Apelin protects sarcoplasmic reticulum function and cardiac performance in ischaemia-reperfusion by attenuating oxidation of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and ryanodine receptor

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Aims
Apelin, an endogenous cytokine, has a number of biological effects on the cardiovascular system, including a cardioprotective effect and calcium modulation. Because the intracellular calcium abnormality is considered to play an important role in cardiac dysfunction induced by ischaemia–reperfusion (I/R), the aim of this study was to examine the effects of apelin-13 on I/R-induced changes in cardiac performance and sarcoplasmic reticulum (SR) function.

Methods and results
Isolated rat hearts were subjected to global ischaemia followed by reperfusion in the absence or presence of apelin-13 and inhibitors of some survival kinases. We found that depressed cardiac performance induced by I/R was attenuated by apelin-13. Furthermore, apelin-13 depressed oxidative stress during I/R. SR function depressed during I/R was partly reversed by apelin-13. SR oxidative modification levels were increased in I/R and reversed by apelin. Inhibitors of phosphatidylinositol-3-kinase and protein kinase C abolished the effects of apelin. Apelin-13 maintained the Ca\(^{2+}\) transient against I/R in cardiomyocytes.

Conclusion
Apelin protects SR function and cardiac performance during I/R by attenuating oxidation of sarcoplas(endo)plasmic reticulum Ca\(^{2+}\)-ATPase and RyR.

Keywords
Ischaemic contracture • PI3K • ROS • Ca\(^{2+}\) • PKC

1. Introduction
Apelin is a peptide ligand for a G-protein-coupled receptor (AP) receptor,\(^{1,2}\), which is widely expressed in brain, heart, stomach, lung, and in the vascular system.\(^{3}\) Apelin regulates cardiovascular function, producing vasodilatory,\(^{4}\) and positive inotropic effects.\(^{5}\) Increasing evidence also suggests that apelin protects the heart against ischaemia–reperfusion (I/R) induced infarction. However, the mechanism remains controversial.\(^{6–10}\)

Besides irreversible injury and cell death, myocardial contractile dysfunction resulting from I/R is a common clinical problem in patients presenting with certain types of heart diseases and in those already undergoing therapies. However, to date the effect of apelin on cardiac dysfunction induced by I/R has not been studied in depth. During I/R, cardiac contractile dysfunction is attributed to impaired calcium handling of cardiomyocytes.\(^{11–13}\) Ca\(^{2+}\) enters cardiomyocytes through the L-type Ca\(^{2+}\) channels, triggering further release of Ca\(^{2+}\) via the ryanodine receptor (RyR) from the sarcoplasmic reticulum (SR). This leads to a large increase in cytosolic-free calcium concentration, known as intracellular \([\text{Ca}^{2+}]\) transient (\([\text{Ca}^{2+}]\)).\(^{14}\) The elevated intracellular calcium concentration, which stimulates contraction of the myofilaments, is removed mainly to the SR by the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and out of the myocytes by the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) to initiate relaxation. On the one hand, these abnormalities in Ca\(^{2+}\) handling have been suggested to explain cardiac contractile dysfunction induced by I/R.\(^{15}\)

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2. Methods

All the animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and were approved by the animal care committee of the Fourth Military Medical University.

2.1 Heart perfusion

Male Sprague–Dawley rats weighing 250 to 350 g were anaesthetized with an i.p. injection of 250 mg/kg sodium pentobarbital containing 2.5 U/g heparin. Adequate anaesthesia was ensured by monitoring the absence of a withdrawal response to a paw pinch. The hearts were rapidly excised, cannulated to the Langendorff’s apparatus and perfused under a constant pressure of 80 mmHg with Krebs – Henseleit (K–H) medium containing (mmol/L) 120 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.25 CaCl2, and 11 glucose (37°C, pH 7.4). The K–H medium was gassed with a mixture of 95% O2 and 5% CO2. The hearts were stimulated at a rate of 300 b. During ischaemia, stimulation was stopped and resumed after reperfusion. A water-filled latex balloon was inserted into the left ventricle and connected to a pressure transducer to measure left ventricular systolic and diastolic pressures. Left ventricular end-diastolic pressure (LVEDP) was continuously recorded and maximum derivatives of the ventricular pressure (∆p/∂tmax) were continuously calculated with the Acknowledge software (Biopac System, Inc., USA). All the hearts were stabilized for a period of 30 min before ischaemia.

2.2 Experimental protocol

Control hearts were perfused with the oxygenated K–H medium for 60 min. Mechanical parameters were not changed during the experiments (data not shown). The hearts were made globally ischaemic by stopping coronary flow for 30 min followed by reperfusion with normal medium in the I/R group (Group 1) for 30 min. Hearts were treated with different concentrations of apelin-13 (1 nmol/L–1 μmol/L) for 10 min before the 30 min of ischaemia and continuously during the 30 min reperfusion in the IR + Ap group (Group 2). In Groups 3 and 4, the PKC inhibitor, chelerythrine (CHE 5 μmol/L; IR + Ap + CHE group), or the selective inhibitor of PI3K, LY-294002 (LY, 50 μmol/L; IR + Ap + LY group) was used to test the involvement of these pivotal kinases in cardioprotection effects of apelin-13. In Group 5, a specific PKCε inhibitor, εV1-2, was used to test the involvement of this kinase (εV1-2 1 μmol/L; IR + Ap + εV1-2 group).

Apelin-13 was used in a concentration of 100 nmol/L (except additional statement) on the basis of results from group 2 (Supplementary material online, Figure S1A). At a concentration of 100 nmol/L, additional apelin-13 did not change the mechanical function parameters significantly in control conditions before ischaemia in the groups above. However, to test whether a time-dependent effect of apelin occurred during the subsequent 60 min, we added a supplemental group (n = 4). In this group, hearts were perfused with the presence of 100 nmol/L apelin-13 in the oxygenated K–H medium for 70 min. Mechanical parameters remained stable during the period and did not differ significantly from those of the control group.

2.3 Determination of myocardial infarct size

Infarct size was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining as described previously. See the details in the Supplementary material online.

2.4 Determination of oxidative stress, redox state, and nitrotyrosine content in isolated hearts

See Supplementary material online.

2.5 Isolation of SR vesicles

SR vesicles were prepared by a method described previously with slight modifications. See details, see Supplementary material online.

2.6 Measurement of calcium uptake by SERCA

Calcium uptake of the SR vesicles was determined by the procedure described elsewhere with slight modifications. For details, see Supplementary material online.

2.7 3H-Ryanodine binding assay

High-affinity ryanodine binding was determined by a procedure described earlier. For details, see Supplementary material online.
2.8 Isolation of adult rat ventricular myocytes and treatment with simulated I/R

Single-rat ventricular myocytes were isolated by methods described previously. Control recordings were performed in normal Tyrode’s solution for 15 min. Cells were then exposed to ischemic Tyrode’s solution for 20 min followed by 30 min reperfusion. For details, see Supplementary material online.

2.9 Measurement of [Ca^{2+}]i transients and cell shortening in the single cardiomyocyte

Mechanical properties and intracellular Ca^{2+} transients of ventricular myocytes were assessed as described previously. For details, see Supplementary material online.

2.10 Measurement of ROS generation in cardiomyocytes

ROS production, which serves as an index for oxidative stress in viable cardiomyocytes, was measured as described previously. For details, see Supplementary material online.

2.11 Immunoprecipitation with anti-SERCA or anti-RyR antibody

Immunoprecipitation was performed as described earlier. For details, see Supplementary material online.

2.12 Statistical analysis

Results are presented as the means ± SEM. For details, see Supplementary material online. P < 0.05 was considered significant.

3. Results

3.1 Cardioprotection effects of apelin-13

Treated with 1 nmol/L to 1 μmol/L of apelin-13, a significant reduction in LDH release in the 100 nmol/L and 1 μmol/L groups (Supplementary material online, Figure S1A) was observed vs. that in the I/R group. We used a concentration of 100 nmol/L apelin-13 for the following assessments. The co-infusion of antagonists of upstream signals, such as LY or CHE (inhibitors of PI3K and PKC, respectively), along with 100 nmol/L apelin-13 completely abolished the cardioprotective effects of apelin-13 on LDH release (Supplementary material online, Figure S1B). However, the co-infusion of apelin-13 with antagonists of downstream signals, such as vV1-2 or 5HD (inhibitors of PKCε or mito K_{ATP} channels, respectively), partially reduced the protective effect of apelin-13. Using TTC staining method, we did not observe a significant infarct area in our protocol in all the groups.

3.2 Apelin improved cardiac function of the I/R hearts

In the present study, the baseline mechanical parameters with 100 nmol/L apelin were not significantly different vs. those of the control condition (Figure 1A–C). In the additional group, we found 100 nmol/L apelin-13 did not change the mechanical parameters significantly within 70 min compared with those of the control group (data not shown). Mechanical dysfunction of the heart induced by I/R was reflected by depressed LVPSP, LVDP and elevated LVEDP, which was reversed by 100 nmol/L of apelin-13 (Figure 1A–C). The hearts of the I/R group showed a marked limitation of LVPD recovery during reperfusion. As can be seen in Figure 1C, apelin-13 markedly improved LVDP recovery during reperfusion. More specifically, at the end of reperfusion LVDP was 97 ± 11% of baseline levels. During I/R, inhibition of PI3K, PKC, PKCε or mito K_{ATP} channels with LY, CHE, vV1-2, or 5HD, respectively, abrogated the beneficial effect of apelin-13 on cardiac function significantly (Figure 1A–C). Furthermore, the effect of apelin on cardiac + dp/dt_{max} was similar, and apelin-13 did not change the coronary flow of the isolated hearts during I/R (Supplementary material online, Figure S2).

3.3 Apelin attenuated ischaemic and reperfused contracture

In our study, the beginning of ischaemic contracture was defined as an increase in LVEDP of 4 mmHg above the baseline level. Ischaemia markedly increased LVEDP (from 5 ± 0.3 mmHg in the baseline to the peak amplitude 24 ± 8 mmHg at the end of ischaemia, P < 0.05, Figure 1B and D). Apelin-13 partly reversed the peak LVEDP to 10 ± 4 mmHg at the end of ischaemia. The time from ischaemia to the onset of contracture was delayed by apelin-13 from 3.4 ± 1.3 to 9.5 ± 0.4 min compared with the same time in the I/R group (P < 0.05, Figure 1B and E). The peak and final LVEDP during reperfusion were attenuated by apelin-13 from 123 ± 15 and 21 ± 2 mmHg to 62 ± 14 and 5 ± 2 mmHg, respectively (P < 0.05, Figure 1B and F). However, inhibition of the kinases or mito K_{ATP} channels abolished the beneficial effect of apelin-13 on cardiac contracture in our study (Figure 1).

3.4 Apelin reduced myocardial oxidative stress caused by I/R

Malondialdehyde (MDA, a type of production from lipid peroxidation) levels in the rat myocardium were significantly lower in the apelin-treated group vs. those of the I/R group (3.9 ± 0.4 vs. 8.1 ± 0.4 mmol/g protein, P < 0.05, Figure 2A). Furthermore, oxidative stress determined by the superoxide level was significantly elevated in I/R hearts, and such increase was attenuated by apelin-13 (Figure 2B). Ten mmol/L Tiron was used as a positive control. The nitrotyrosine level was much higher in the I/R hearts than in the control group, indicating that I/R led to large increases in peroxynitrite. Apelin-13 effectively reduced the level of nitrotyrosine (Figure 2C). The level of GSH, the major cellular antioxidant, was reduced in I/R hearts and normalized in the presence of apelin-13 (Figure 2D). The cytosolic lactate/pyruvate (L/P) ratio was used as surrogate assay for NADH/NAD^{+} ratio, a redox indicator. We found that the L/P ratio was increased in I/R hearts (Figure 2E) and attenuated by treatment with apelin-13. The inhibitors or blockers of PI3K, PKC, PKCε, or mito K_{ATP} channels, respectively, abolished the effect of apelin-13 on redox state in isolated hearts during I/R (Figure 2).

3.5 Apelin-13 restored the activities of SERCA and RyR during I/R

Both the rate and capacity of SR Ca^{2+} uptake were significantly depressed in hearts from I/R. This change was attenuated by perfusion of the hearts with apelin-13 (Figure 3A).

The results showed a depression in the maximal binding of (3H)-ryanodine with SR preparations from the ischaemic-reperfused hearts (Figure 3B and C). Perfusion of the hearts with apelin-13 was observed to attenuate the depression of the ability of (3H)-ryanodine binding to SR (Figure 3B and C). The level of SERCA and RyR protein was not altered by I/R and apelin-13 had no effect on its protein level (data not shown). The effects of apelin-13 on SERCA Ca^{2+} uptake and RyR, 3H-ryanodine binding were abolished by the pathway inhibitors, as shown in Figure 3.
Figure 1  Effect of apelin-13 on cardiac performance using Langendorff’s preparation. Ischaemia was induced by occluding coronary flow for 30 min, and hearts were then reperfused for 30 min. (A) Left ventricular systolic pressure (LVSP), (B) left ventricular end-diastolic pressure (LVEDP), (C) left ventricular developed pressure (LVDP), (D) Peak amplitude of ischaemic contracture, (E) Time to onset of 4 mmHg elevation of LVEDP in ischaemia, (F) Peak amplitude and final LVEDP in reperfusion in different groups. n > 13 in each group. *P < 0.05 vs. I/R.
3.6 Apelin attenuated the tyrosine nitration of SERCA and maintained the S-glutathiolation of SERCA and S-glutathiolation of RyR in I/R rat hearts

The protein was immunoprecipitated from heart extracts using anti-SERCA antibody and analysed by western blot using anti-nitrotyrosine antibody (Figure 4A). The level of nitrotyrosine on SERCA was significantly increased in I/R hearts and apelin-13 attenuated tyrosine nitration of SERCA.

Besides tyrosine nitration of SERCA, S-glutathiolation of SERCA also affected its activity. As shown in Figure 4B, the amount of GSH on the immunoprecipitated SERCA was significantly decreased in the I/R heart and apelin-13 restored the level of S-glutathiolation. We investigated whether apelin-13 influenced the level of S-glutathiolation of RyR in I/R hearts. To assess the effect of apelin-13 on the level of S-glutathiolation of RyR in I/R hearts, RyR was immunoprecipitated and analysed by western blotting with anti-GSH antibody (Figure 4C). The level of RyR S-glutathiolation was significantly decreased in I/R hearts, and apelin-13 restored the level of S-glutathiolation. Inhibition of the pathway abolished the effects of apelin-13 (Figure 4), suggesting that PI3K, PKC, PKCe, and mito KATP channels were involved in the mechanism.

3.7 Effect of apelin-13 on ROS generation during I/R in cardiomyocytes

To assess ROS generation during simulated I/R, we examined the fluorescence of DCFH-loaded cells. The ROS level was found to be increased after reperfusion and apelin-13 reduced this increase (Figure 2F).
3.8 Apelin-13 improved the impairment of Ca\(^{2+}\) homoeostasis and cell shortening induced by I/R

Contraction of the cells was induced by electrical pacer at 0.5 Hz. The representative tracings of electrically stimulated [Ca\(^{2+}\)]\(_i\) transients are shown in Figure 5A. The amplitude of the electrically induced Ca\(^{2+}\) transient was greatly reduced after I/R, which was restored by the continuous presence of apelin-13 during simulated I/R (Figure 5B). The basal Ca\(^{2+}\) level, representing the diastolic cytosolic Ca\(^{2+}\) content, was increased from 102.5 ± 15 to 137.5 ± 10 nmol/L after reperfusion. Apelin-13 maintained the basal Ca\(^{2+}\) level close to the pre-ischaemic level (P < 0.05, Figure 5C).

The time to peak of [Ca\(^{2+}\)]\(_i\) represents the rate of Ca\(^{2+}\) release from the SR, mainly via RyR. After I/R, the time to peak of [Ca\(^{2+}\)]\(_i\) was increased to 208% (P < 0.05), suggesting that I/R reduced RyR activity. Apelin reversed the time to peak of [Ca\(^{2+}\)]\(_i\) to 112% of control (Figure 5D). The decay of [Ca\(^{2+}\)]\(_i\) was mainly by Ca\(^{2+}\) uptake via SERCA, which was responsible for the removal of ~90% of Ca\(^{2+}\) from the cytoplasm. After I/R, the time to reduce 50% of the peak [Ca\(^{2+}\)]\(_i\) (T\(_{50}\)) was increased to 116% of control cells. Apelin-13 reduced T\(_{50}\) of the I/R cells to 103% of the control cells (Figure 5E).

Specimen records of contraction in control and following I/R are displayed in Figure 5F. A decrease in the degree of shortening during systole along with a decrease in diastolic myocyte length was apparent. On average, shortening during systole (Figure 5G) was decreased by 42% (control, 4.3 ± 0.3; I/R, 2.5 ± 0.3%, P < 0.05) after I/R. Apelin-13 increased shortening to 97 ± 1.7% of control cells.

We added a supplemental group of myocytes subjected to the same simulated I/R condition but added apelin-13 after the end of reperfusion.
We found apelin-13 could not improve the calcium transient and cell shortening within 30 min vs. The I/R group (n = 5). This result indicated that the beneficial effect of apelin on calcium transient and cell shortening in I/R cells was not simply due to its positive inotropic action per se.

### 3.9 The effects of apelin on the $[\text{Ca}^{2+}]_{i}$ removal

The rate of the decay of $\text{Ca}^{2+}$ transients ($K_{\text{sys}}$) depends on both SERCA and sarcolemmal $\text{Ca}^{2+}$ transporters (NCX and plasmalemmal $\text{Ca}^{2+}$ ATPase), whereas the rate of the decay of the $\text{Ca}^{2+}$ release by 10 mmol/L caffeine ($K_{\text{caf}}$) depends only on sarcolemmal transporters. Therefore, $K_{\text{SERCA}} = K_{\text{sys}} - K_{\text{caf}}$ can be used to calculate the SERCA activity in the same cell. $^{36}$ Figure 6A shows normalized systolic $[\text{Ca}^{2+}]_{i}$ transients. A slowing of the descending phase was clearly apparent, indicative of a reduction in the rate of systolic $[\text{Ca}^{2+}]_{i}$ removal. On average, the rate constant of systolic calcium removal ($K_{\text{sys}}$) (Figure 6B) was reduced by 31% (control, 4.5 $\pm$ 0.3 s$^{-1}$; I/R, 3.2 $\pm$ 0.3 s$^{-1}$, $P < 0.05$). Apelin-13 (100 nmol/L) improved $K_{\text{sys}}$ to 97.7% of control. The rate constant of the decay of caffeine-evoked $[\text{Ca}^{2+}]_{i}$ transient ($K_{\text{caf}}$) (Figure 6C and D) was reduced by 13.1% (control, 1.33 $\pm$ 0.01 s$^{-1}$; I/R, 1.18 $\pm$ 0.04 s$^{-1}$, $P < 0.05$), indicating 13.1% decrease in NCX activity in I/R. Apelin-13 increased $K_{\text{caf}}$ to 101.5% of control. The activity of SERCA can be calculated as $K_{\text{SERCA}} = K_{\text{sys}} - K_{\text{caf}}$ and was reduced by 37% in the I/R group (control, 3.17 $\pm$ 0.3 s$^{-1}$; I/R, 2.02 $\pm$ 0.3 s$^{-1}$, $P < 0.05$) (Figure 6E). However, apelin-13 improved $K_{\text{SERCA}}$ to 96.4% of control cells.

### 4. Discussion

In the present study, we have shown that cardiac dysfunction induced by I/R was attenuated by apelin-13. Furthermore, apelin-13 depressed oxidative stress during I/R. On the other hand, cardiac SR function depressed during I/R was partially reversed by apelin-13. To better understand the mechanism, the oxidative modification levels of SERCA and RyR were analysed. We found that SERCA and RyR oxidative modification levels were increased in I/R and apelin-13 improved the redox states of these SR $\text{Ca}^{2+}$ modulators. Using classic antagonists, we demonstrated that the beneficial effects of apelin depend on the activation of PI3k, PKCs and mito KATP channels. Furthermore, rat cardiomyocytes subjected to simulated I/R showed significant alterations in a $\text{Ca}^{2+}$ signal profile and shortening function. These changes are most likely consequences of the dysfunction of RyR and SERCA and were reversed by apelin, indicating that apelin-13 protected SR function during I/R.

### 4.1 Infarct size and LDH release

Part of the beneficial effect of apelin on cardiac function may be due to its protection against necrotic reperfusion injury as indicated by the release of LDH. However, we observed no infarction with TTC staining. There are two possible explanations for this paradox: (i) the quantity and severity of sarcolemmal integrity damage are not extensive enough to be detected after 30 min reperfusion and the quantity or severity of injury would increase to an extent which could be defined as infarct size in isolated hearts with a longer reperfusion time; (ii) the TTC staining technique per se requires a longer time of reperfusion. This technique relies on the ability of dehydrogenase enzymes and cofactors in the tissue to react with tetrazolium salts to form a formazan pigment. Tissue that stains positively is not necessarily healthy and may succumb hours or even days later. For that reason, the longer the reperfusion period after an ischaemic insult, the more reliable the method becomes for
discriminating between dead and viable tissue. Although it is difficult to distinguish non-specific damage from functional disability in the early phase of injury, the beneficial effects of apelin on cardiac performance and SR function during ischaemia reperfusion are unlikely to be completely due to its anti-necrosis effect.

4.2 Mechanical function

The abnormalities of cardiac performance induced by I/R were significantly attenuated by apelin-13. In view of the important role of RyR calcium-release and SERCA calcium uptake activities in the process of cardiac contraction and cardiac relaxation, respectively, it is evident that the observed protection of SR by apelin may contribute to the beneficial effect. In our present study, 100 nmol/L apelin-13 did not produce a significant effect on mechanical parameters before ischaemia. Consequently, we added a supplemental group to examine whether apelin produced a delayed positive inotropic effect during the next 60 min. We found that 100 nmol/L apelin-13 did not have a significant effect on LVSP and LVDP in this situation within 70 min. Hence, the beneficial effect of apelin on mechanical function is not due to the positive inotropic effect of apelin per se. Another additional group in

Figure 5 Effect of apelin-13 on Ca\textsuperscript{2+} transient [Ca\textsuperscript{2+}i] and cell contraction during I/R. (A) Representative tracings of electrically stimulated intracellular [Ca\textsuperscript{2+}i] transients. (B) Amplitude of transient. (C) Diastolic Ca\textsuperscript{2+} level. (D) Time to peak of Ca\textsuperscript{2+} transient. (E) Decay time of Ca\textsuperscript{2+} transient. (F) Representative tracings of electrically stimulated cell contraction. (G) Cell contractions normalized by its original length. n > 10, #P < 0.05 vs. control, *P < 0.05 vs. I/R.
cardiomyocytes showed that apelin-13 administered after the end of simulated reperfusion could not improve the intracellular calcium transient and cell shortening, indicating that apelin protected the cellular function against I/R injury rather than directly increasing the calcium transient. The ischaemic contracture might be caused by an abnormal increase in intracellular calcium accumulation, which activates the contractile process, or by depletion of intracellular ATP, which leads to rigour or a state of contracture.32 Although the exact mechanisms of cardiac ischaemic contracture attenuated by apelin are not known, several possibilities including modulation of intracellular calcium and preservation of the ATP content can be found.21,33 In the present study, we found apelin-13 could preserve SERCA activity, reversing the elevated diastolic cytosolic Ca2+ concentration after reperfusion, which may also occur during ischaemia and contribute to attenuate ischaemic contracture by alleviation of calcium overload. On the other hand, there is evidence suggesting that apelin could maintain high energy phosphates, particularly ATP, in reperfused myocardium.33 This could also explain the effect of apelin relieving cardiac ischaemic contracture. However, our study had some limitations. As we did not observe the cytosolic-free calcium concentration during ischaemia, the exact effect of apelin on calcium overload in the course of ischaemia is not clear. Furthermore, it is not known effect apelin has on cardiac substrate metabolism during ischaemia and reperfusion. How apelin maintains myocardial ATP content still needs to be investigated. Therefore, further studies are required to answer these questions and to provide further insight into the underlying biochemical processes of the mechanical events we have demonstrated in this study.

4.3. Redox state and SR

Oxygen free radicals are a key factor involved in I/R injury. RyR and SERCA activity were most likely impaired by increasing oxidative
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stress during I/R. Our study revealed that apelin decreased the MDA content, superoxide level, and nitrotyrosine content elevated in I/R hearts and increased GSH level reduced in I/R hearts. GSH is required for glutathione peroxidase to remove ROS. Thus, a low level of GSH would lead to an increased level of ROS. Furthermore, one of the major sources of ROS is superoxide produced by the membrane-associated NADH oxidase, an enzyme controlled by the cytosolic NADH/NAD⁺ ratio and oxygen pressure. During I/R, increased NADH stimulates NADH oxidase, which is in close proximity to SR, to generate ROS. Increased ROS inhibits SERCA by oxidizing cysteine thiols, interfering with the ATP-binding site, making it unable to hydrolyse the ATP. In the I/R hearts, the increased level of peroxynitrite led to increased tyrosine nitration of SERCA, and was accompanied by a decreased GSS-SERCA level. We showed that apelin-13 normalized the levels of nitrotyrosine in SERCA and GSS-SERCA.

Ryanodine at low concentrations activates calcium efflux from SR by increasing the probability of channel opening. The channel can be closed by micromolar concentrations of ryanodine. Saturation of the high-affinity binding site is associated with an increased efflux through the channel, and occupancy of a low-affinity site results in the closure of the channel. It has been shown that the gating property of cardiac RyR is modulated by the cytosolic NADH/NAD⁺ ratio. NADH inhibits the current through RyR channels by a mechanism that requires oxidation of NADH and is reversed by NAD⁺. We showed that the NADH/NAD⁺ ratio, as reflected by the L/P ratio, was increased in I/R hearts, contributing to the impairment of RyR. It has been shown that S-glutathiolation of RyR causes a significant stimulation of channel activity in skeletal RyR, and S-glutathiolation of cardiac RyR has been observed under normal and myocardial preconditioning. We found that S-glutathiolation of the cardiac RyR was reduced during I/R, and apelin-13 attenuated the decrease in S-glutathiolation of cardiac RyR. This provides another explanation for apelin protecting contractile function in I/R hearts.

4.4 Signal pathway

The molecular mechanism of cardioprotection against I/R is very complex. In our present study, antagonists of upstream elements (PI3K and PKC), as well as a blockade of PKCe and mitochondrial KATP channels abrogated apelin-13 protective effects. PI3K is the upstream kinase of the so-called ‘reperfusion injury survival kinase pathway’. Several studies have demonstrated that activation of G protein-coupled receptors may lead to PI3K/Akt activation and cardioprotective effects. Apelin, as an endogenous peptide ligand for a G protein-coupled receptor (AP) receptor, was reported to protect heart against I/R via activation of PI3K. We here demonstrated that apelin stimulates glucose uptake through the PI3K/Akt pathway and improves insulin resistance in 3T3-L1 adipocytes. This indicated that PI3K is one of the important intracellular signal molecules involved in the effect of apelin. In addition, there is evidence demonstrating that in hearts, PI3K is located upstream of the activation of PKC during the cardioprotective signal pathway. PKC plays a key role in the ischaemic preconditioning signalling cascade in cardiomyocytes. In the present study, inhibition of the PI3K or PKC signalling pathway by LY-294002 or CHE almost completely abolished the cardioprotective effect (both on LDH release and on cardiac contractile function) of apelin. The data suggest that apelin-induced protection against myocardial I/R injury might be mediated at least partially via a PI3K/PKC-dependent mechanism. In contrast to CHE, the PKCe selective inhibitor eV1-2 only partially abolished the effect of apelin, reducing LDH release after I/R injury but completely abrogated the contractile protection induced by apelin, indicating that besides PKCe, other isoforms of PKCs were also involved in the cardio-protective effect of apelin, particularly in the protection of sarcocellular integrity during I/R injury. PKCe may act on mito KATP channels to reduce both cell death and dysfunction. At the same time, mito KATP channels activation may facilitate PKCe reactivation via ROS signalling. This PKCe-dependent circuit of activation and re-activation plays a role in the so-called ‘memory-associated protection’. Our study revealed that apelin-dependent cardioprotection was mediated by PKCe and mito KATP channels. During reperfusion, a large burst of ROS has been shown to occur. It was proposed that ROS, particularly ROS generated during early reperfusion, would lead to extensive oxidative damage to the cell resulting in the loss of cell function and viability.

In the present study, we found apelin-13 could decrease the ROS content and modulate the SR redox state during I/R. The beneficial effect could be abolished by LY-294002, CHE, eV1-2, and SHD, respectively. Although the exact mechanism is not fully clear yet, the most plausible interpretation of our observations is that apelin reduced the oxidative modification of SR proteins by decreasing ROS overproduction during I/R, which is mediated by the PI3K-PKCs (PKCe)-mito KATP channels pathway.

In conclusion, apelin-13 attenuated the oxidative modification of SERCA and RyR during I/R and not only reduced infarct size as demonstrated earlier, but also improved cardiac performance during I/R, in which PI3K, PKC, and downstream targets, such as PKCe and mito KATP channels, were involved.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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