP), caspase-12, and JNK activation (CHOP: 2.49-fold increase, caspase-12: 2.09-fold increase, and JNK: 3.38-fold increase, P < 0.05, respectively). Administration of apelin at 1 μg/kg not only completely abolished the activation of ER stress-induced apoptosis signaling pathways at 2 h of reperfusion but also significantly attenuated time-related changes at 24 h of reperfusion. Using pharmacological inhibition, we also demonstrated that PI3K/Akt, AMPK, and ERK activation were involved in the protection against I/R injury via inhibition of ER stress-dependent apoptosis activation. In contrast, although eNOS activation played a role in decreasing IS at 2 h of reperfusion, it failed to modify either IS or ER stress-induced apoptosis signaling pathways at 24 h after reperfusion.

The endoplasmic reticulum (ER) is an important cellular organelle for protein synthesis, folding, maturation, and transport. Normal function of the ER depends on a stable microenvironment, which can be disrupted, for example, by nutrient deprivation, redox imbalance, and impaired Ca²⁺ homeostasis, all of which may lead to the accumulation of unfolded or misfolded proteins in the ER, a pathological process termed ER stress (22). In the case of mild ER stress, the unfolded protein response (UPR) including induction of molecular chaperones is induced to attenuate cellular dysfunction or damage. However, if ER stress is prolonged or too severe, the UPR may lead to the activation of apoptosis through CCAAT/enhancer-binding protein homologous protein (CHOP), caspase-12, and JNK pathways (5).

ER stress is involved in various cardiovascular diseases (7, 9, 34, 43) for which the detailed pathological process has been widely investigated (31, 40). Furthermore, modification of ER stress has become an important therapeutic target for the treatment of cardiovascular diseases (32). In the setting of ischemia-reperfusion (I/R) injury, different strategies have been used to attenuate ER stress to protect the heart. For example, ischemic postconditioning is associated with a decrease in the expression of the chaperone calreticulin and reduced JNK activation, possibly through enhanced expression of p38 MAPK (28). In contrast, enhancing the levels of ER resident chaperones, 78-kDa glucose-regulated protein (GRP78) and 94-kDa glucose-regulated protein via activating transcription factor 6 overexpression, a component of the UPR, can also protect the heart against I/R injury secondary to ER stress (30). The benefit from induction of chaperone expression has also been validated in other studies (12, 26, 44). Other strategies have also been pursued to modify this pathway. Belmont and colleagues (3) showed that enhancing the activity of the ER response gene derlin-3, a component of ER-associated protein degradation through activation of activating transcription factor 6, successfully rescued cardiomyocytes from ischemia-induced death.

To date, studies of the role of ER stress in I/R injury have concentrated predominantly on early (2–4 h) responses, and the later time course of changes in ER stress activation has not been rigorously examined. This is important, as clearly the responses to I/R injury are dynamic and continue to evolve long after the primary insult.

Apelin, as a relatively recently discovered endogenous ligand for the G protein-coupled APJ receptor (41), is not only involved in cardiovascular development (6, 8) but also plays multiple roles in cardiovascular pathological processes (21). Studies have shown that apelin acutely protects the heart against I/R injury via the activation of phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial nitric oxide (NO) synthase (eNOS) and ERK signaling pathways (25, 37) and may have more sustained effects to enhance cardiac performance and improve hemodynamics in heart failure (1, 19). Furthermore, apelin has been shown to stimulate glucose metabolism via the modulation of AMP-activated protein kinase (AMPK) (10), and activation of AMPK has also been shown to improve ER

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stress (42), which might account for the protection against I/R injury conferred by AMPK (39, 45).

Therefore, in the present study, we used a rat in vivo I/R injury model to examine several components of the ER stress response. First, we studied the temporal responses of ER stress during the first 24 h after I/R injury. Second, we examined whether apelin protects against I/R injury via modulation of ER stress. Finally, we examined the role of activation of PI3K/Akt, eNOS, ERK, and AMPK in the modulation of ER stress-dependent apoptosis.

MATERIALS AND METHODS

Animals

Eight-week-old male Sprague-Dawley rats, weighing between 250 and 280 g (Experimental Animal Center, Fudan University, Shanghai, China) were studied. The animal research study protocol was in compliance with National Institutes of Health (NIH) Guide for the Care of Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and was approved by the Animal Care Committee of Shanghai Sixth Hospital, Shanghai Jiao Tong University School of Medicine. All rats were housed for 2 wk for an acclimatization period before the experiments.

In Vivo I/R Injury Model

The in vivo rat I/R injury model has been established in our laboratory and has been previously described (46). Briefly, after anesthesia with pentobarbital sodium (50 mg/kg ip) and endotracheal intubation, animals were ventilated (Animal Respirator DW-2000, Alcott Biotech, Shanghai, China) with room air at 45–60 breaths/min with the tidal volume set to 1.0 ml/100 g body wt with body temperature maintained at 37°C using a heating pad and monitored with a thermometer. Using sterile surgical procedures, the right carotid artery was cannulated with a 24-gauge angiocatheter for drug infusion. A left thoracotomy was performed through the fourth 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. Before LCA occlusion, animals were anticoagulated (150 U/kg sodium heparin). Cardiac ischemia was confirmed by an initially pale and later cyanotic area below the suture and ST-T elevation shown in ECG, whereas reperfusion was characterized by the rapid disappearance of cyanosis followed by vascular blush. The duration of ischemia was 30 min, and hearts were examined 2, 12, and 24 h after reperfusion to assess the temporal changes in I/R injury. For rats where I/R injury was assessed at 12 or 24 h after reperfusion, the chest was closed in layers at 30 min after reperfusion. Rats were allowed to recover.

Area at Risk and Infarct Size Determination

After the animals had been killed, the LCA was reoccluded, and 2% Evans blue dye was retrogradely injected into the ascending aorta to delineate the area at risk. The heart was then cut into five to six transverse slices followed by an incubation for 15 min at 37°C in a phosphate-buffered 1% 2,3,5-triphenyltetrazolium chloride solution to determine the infarcted myocardium. The extent of the area of necrosis was quantified by computerized planimetry and corrected for the weight of the tissue slices. Infarct size (IS) was expressed as a percentage of the total weight of the left ventricular (LV) area at risk (46).

Western Blot Analysis

Western blot analysis was performed on myocardium from the area at risk. After homogenization and protein quantification, 40 μg of protein from each sample were separated using 12% Tris-glycine SDS gels by electrophoresis and then transferred to polyvinylidene difluoride membranes. After being blocked with 5% BSA in Tris buffer, membranes were incubated with primary antibodies. Primary antibodies targeting phospho-Akt (Ser473), total Akt, phospho-eNOS (Ser1177), total eNOS, phospho-ERK1/2 (Thr202/Tyr204), total ERK, phospho-JNK (Thr183/Tyr185), JNK, phospho-AMPK-α (Thr172), and total AMPK were obtained from Cell Signaling Technology. GRP78 and caspase-12 were from Sigma-Aldrich, and CHOP and β-actin were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary antibody was then used. An ECL Western blotting detection kit (Pierce) was finally used to measure the immunoreaction with a light-sensitive film (Kodak). The band for each protein was then quantified by densitometry using ImageJ software (version 1.41, NIH) and normalized to the expression of β-actin for protein loading.

Evaluation of Apoptosis Activity

TUNEL staining was performed using a commercially available kit (Roche), according to the manufacturer’s instructions, on heart tissue slices incorporating the infarct zone that were randomly selected.

Table 1. Mortality rates for each group recorded for the different protocols

<table>
<thead>
<tr>
<th>Protocol I</th>
<th>Mortality Rate, numbers of dead/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/R, 30 min of ischemia</td>
<td>1/6</td>
</tr>
<tr>
<td>I/R, 30 min of reperfusion</td>
<td>1/6</td>
</tr>
<tr>
<td>I/R, 2 h of reperfusion</td>
<td>2/14</td>
</tr>
<tr>
<td>I/R, 12 h of reperfusion</td>
<td>3/14</td>
</tr>
<tr>
<td>I/R, 24 h of reperfusion</td>
<td>3/14</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Protocol IIA</th>
<th>Mortality Rate, numbers of dead/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/R</td>
<td>2/8</td>
</tr>
<tr>
<td>I/R + apelin (0.1 μg/kg)</td>
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</tr>
<tr>
<td>I/R + apelin (1 μg/kg)</td>
<td>1/8</td>
</tr>
<tr>
<td>I/R + apelin (10 μg/kg)</td>
<td>2/8</td>
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</table>

<table>
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<tr>
<th>Protocol IIB</th>
<th>Mortality Rate, numbers of dead/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/R, 2 h of reperfusion</td>
<td>2/14</td>
</tr>
<tr>
<td>I/R + apelin, 2 h of reperfusion</td>
<td>2/14</td>
</tr>
<tr>
<td>I/R, 12 h of reperfusion</td>
<td>3/14</td>
</tr>
<tr>
<td>I/R + apelin, 12 h of reperfusion</td>
<td>3/14</td>
</tr>
<tr>
<td>I/R, 24 h of reperfusion</td>
<td>2/14</td>
</tr>
<tr>
<td>I/R + apelin, 24 h of reperfusion</td>
<td>3/14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protocol IIIA</th>
<th>Mortality Rate, numbers of dead/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/R + LY-294002</td>
<td>3/21</td>
</tr>
<tr>
<td>I/R + apelin + LY-294002</td>
<td>2/21</td>
</tr>
<tr>
<td>I/R + compound C</td>
<td>2/21</td>
</tr>
<tr>
<td>I/R + apelin + compound C</td>
<td>3/21</td>
</tr>
<tr>
<td>I/R + l-NAME</td>
<td>3/21</td>
</tr>
<tr>
<td>I/R + apelin + l-NAME</td>
<td>3/21</td>
</tr>
<tr>
<td>I/R + PD-98059</td>
<td>3/21</td>
</tr>
<tr>
<td>I/R + apelin + PD-98059</td>
<td>2/21</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Protocol IIIB</th>
<th>Mortality Rate, numbers of dead/total</th>
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<tbody>
<tr>
<td>I/R + LY-294002</td>
<td>1/5</td>
</tr>
<tr>
<td>I/R + apelin + LY-294002</td>
<td>0/5</td>
</tr>
<tr>
<td>I/R + compound C</td>
<td>0/5</td>
</tr>
<tr>
<td>I/R + apelin + compound C</td>
<td>1/5</td>
</tr>
<tr>
<td>I/R + l-NAME</td>
<td>0/5</td>
</tr>
<tr>
<td>I/R + apelin + l-NAME</td>
<td>1/5</td>
</tr>
<tr>
<td>I/R + PD-98059</td>
<td>1/5</td>
</tr>
<tr>
<td>I/R + apelin + PD-98059</td>
<td>0/5</td>
</tr>
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Events of sudden cardiac death were recorded for each protocol during the ischemia-reperfusion (I/R) injury process. The occurrence of cardiac death between groups with different dose of apelin (protocol IIB) or different pharmacological interventions (protocol IIIa and protocol IIIB) was similar (a χ²-test was used to test for the occurrence of sudden death between different time points, different doses of apelin, or different pharmacological blockers). l-NAME, Nω-nitro-l-arginine methyl ester.
selected from each group. Tissue sections were examined microscopically at ×400 magnification, and at least 100 cells from the peri-infarct area were counted for each field of view, with a total of 10 fields examined (Leica Q500MC). The peri-infarct area was predetermined by hematoxylin and eosin staining performed on the adjacent tissue slide. The percentage of TUNEL-positive cells was calculated as follows: number of apoptotic cells/total number counted × 100%.

Using previously described methods (27), caspase-3 activity was measured using a commercial available kit (CASP3C, Sigma) for heart tissue based on the amount of p-nitroanilide cleaved from peptide (acetyl-Asp-Glu-Val-Asp p-nitroanilide) by caspase-3 per minute, which can be calculated from the absorbance value at 405 nm. All samples were analyzed as the percent increase compared with controls.

Confocal Laser Scanning Microscopy Experiments

To evaluate whether ER stress signaling activation occurs in cardiomyocytes, immunofluorescence staining was carried out. Frozen OCT-embedded heart tissue was cut into 5-μm sections using a cryostat. Double staining was performed using antibodies against ER stress markers [rabbit anti-rat antibodies targeting either GRP78 (G9043, Sigma-Aldrich) or CHOP (AP00161PU-N, Acris Antibodies)] and α-actinin [mouse anti-rat antibody (A7811, Sigma-Aldrich)]. Secondary fluorescent antibodies were used at a dilution of 1:1,000 [goat anti-rabbit IgG (Alexa fluor 488) and goat anti-mouse IgG (Alexa fluor 555), Cell Signaling]. 4',6-Diamidino-2-phenylindole (D9542, Sigma-Aldrich) staining was also done to label nucleic acid. All images were acquired using a Zeiss LSM710 laser scanning confocal microscope.

Pharmacological Interventions

To test whether apelin/APJ signaling activation can protect against ER stress, apelin-13, a potent ligand for the apelin APJ receptor (synthesized by GL Biochem Shanghai, Shanghai, China) was given intravenously 15 min before reperfusion. Pharmacological blockers (Calbiochem), including LY-294002 (PI3K/Akt inhibitor, 0.3 g/kg), PD-98059 (ERK inhibitor, 0.3 mg/kg), and compound C (AMPK inhibitor, 250 μg/kg), were all dissolved in DMSO (<300 μg/kg), and Nω-nitro-l-arginine methyl ester (L-NAME; Sigma-Aldrich, nonselective eNOS inhibitor, 10 mg/kg) was dissolved in saline and administered intravenously 10 min before coronary artery occlusion to test the role for each specific signaling pathway.

Monitoring of Hemodynamic Parameters

Hemodynamic parameters were measured using the same methods described in our previous study (46). Briefly, during the surgical procedure, right carotid artery cannulation was performed,
Fig. 2. Double immunofluorescent staining was done for I/R rats that completed 2, 12, and 24 h of reperfusion (n = 4 for each time point). Confocal laser scanning microscopy was then performed on tissue at the area of risk, with α-actinin stained in red and GRP78 (A) or CHOP (B) stained in green. Nucleic acid was labeled by 4′,6-diamidino-2-phenylindole (DAPI). A merged image was also obtained to evaluate the correlation of α-actinin with either GRP78 or CHOP (stained with yellow) with detailed structure further shown at a higher magnification (right column). Time-related changes in expression at 2 h (top row), 12 h (middle row), and 24 h (bottom row) of reperfusion was demonstrated, thus indicating that cardiomyocytes are an important component of ER stress activation, even though ER stress activation can be observed in noncardiomyocytes (as shown in the merged images).
and a 24-gauge angiocatheter was introduced retrogradely into the LV. LV pressure tracings were digitized with an analog-to-digital converter and stored in a personal computer (sampling rate of 1 kHz, MPA-CFS, Alcott Biotech, Shanghai, China). LV end-diastolic pressure and the maximal rates of pressure rise/fall were analyzed in a blinded fashion with dedicated software (MPC Systems, 2000M). Mean arterial pressure (MAP) was also monitored with a femoral artery cut down approach using the same methods as described above. Hemodynamic parameters were monitored until 2 h after reperfusion.

For rats assigned to protocols with 12 or 24 h of reperfusion, the chest was closed at 30 min after reperfusion, as described above in the surgical procedures. The hemodynamic parameters were obtained again via a left carotid and femoral artery cut down approach just before rats were euthanized.

**Experimental Protocols**

**Protocol I.** This protocol aimed to test the time course of changes in ER stress-induced apoptosis activation and IS. At the end of the 30-min ischemic period, Sprague-Dawley rats were randomly assigned to the following five subgroups according to the different observation periods: 1) nonreperfusion group (n = 6; rats were killed and hearts were harvested after 30 min of coronary occlusion only), 2) 30-min reperfusion group (n = 6), 3) 2-h reperfusion group (n = 14), 4) 12-h reperfusion group (n = 14), and 5) 24-h reperfusion group (n = 14).

Heart tissue was obtained for Western blot analysis to quantify ER stress-induced apoptosis signaling activation for each group (n = 6 rats/group), and IS was measured for rats that completed 2-, 12-, and 24-h reperfusion periods (n = 8 rats/group). TUNEL staining and a caspase-3 activity assay were also performed for the three time points.

There was one sham-operated group of rats (n = 6) that were killed at the end of 24 h of reperfusion; heart tissue was obtained and served as the sham control group. Another 12 I/R rats were euthanized at 2, 12, and 24 h of reperfusion, respectively (n = 4 for each time point), and heart tissue was obtained for fluorescent immunostaining.

**Protocol IIA.** This protocol aimed to test if apelin exerts its protection against I/R injury in a dose-dependent fashion. At the end of the 30-min ischemic period, Sprague-Dawley rats were randomly assigned to the following four subgroups according to the dose of apelin infusion: 1) I/R group without any treatment (n = 8), 2) I/R group treated with apelin (apelin was delivered intravenously as a bolus at a dose of 0.1 μg/kg 15 min before reperfusion, n = 8), 3) I/R group treated with apelin at a dose of 1 μg/kg (n = 8) and delivered the same way as described above, and 4) I/R group treated with apelin at a dose of 10 μg/kg and delivered as described above (n = 8). All rats were killed at the end of 2 h of reperfusion, and the LV was processed for IS quantification.
Hemodynamic parameters were obtained from these four groups of rats at different time points to assess whether different doses of apelin infusion result in significant changes in hemodynamic parameters during the I/R injury process.

Protocol III. Based on protocol II, our preliminary data indicated that the optimal effect was obtained with an apelin dose of 1 μg/kg. Therefore, protocol III aimed to test whether this dose of apelin protects the heart against ER stress-induced apoptosis activation in a time-dependent fashion. A total of 84 SD rats were randomly assigned to the I/R group or the I/R + apelin group (n = 42 rats/group). Rats from each group were then further randomly assigned to the following three subgroups according to the observation period: 1) 2 h of reperfusion (n = 14), 2) 12 h of reperfusion (n = 14), and 3) 24 h of reperfusion (n = 14). Sprague-Dawley rats were euthanized at the completion of the observation period. IS was quantified for eight rats from these six subgroups where the hemodynamic parameters were also recorded for rats that underwent either 12 or 24 h of reperfusion and were compared with those obtained from protocol II. Heart tissue obtained from the remaining rats (6 rats/group) were processed for TUNEL staining, Western blot analysis, and caspase-3 activity quantification.

Another 10 I/R rats (5 rats with apelin infusion and 5 rats without apelin therapy) were killed at 30 min of reperfusion to quantify JNK activation. Data obtained from six sham-operated rats (five rats with apelin infusion and five rats without apelin) were killed at 30 min of reperfusion to quantify JNK activation. Data obtained from six sham-operated rats (in protocol I) after completion of 24 h of reperfusion were used to compare differences of protein expression and apoptosis activation between groups.

Fluorescent immunostaining was done again in eight I/R rats with apelin infusion at 1 μg/kg to assess whether apelin infusion can attenuate ER stress in a time-dependent fashion (rats were euthanized at 2 and 24 h of reperfusion, respectively) using the same methodology described above. Data were compared with those obtained from I/R rats without apelin therapy in protocol I.

### Protocol III. This protocol aimed to assess how the cardiac protection afforded by apelin infusion was modified by PI3K, AMPK, eNOS, and ERK1/2 inhibitors.

Sprague-Dawley rats were randomly assigned to receive one of four pharmacological blockers: 1) LY-294001 (a PI3K/Akt inhibitor), 2) compound C (an AMPK inhibitor), 3) 1-NAME (a nonselective eNOS inhibitor), and 4) PD-98059 (an ERK1/2 inhibitor). Drugs were delivered as described above. Ten minutes before reperfusion was reinstated, rats in each inhibitor therapy subgroup were then further randomly assigned to receive either apelin infusion or placebo treatment (a total of 8 groups, n = 21 rats/group). IS was quantified at both 2 and 24 h of reperfusion (n = 8 rats/group at each time point). Time course changes in hemodynamic parameters were monitored for these rats from the eight subgroups using the same methodology as described above.

Other rats were killed at 24 h of reperfusion (n = 5 rats/group), and heart tissue was collected for TUNEL staining and Western blot analysis targeting CHOP, phospho-JNK, and cleaved caspase-12. Data from the sham-operated group (from protocol I) as well as the I/R and I/R + apelin groups (from protocol II) were used for comparison.

Protocol IIIb. To assess the roles of PI3K/Akt, AMPK, eNOS, and ERK signaling pathways, 40 Sprague-Dawley rats underwent the same experiments as described above in protocol III (a total of 8 groups, n = 5 rats/group) and were killed at 30 min of reperfusion. LV tissue from the area at risk was obtained and used for Western blot analysis to quantify the phosphorylation levels of Akt, AMPK, eNOS, and ERK1/2.

Finally, another three groups (sham-operated, I/R, and I/R + apelin groups, n = 5 rats/group for each) underwent the same experiments as described in protocol II and were also killed at 30 min of reperfusion for Western blot analysis.

### Table 2. Effects of different doses of apelin on hemodynamic parameters at different time points during the I/R process in rats

<table>
<thead>
<tr>
<th>Time after reperfusion (min)</th>
<th>n</th>
<th>HR, beats/min</th>
<th>MAP, mmHg</th>
<th>LVEDP, mmHg</th>
<th>dP/dt max, mmHg</th>
<th>dP/dt min, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min after ischemia</td>
<td>21</td>
<td>358 ± 26</td>
<td>115 ± 8</td>
<td>6.26 ± 1.69</td>
<td>4,488 ± 390</td>
<td>3,773 ± 391</td>
</tr>
<tr>
<td>2 h after reperfusion</td>
<td>7</td>
<td>360 ± 21</td>
<td>117 ± 9</td>
<td>6.81 ± 1.84</td>
<td>4,511 ± 422</td>
<td>3,808 ± 331</td>
</tr>
<tr>
<td>12 h after reperfusion</td>
<td>7</td>
<td>367 ± 22</td>
<td>115 ± 8</td>
<td>6.61 ± 1.83</td>
<td>4,523 ± 392</td>
<td>3,818 ± 337</td>
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<tr>
<td>24 h after reperfusion</td>
<td>7</td>
<td>365 ± 21</td>
<td>116 ± 7</td>
<td>6.65 ± 1.84</td>
<td>4,509 ± 289</td>
<td>3,861 ± 351</td>
</tr>
</tbody>
</table>
| Values are means ± SD; n, number of rats/group. All data were obtained from rats assigned to protocols IIA and IIB (data obtained from rats with sudden cardiac death were excluded). For 12 and 24 h of reperfusion, data were only from rats that were assigned to protocol II. HR, heart rate; MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt max, maximal rate of increase in intraventricular pressure; dP/dt min, maximal rate of decrease in intraventricular pressure. *P < 0.05 vs. the I/R group at the same time point (two-way ANOVA followed by a least-significant-difference corrected multiple-comparison test was used to compare differences between groups at the same time points or between different time points within the same group).
Role of ER Stress in Dynamic Ischemia-Reperfusion Injury

Statistical Analysis

All the data are expressed as means ± SD. Time course changes in hemodynamic indexes with different pharmacological blockers were analyzed by two-way ANOVA followed by a least-significant-difference corrected multiple-comparison test. One-way ANOVA followed by a least-significant-difference corrected multiple-comparison test was used to compare all other variables between groups or at different time points. A χ²-test was used to assess differences in mortality between groups with different treatments. P values of <0.05 were considered as statistically significant. Statistical analyses were performed using SPSS 13.0 statistics software (SPSS, Chicago, IL).

RESULTS

Mortality Rate

Sudden cardiac death occurred during the I/R injury process; however, different doses of apelin infusion did not significantly improve the survival rate during the 24-h reperfusion process. In addition, different pharmacological inhibitors, including LY-294002, compound C, l-NAME, and PD-98059, resulted in similar mortality as the I/R group or the I/R + apelin group (Table 1).

I/R Injury Is Associated With a Time-Related Increase in ER Stress-Dependent Apoptosis Activation

After I/R injury, we observed dynamic changes in ER stress-dependent apoptosis signaling activities. ER stress-specific protein markers, including GRP78, CHOP, phospho-JNK, and caspase-12, remained unchanged at the end of the 30-min index ischemia period, exhibiting similar levels as in sham-operated controls (P > 0.05). However, during the 24-h observation period after reperfusion, there was a time-dependent change in ER stress-dependent apoptosis signaling activation, demonstrating a peak in protein expression at 12 h of reperfusion, which remained raised but tended to fall at 24 h. The pattern of JNK phosphorylation was slightly different compared with GRP78, CHOP, and caspase-12 in that there was an earlier increase at 30 min of reperfusion, which fell at 2 h and again peaked at 12 h of reperfusion (Fig. 1). Double fluorescent immunostaining showed that GRP78 and CHOP were associated with α-actinin expression, indicating that cardiomyocytes are an important component of ER stress responses during the I/R injury process (Fig. 2). However, ER stress in noncardiomyocyte cells was also observed in confocal immunostaining images at higher magnification when α-actinin staining was merged with either GRP78 or CHOP.

Consistent with ER stress-related apoptosis signaling activation, both caspase-3 activity and the percentage of TUNEL-positive cells exhibited the same pattern of changes as ER stress-induced apoptosis activation, being significantly increased from 2 to 12 h of reperfusion. This augmented activation was, however, downregulated at 24 h of reperfusion. Importantly, IS increased from 2 to 12 h of reperfusion (2 h: 34.85 ± 2.14% and 12 h: 48.98 ± 3.35%, P < 0.05) with no further increase at 24 h (50.57 ± 3.24%, P > 0.05 vs. 12 h; Fig. 3).

Apelin Infusion Protects the Heart Against I/R Injury Partly Through Attenuation of ER Stress-Dependent Apoptosis Activation

Apelin infusion at 0.1, 1, or 10 μg/kg when delivered 15 min before the end of the ischemic period did not affect hemodynamic parameters as there were no significant changes in heart rate, blood pressure, and cardiac performance measured at the end of ischemia or 30 min after reperfusion (Table 2). The data obtained in the present study also confirmed previous observations showing that apelin can reduce IS (P < 0.05 vs. the I/R group). Moreover, apelin exerted cardiac protection in a dose-dependent fashion (Fig. 4). The higher dose of apelin delivered at 1 μg/kg exhibited enhanced protection, resulting in a further decrease in IS when measured at 2 h of reperfusion (18.68 ± 1.99) compared with 0.1 μg/kg (25.54 ± 1.80, P < 0.05). However, this benefit was lost when apelin was given at 10 μg/kg, where there was a similar IS to that at 0.1 μg/kg (26.62 ± 1.53, P > 0.05; Fig. 4). Improved hemodynamic parameters at 24 h of reperfusion were only observed when apelin was given at 1 μg/kg but not at other doses of apelin (Table 2).

We then tested whether 1 μg/kg apelin protects the heart via modulation of ER stress-dependent apoptosis. Apelin infusion completely blocked the activation of ER stress-dependent apoptosis at 2 h of reperfusion, with CHOP, phospho-JNK, and cleaved caspase-12 showing similar levels to those in sham-operated controls (P > 0.05). Time-dependent increases in CHOP, phospho-JNK, GRP78, and cleaved caspase-12 levels at 24 h of reperfusion were only observed when apelin was given at 1 μg/kg but not at other doses of apelin (Table 2).

Fig. 4. Dose-dependent protection by apelin infusion against I/R injury was tested. An optimal effect was achieved when apelin was delivered at 1 μg/kg 15 min before reperfusion began. A: representative sequential LV slices from the I/R group (n = 6), I/R + apelin (0.1 μg/kg) group (n = 7), I/R + apelin (1 μg/kg) group (n = 7), and I/R + apelin (10 μg/kg) group (n = 6). B: bar graph showing percent IS (the same as described in Fig. 2). *P < 0.05 vs. the I/R group; †P < 0.05 vs. the I/R + apelin (0.1 μg/kg) group; ‡P < 0.05 vs. the I/R + apelin (1 μg/kg) group (one-way ANOVA followed by a least-significant-difference corrected multiple-comparison test was used to compare differences between groups with different doses of apelin infusion).
at 12 or 24 h of reperfusion compared with 2 h were also significantly attenuated by this therapy; however, protein levels were still higher than at 2 h (P < 0.05; Fig. 5). Double fluorescent immunostaining demonstrated that time-related increases in GRP78 and CHOP expression were attenuated by apelin infusion, which was consistent with Western blot analysis findings (Fig. 6).

Consistent with ER stress-dependent apoptosis activation, both caspase-3 activity and the percentage of TUNEL-positive cells were significantly reduced while manifesting a similar temporal pattern. Of note, these beneficial effects on ER stress-related apoptosis were not only associated with a significant decrease in IS at 2 h of reperfusion but also resulted in a significant reduction at 12 and 24 h, albeit with a higher IS than at 2 h of reperfusion (Fig. 7).

**Signaling Pathways Involved in Protection Against ER Stress by Apelin Infusion**

Different pharmacological blockers were used to test whether PI3K/Akt, eNOS, AMPK, and ERK activation were involved in the underlying mechanisms for heart protection mediated by apelin infusion. Whereas delivery of PI3K/Akt, AMPK, and ERK inhibitors before ischemia did not cause any significant changes in hemodynamic parameters, L-NAME, a nonspecific eNOS inhibitor, resulted in significant increases in MAP at the end of ischemia compared with either the I/R group or the I/R + apelin group of rats (Table 3). Expression levels of ER stress-specific markers were measured using tissue obtained at 24 h of reperfusion (Fig. 8, A–C). Inhibition of PI3K/Akt, AMPK, and ERK activation significantly attenuated the decreases in CHOP, JNK, and caspase-12 expression induced by apelin infusion. Although the trend was for AMPK inhibition to have less effect on protein expression, the difference did not reach statistical significance. Surprisingly, eNOS inhibition had no significant effects on the apelin-induced protection against ER stress-related apoptosis. Again, the quantification of TUNEL-positive cells further supported the notion that eNOS was not involved in the protection against ER stress-induced apoptosis, whereas PI3K/Akt, AMPK, and ERK inhibition partially restored ER stress-induced apoptosis levels to those of untreated controls (Fig. 8D).

Interestingly, at 2 h of reperfusion, inhibition of each signaling pathway except for AMPK completely abolished the protection against I/R injury by apelin infusion. In contrast, AMPK inhibition only resulted in a slight increase in IS at 2 h of reperfusion. Importantly, when IS was compared between 2 and 24 h of reperfusion after PI3K/Akt, ERK, and AMPK, and eNOS had been inhibited, PI3K/Akt, ERK, and AMPK inhibition re-

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Fig. 5. Attenuation of ER stress by apelin was assessed by the quantification of GRP78 (A), CHOP (B), phosphorylated and total JNK (C), and cleaved bands of caspase-12 (D) at 2 h (n = 5 for the I/R and I/R + apelin groups), 12 h (n = 4 for the I/R and I/R + apelin groups), and 24 h (n = 5 for the I/R group and n = 4 for the I/R + apelin group) of reperfusion compared with sham controls (the same rats from protocol I). JNK activation was also measured at 30 min of reperfusion (n = 5). All data in bar graphs are means ± SD. *P < 0.05 vs. the I/R group at the same time point; †P < 0.05 vs. the I/R + apelin group at 2 h of reperfusion (two-way ANOVA followed by a least-significant-difference corrected multiple-comparison test was used to compare differences between groups at the same time points or between different time points).
Fig. 6. A: the time-related increase in GRP78 (stained in green) from 2 to 24 h of reperfusion (n = 4 for the I/R and I/R + apelin groups at each time point) can be suppressed by apelin infusion. Double staining was performed for α-actinin (stained in red), whereas DAPI was used to label nucleic acid. B: immunofluorescent double staining showing α-actinin (in red) and CHOP (in green), which was also suppressed by apelin infusion (numbers of rats are the same as for GRP78 staining). The data for I/R rats without apelin therapy for either at 2 or 24 h after reperfusion were obtained from the same I/R rats as in Fig. 2.
sulted in increased IS, whereas the IS between 2 and 24 h of reperfusion was unaffected by eNOS inhibition, suggesting that eNOS was only involved in early protection (Fig. 9).

Finally, the potential interaction between signaling pathways was also tested by measuring the phosphorylation level of each kinase at 30 min of reperfusion. Apelin induced a significant increase in the phospho-Akt level at 30 min of reperfusion, which was completely abolished by the PI3K/Akt inhibitor LY-294002. The phospho-Akt levels induced by apelin were, however, only partially attenuated by the specific AMPK inhibitor compound C but not affected by eNOS or ERK inhibition. Compound C completely blocked the upregulated phospho-AMPK levels, which were also partially attenuated by the PI3K inhibitor but not affected by ERK inhibition. In contrast, ERK activation was not affected by PI3K/Akt, AMPK, or eNOS inhibition, suggesting that ERK does not interact with other apelin-induced protective signaling pathways (Fig. 10).

DISCUSSION

The importance of ER stress responses in a variety of cardiovascular diseases is now well established, but the understanding of its mechanisms and signaling in response to I/R injury is incomplete. This study described several important new observations that both help to clarify the mechanisms of the cellular response to I/R-induced ER stress as well as describing potential therapeutic targets to modify them. First, we demonstrated that dynamic changes in responses occur beyond the commonly investigated early responses and continue to evolve during the first 24 h after I/R injury. This novel observation was not only manifested at the protein level but also was associated with increased IS at 24 h compared with 2 h after reperfusion. We went further to show that apelin infusion starting 15 min before reperfusion completely abolished ER stress-dependent apoptosis activation at 2 h of reperfusion and significantly attenuated its activation at 24 h of reperfusion. Finally, we demonstrated that apelin exerts its protective effects via PI3K/Akt, ERK, MAPK, and eNOS signaling pathways, with each signaling pathway showing their protective roles against I/R injury, especially in the modulation of ER stress-induced apoptotic activation during the first 24 h of reperfusion.

The Time-Related Increase in IS Is Associated With Temporally Related ER Stress-Induced Apoptosis

While it is well known that I/R injury is a dynamic pathological process, very few studies (33, 49, 50) have addressed time-dependent changes in IS. In the present study, we showed that IS continues to increase between 2 and 12 h of reperfusion.
Interestingly, no further increase in IS was present at 24 h of reperfusion. This is important when assessing the results of other studies where the examination of events may vary in relation to the timing of reperfusion but also suggests that a plateau is reached, at least in the immediate responses within a few hours. That is not to say that the extent of infarction is complete at this time, as it is well known that local inflammatory responses can drive extension and remodeling for days and even weeks after I/R injury (16), which, in turn, are reflected by changes in IS. Moreover, we observed similar increases in the percentage of TUNEL-positive cells and caspase-3 activity. Notably, these dynamic changes were associated with ER stress-dependent apoptosis activation in the same time-dependent fashion, including CHOP, caspase-12, and JNK signaling pathways.

Modification of ER stress has become a therapeutic target for treatment of various cardiovascular diseases, including atherosclerosis (40), and has been the subject of several important reviews (14, 23, 31, 32). In the setting of I/R injury, attenuation of ER stress has been shown to be cardioprotective (28, 32). However, as oxidative stress can induce both ER stress (29) and I/R injury (2, 4), it is difficult to separate these two pathological processes to elucidate whether cardioprotection by reducing ER stress is a primary or secondary phenomenon. In the present study, we showed dynamic ER stress-induced apoptosis signaling activation with CHOP, JNK, and caspase-12 activation as early as 2 h of reperfusion, which continued to increase during the first 12–24 h after injury. This is strikingly different to the data observed in a previous transaortic banding animal model.
where only CHOP signaling was activated. This indicates that the pattern of ER stress-induced apoptosis signaling activation is related to the phase of the pathological process and its related model. Interestingly, the protection offered by apelin exhibited similar time-dependent effects. Whereas CHOP, JNK, and caspase-12 activation were significantly attenuated by apelin infusion, the beneficial effect of apelin was much less at 24 h of reperfusion compared with at 2 h of reperfusion, in terms of the percent decrease in levels of CHOP, JNK, and cleaved caspase-12. This could be that the apoptosis related to ER stress has not been fully activated at 2 h of reperfusion or that apelin infusion might not be able to fully suppress ER stress at this late phase of reperfusion. Coincidently, we also observed that the increased GRP78 level during the I/R process was downregulated by apelin infusion, which was consistent with a previous study (28) where attenuated ER stress was associated with lower chaperone expression. These data suggest that reduced chaperone expression is probably secondary to the improved ER stress offered by apelin. Thus, apelin does not improve ER stress via the induction of ER resident chaperones.

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Of note, in the present study, we were not able to determine what percentage of time-related ER stress activation contributed to the increase in apoptotic cells, which reflects one point of pathological activity during the dynamic necrosis process, nor were we able to show what percentage of apoptotic cells were directly caused by ER stress activation. However, we demonstrated that this dynamic activation of ER stress-induced apoptosis signaling (i.e., JNK, caspase-12, or CHOP activation) was associated with a higher percentage of TUNEL-positive cells and caspase-3 activity, which were related to an increase in IS from 2 to 12 h of reperfusion. Importantly, attenuation of ER stress activation was associated with a decrease in IS, i.e., reduced I/R injury.

Fig. 8. Using pharmacological inhibitors, the signaling pathways involved in the attenuation of ER stress by apelin was explored. CHOP (A), phosphorylated and total JNK (B), and cleaved bands of caspase-12 (C) as well as the percentage of TUNEL-positive myocytes (D) were assessed at 24 h of reperfusion when LY-294002 (n = 5 with apelin and n = 4 without apelin), compound C (n = 4 with apelin and n = 5 without apelin), N\textsuperscript{\textendash}nitro-L-arginine methyl ester (L-NAME; n = 4 with or without apelin), and PD-98059 (n = 4 with or without apelin) were used to inhibit phosphatidylinositol 3-kinase (PI3K)/Akt, AMP-activated protein kinase (AMPK), endothelial nitric oxide synthase (eNOS), and ERK activity, respectively. Data for sham, I/R, and I/R + apelin groups are the same as in Fig. 5. All data in bar graphs are means ± SD. *P < 0.05 vs. the I/R group without apelin therapy; †, P < 0.05 vs. the I/R + apelin group (one-way ANOVA followed by a least-significant-difference corrected multiple-comparison test was used for the comparison between different groups).
Apelin Attenuates ER Stress via Different Signaling Pathways in a Time-Dependent Fashion

Previous studies have demonstrated the cardioprotective effects of apelin (25, 37, 48) using a Langendorff-perfused heart model (25, 37, 48), an in vivo I/R injury model (37), and even isolated myocytes (37, 48). While activation of both PI3K/Akt and ERK signaling pathways [the key components of reperfusion injury salvage kinase pathways (15)] has been demonstrated to play a role in cardiac protection by apelin (37, 48), Kleinz et al. (25), however, showed that the protection by apelin was independent of PI3K/Akt and P70S6 kinase. In our study, we showed that inhibition of these two signaling pathways completely abolished the protection by apelin infusion at 2 h of reperfusion but only resulted in the partial reversal of suppression of ER stress and a reduction in IS at 24 h of reperfusion. Thus, our data further support the notion that both PI3K/Akt and ERK activation are involved in the protection against I/R injury by apelin and that PI3K/Akt and ERK activation can attenuate ER stress, as shown in previous studies (17, 18), but in a time-dependent fashion. The decreased role of PI3K/Akt in cardiac protection by apelin infusion shown in one previous study (25) could be related to the different time phase or experimental setting.

Although apelin also can activate AMPK to improve glucose metabolism (10), which, in turn, can improve ER stress (41), the role of AMPK activation in the cardiac protection offered...
by apelin has been questioned by Simpkin et al. Our data showed that AMPK activation modulated by apelin also exhibits time dependency. Inhibition of AMPK only partially blocked the beneficial effects by apelin infusion at 2 h of reperfusion, showing less effect compared with that of PI3K/Akt or ERK inhibition. However, this difference was lost at 24 h of reperfusion, with the effect of AMPK inhibition being similar to that seen with inhibitors of PI3K/Akt and ERK signaling pathways on mediating the protection by apelin therapy.

Cardioprotection by apelin was also abrogated by eNOS inhibition at 2 h of reperfusion, to a similar degree as that seen with the PI3K/Akt and ERK inhibitors. Surprisingly, however, inhibition of eNOS was associated with only a partial reduction in IS at 24 h, suggesting a much decreased role for eNOS signaling in the later modulation of ER stress-induced apoptosis activation. While the beneficial role of eNOS against I/R injury has been well demonstrated (11, 20, 36), its role in ER stress has been controversial (13, 24, 47). Our study suggests that the role of eNOS in I/R injury is time dependent, and, at 2 h of reperfusion, both NO-dependent and NO-independent signaling pathways (via PI3K/Akt and ERK pathways, respectively) were involved in the cardiac protection conferred by apelin (35). Unfortunately, in the present study, we are not able to explain why at this late phase (i.e., 24 h) of reperfusion inhibition of either PI3K/Akt or ERK can abolish the protective effects of apelin without having eNOS activation being involved, indicating that a different signaling pathway is involved at the late phase of reperfusion. However, the significant decrease in MAP has to be taken into account when we use L-NAME to assess the role for eNOS in the cardioprotection induced by apelin, in addition to its effect on the downward signaling cascade of eNOS.

**Interrelationship Between the Different Signaling Pathways Involved in Cardiac Protection by Apelin**

Using pharmacological blockers for each signaling pathway, we also observed an interrelationship between different signals involved in the cardiac protection offered by apelin. Our data clearly suggest that PI3K/Akt and AMPK signals are mutually dependent for activation, as the phosphorylation level of each kinase was partially blocked by inhibition of the other. In addition, inhibition of either PI3K/Akt or AMPK also partially attenuated eNOS activation, indicating that both signals function upstream of eNOS. These findings are consistent with the quantification of both ER stress signaling activation and IS with different pharmacological inhibitors. Moreover, ERK activation appears to be independent of PI3K/Akt, AMPK, or eNOS signals. This is also in line with the fact that the modulation of ER stress signaling by ERK induced by apelin was not affected by inhibition of these signals. Again, the same was true with IS quantification.
Limitations of the Study

Due to the experimental design, we cannot control or measure coronary perfusion pressure, which is an important factor for determining the fate of cell survival at the early phase of reperfusion. Therefore, the inhibitory effect of t-NAME on the eNOS signaling cascade could be also affected by a significant change in MAP. In addition, we only used pharmacological antagonists to test whether PI3K/Akt, AMPK, eNOS, and ERK are involved in mediating the protection against I/R injury by apelin infusion. However, while the relative specificity of these agents has been confirmed in other experiments, we cannot exclude nonspecific effects, given the nature of our in vivo experiments. Activation of each signaling pathway was only assessed by their phosphorylation levels, which might not reflect their actual kinase activity (38); however, this was only observed for the endogenous kinase phosphorylation induced by ischemic insult. Importantly, in the present study, the reversal of each kinase phosphorylation level by their specific pharmacological blocker was associated with loss of protection; thus, we reason that the phosphorylation level induced by apelin infusion reflects their actual kinase activity. In addition, the underlying mechanisms by which these signaling pathways attenuated ER stress-dependent apoptosis activation are still not fully understood, especially to what extent ER stress contributes to further increase in IS. Future study is certainly warranted using transgenic mice models where ER stress-related apoptosis signaling pathways (i.e., JNK, caspase-12, or CHOP) can be manipulated to measure to what extent ER stress activation leads to an increase in apoptosis activation or using transgenic animal models where protective kinases (i.e., Akt, AMPK, or ERK) can be conditionally induced or downregulated to elucidate the molecular mechanisms involved in protection by apelin therapy. Finally, we have to point out that 2,3,5-triphenyltetrazolium chloride staining might have underestimated the actual IS at the early phase of reperfusion due to a slow or delayed washout of dehydrogenase, which certainly should be taken into account, even though a higher IS value can be observed at this early stage when ischemia is further prolonged.

In summary, we demonstrated a time-dependent response to I/R injury that was associated with the dynamic activation of ER stress-induced apoptosis and could be attenuated by apelin infusion.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


ROLE OF ER STRESS IN DYNAMIC ISCHEMIA-REPERFUSION INJURY


