Cardioprotection induced by Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activation involves extracellular signal-regulated kinase 1/2 and phosphoinositide 3-kinase/Akt pathway

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Aims
Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (NKA) has recently been found to relay extracellular signals to intracellular compartments. Activation of NKA with polyclonal antibody produces positive inotropic effects. The present study was designed to examine whether DRRSAb, a NKA DR region-specific antibody, also produces cardioprotective effects.

Methods and results
Contractile function was examined in both isolated cardiomyocytes and hearts subjected to ischaemic injury. We found that DRRSAb (0.125–2.0 \textmu M) concentration-dependently stimulated the activity of NKA in rat or mouse kidney tissues. Moreover, DRRSAb increased the amplitudes of cell shortening and electrically induced [Ca\textsuperscript{2+}]i transients in rat or mouse cardiac myocytes. These effects were significantly attenuated by blockade of either extracellular signal regulated kinase 1/2 (ERK1/2) with PD98059 or Src with herbimycin A, suggesting a role of ERK1/2 and Src kinases in the positive inotropic effect of DRRSAb. More importantly, DRRSAb significantly increased cell survival rates for at least 24 h after isolating from the heart. Activation of NKA also protected hearts against ischaemic injury in both cardiomyocytes and isolated hearts. The protective effect was reversed by blockade of ERK1/2 or phosphoinositide 3-kinase (PI3K)/Akt but not by inhibition of protein kinase C. The involvement of ERK1/2 and PI3K/Akt was further confirmed by examining the phosphorylation of these kinases with western blot analysis.

Conclusion
Activation of NKA with DRRSAb induces both positive inotropic and cardioprotective effects via stimulation of Src/PI3K/Akt/ERK1/2 pathways. The unique properties of DRRSAb may make NKA antibody a promising drug to treat heart failure.

Keywords
Chronic heart failure • Na\textsuperscript{+}/K\textsuperscript{+}-ATPase • Antibody • Cardioprotection

1. Introduction

Chronic heart failure (CHF) is a common, costly, disabling, and deadly condition in which a problem with the structure or function of the heart impairs its ability to supply sufficient blood flow to meet the bodies needs. The serious hazard of CHF is its symptoms, which are associated with significantly reduced physical and mental health, resulting in a markedly decreased quality of life. For centuries, cardiac glycosides (e.g. ouabain, digitalis, and digoxin) were used to treat CHF. These drugs bind to the \alpha-subunit of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (NKA) and inhibit the pump activity to elevate intracellular Na\textsuperscript{+} concentration, which further inhibits the forward model of Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger, thus increasing intracellular Ca\textsuperscript{2+} and improving the cardiac function (positive inotropic effect) during cardiac failure. However, these drugs also produce severe toxic effects including cardiac arrhythmias and disturbances of atrioventricular conduction, gastrointestinal disorders, neurological effects, anorexia, blurred vision, nausea, and vomiting.

NKA is an energy-transducing ion pump located in the plasma membrane in most mammalian cells. Within the last decade,
several groups have identified that in addition to the classical ion transporting, NKA also relays an extracellular ouabain binding signal- ing into the intracellular compartments through regulation of differ- ent protein tyrosine phosphorylation.6 In recent years, an activation site that resides in the H7–H8 domain of α-subunit of NKA, which is different from the ouabain binding site, has been demonstrated7 SSA412, a site-specific antibody against the H7–H8 domain, signifi- cantly activates NKA when it binds to the DR region (897DVEDSYGQQWTYEQR911) of the H7–H8 domain. It has been found that activation of NKA with SSA412 augments mouse heart contraction through increasing calcium influx via activation of Src/extracellu- lar signal kinase 1/2 (ERK1/2) signalling cascade.8,9 ERK1/2, phosphoinositol 3-kinase (PI3K)/Akt and protein kinase C (PKC) are three well-known important pro-survival protein kinases in the heart. All of them have been reported to be activated upon treat- ment with ouabain.10,11 The present study was therefore designed to study the cardioprotective effect of the DR region-specific antibody (DRRSAb), which is against the same DR region as SSA412, and its underlying mechanism.

2. Methods

2.1 Animals

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Insti- tutes of Health [NIH Publication No. 85 (23), revised 1996]. The animal study protocols were approved by the Institutional Animal Care and Use Committees of National University of Singapore.

2.2 Chemicals and reagents

Polyclonal antibody DRRSAb against 897DVEDSYGQQWTYEQR911 region of α-subunit of NKA, was kindly provided by Drs. Casey Chan and James W. Larrick (Humanyx Pte. Ltd, Singapore and Panorama Research Institute, Sunnyvale, CA, USA). Phospho-ERK1/2, total-ERK1/2, phospho-Akt, and total-Akt antibodies were purchased from Cell signal- ing Research Institute, Sunnyvale, CA, USA). Goat anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 897DVEDSYGQQWTYEQR911 polyclonal antibody DRRSAb (0.25 m) or normal rabbit serum (NRS, control) and thereafter

2.3 Immunofluorescent staining

The tissue of rat heart was fixed with 4% paraformaldehyde for over 24 h, and then cryoprotected in a high concentration of sucrose followed by freezing and cutting on a cryostat. Sections (10 μm) were mounted onto poly-lysin coating slides. After blocking with 3% bovine serum albumin (BSA) for 30 min, slides were then incubated with DRRSAb (1:1000) for 60 min at room temperature. The primary antibodies were detected with goat anti-rabbit IgG (H + L) conjugated to Alexa Fluor 468.

2.4 Purification of NKA

NKA was purified from the outer medulla of rat and mouse kidney as described previously.12 Medulla were homogenized in homogenizing medium, then centrifuged at 6000 g for 15 min. The supernatant was resup 15 000 g for 30 min at 4 °C and the resultant supernatant was centrifuged at 148 000 g for 90 min. The pellet was suspended in the homogenizing medium and incubated with SDS 0.4 mg/mL for 30 min at room temperature in the presence of 3 mmol/L ATP, 2 mmol/L EDTA, and 50 mmol/L imidazole, pH 7.5. The resultant suspension was applied to dis- continuous sucrose gradients and centrifuged at 148 000 g for 120 min. The pellet appearing at the bottom was resuspended in the homogenizing medium to a protein concentration of 2 mg/mL, and stored in −80 °C.

2.5 Measurement of NKA activity

NKA activity was determined on the basis of Jack Kyte’s method with modifications as previously described.13 Purified rat NKA (10 μg/mL) and mouse NKA (1 μg/mL) were incubated with different concentrations of DRRSAb at 37 °C for 60 min. The reaction was initiated by adding Mg-ATP (3 mM) in a final volume of 0.2 mL at 37 °C for 30 min and ter- minated by adding 0.75 mL quench solution and 0.02 mL developer. The concentration of phosphate was then determined at 700 nm using a spectrophotometer.

2.6 Isolation of cardiac myocytes

Cardiac myocytes were isolated from adult Sprague–Dawley rats and C57BL/6J mice, using standard enzymatic methods.14 Briefly, a central thoracotomy was performed after the animals were anesthetized. The heart was quickly excised and perfused in a Langendorff system with calcium-free Tyrode’s. After perfusion for 5 min, the solution was changed to the Tyrode’s solution containing 1 mg/mL collagen (type I) and 0.28 mg/mL protease (type XIV) and perfused for a further 30 min. Left ventricular tissue was gently minced, filtered, and washed three times in Ca2+-free Tyrode’s solution. The cells were allowed to stabilize at room temperature for 30 min.

2.7 Measurement of cell contraction

Experiments were conducted at room temperature on a Nikon TS100 inverted microscope. Isolated rat myocytes were resuspended in Krebs’ bicarbonate buffer. Rod-shaped cardiac myocytes with clear striation were chosen. The motion of the selected myocyte was continuously recorded online through an × 20 objective lens (Nikon) and transmitted to a charge-coupled device (CCD) black and white (B/W) video camera (NL-2332; National Electronic, Canada). The output from the CCD camera was displayed on a video monitor (National Electronic). Cardiac myocyte edge was measured using a video motion edge detector (VED-105; Crescent Elec- tronics, Canada). Light–dark contrast of the edge of the myocyte provided a marker for measurement of the amplitude of motion. Contraction amplitude was indexed by the percentage shortening of cell length.

2.8 Measurement of intracellular Ca2+ transients

The ventricular myocytes were washed twice with Dulbecco’s modified Eagle’s medium (DMEM) after being isolated and stabilized for 30 min, then the cells were resuspended with DMEM supplemented with DRRSAb (0.25 μM) or normal rabbit serum (NRS, control) and thereafter incubated at humidified CO2 incubator at 37 °C for 30 min. Cells were then incubated with 4 μM Fura 2-AM for 30 min at room temperature. Ventricular myocytes loaded with fura-2/AM were transferred to the stage of an inverted microscope (Nikon) in a superfusion chamber at room temperature. The Fura-2/AM loaded rat cardiac myocytes were measured using the dual-wavelength excitation spectrofluorometer (PTI, USA). Fluorescent signals obtained at 340 and 380 nm excitation wave- length were used to represent [Ca2+]i changes in myocyte.

2.9 Measurement of cell viability

After the ventricular myocytes were isolated and stabilized for 30 min, cells were washed and resuspended in DMEM supplemented with 10% fetal bovine serum. Cell viability was measured after incubation of the cells with or without DRRSAb (0.25 μM) treatment for different periods (0, 3, 6, 12, or 24 h) at a humidified CO2 incubator at 37 °C.
Cardioprotection induced by Na⁺/K⁺-ATPase activation

Tryptan Blue exclusion was used as an index of myocyte viability. After the live cells were incubated with 0.4% (w/v) Tryptan Blue dye for 3 min, those that were unstained were termed to be non-viable cells. Non-viable cells/total cells were determined in a haemocytometer chamber using a light microscope (10 × magnification). A total of 200–300 cells in each of five cultures were tested for each group.

To detect the cardioprotection of DRRSAb against ischaemic reperfusion-induced cell injury and the underlying signalling mechanisms, ventricular myocytes were treated with PD98059 (30 μM, 30 min), herbimycin A (1 μM, 2 h), LY 294002 (15 μM, 15 min), chelerythrine (3 μM, 30 min), Go6976 (2 μM, 30 min), or EAVSKPK (200 μM, 30 min) before application of DRRSAb (0.25 μM 3 h). Cells were then subjected to ischaemia and reperfusion as described before. Briefly, to mimic ischaemia, a simulated ischaemia solution [i.e. glucosefree Krebs buffer containing 5 mM sodium lactate, 20 mM 2-DOG (an inhibitor of glycolysis), 16 and 20 mM sodium dithionite (Na2S2O4, an oxygen scavenger), 17 pH 6.6 was used]. The cells were subjected to simulated ischaemia solution for 10 min and then washed with normal culture solution for reperfusion (RE). After 10-min reperfusion, cell viability was examined.

2.10 Western blot analysis
Cardiomyocytes were washed with PBS twice and lysed with 150 μl ice-cold lysis buffer. The cell lysate was centrifuged at 10 000 g for 10 min at 4 °C. Proteins or purified NKA were loaded on an 8% SDS–polyacrylamide gel. After being transferred to a nitrocellulose membrane, proteins were blocked with 10% non-fat milk for 1 h and probed first with the primary antibody A277 (1:2000), DRRSAb (1:1000), phospho-ERK1/2, total-ERK1/2, phospho-Akt, or total-Akt antibodies at 4 °C overnight, and then washed three times with TBST buffer before incubating with a horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:10 000) 1 h at room temperature. Membranes were detected using an ECL advance western blot detection kit (Amersham Biosciences Biotech, Piscataway, NJ, USA).

2.11 Measurement of cardiodynamic functions in the isolated heart
Cardiodynamic parameters were measured with a pressure transducer connected to a PowerLab system (ADInstruments). An incision was made in the left atrium, and a fluid-filled latex balloon connected to the pressure transducer was inserted and positioned in the left ventricular cavity for the continuous assessment of cardiodynamic function. The balloon was initially inflated to an end-diastolic pressure of 5–10 mmHg, and thereafter the balloon volume was held constant. The hearts were perfused at a constant flow rate of 12 ml/min. Isolated rat hearts were perfused with Krebs solution and stabilized for at least 20 min before data recording. Hearts were then perfused with Krebs, NRS (Control), and DRRSAb (0.25 μM) for 1 h, respectively. Global ischaemia was induced by no-flow perfusion followed by reperfusion with Krebs bicarbonate buffer for 1 h. Cardiodynamic data were collected and analysed using a Data Acquisition System (PowerLab System; ADInstruments). Left ventricular end-diastolic pressure (LVdP/dt) was represented by the minimum pressure recorded during diastoles, left ventricular developed pressure (LVDP) was calculated as the difference between left ventricular systolic pressure and left ventricular diastolic pressure, contractility (+dP/dt) was represented by the maximum gradient during systoles, and compliance (−dP/dt) was represented by the minimum gradient during diastoles. Heart tissues from different groups were harvested to examine phosphorylation of ERK1/2 and Akt level by western blot at the end of 1 h reperfusion.

2.12 Statistical analysis
Values presented are mean ± standard error of mean (SEM). One-way analysis of variance was employed to determine the difference among groups. Significance level was set at P < 0.05.

3. Results
3.1 Binding of DRRSAb to DR region increases NKA activity
We first detected the binding of DRRSAb to DR region with immunofluorescent staining and western blots. As shown in Figure 1A, strong immunofluorescent signals were detected on the membrane surface of cardiac myocytes treated with DRRSAb. These data suggest that DRRSAb can bind to cell membrane in cardiac myocytes. A277 is a monoclonal antibody against amino acids 496 and 506 in the α1 subunit of NKA. In cell membrane permeabilized cardiac myocytes, A277 also binds to cardiac myocytes (Figure 1A, lower panel images). Western blot analysis showed that similar to A277, DRRSAb (1:1000 dilution) was able to detect NKA protein collected from rat or mouse kidneys (Figure 1B).

We further investigated whether the binding of DRRSAb could increase NKA activity. The activities of NKA in rat (ouabain-resistant) and mouse (ouabain-sensitive) kidney tissues were examined. As shown in Figure 1C and D, compared with normal rabbit sera (NRS, control), DRRSAb at 0.125–2.0 μM concentration-dependently stimulated NKA in both rat (C) and mouse (D) kidney tissues. The half effective concentrations (EC50) of DRRSAb for rat NKA and mice NKA were 0.16 and 0.29 μM, respectively.

3.2 Effects of DRRSAb on the amplitudes of myocyte contraction and Ca²⁺ transients in the electrically stimulated cardiomyocytes
Both rat (Figure 2A) and mouse (Figure 2C) single cardiomyocytes were chosen to study the cell contractility. Compared with that caused by NRS, a gradual and significant increase in the contraction was found in the rat cardiomyocytes treated with DRRSAb (Figure 2A). The concentration-dependent response of DRRSAb (0.05–0.25 μM) was shown in Figure 2B. At 0.25 μM, DRRSAb produced the strongest effect. For this reason, 0.25 μM was chosen for the subsequent experiments. DRRSAb at 0.25 μM also significantly improved mouse myocyte contraction (Figure 2C). These data suggest that DRRSAb can stimulate myocyte contraction via activation of both ouabain-sensitive and resistant NKA.

Similarly, DRRSAb (0.25 μM) also significantly increased the amplitudes of electrically induced calcium transients in rat cardiac myocytes (Figure 2D). These data suggest that DRRSAb may improve the calcium handling in cardiomyocytes.

3.3 Involvement of Src and ERK1/2 in the positive inotropic effect of DRRSAb
This series of experiments was undertaken to examine the involvement of ERK1/2 and Src in the positive inotropic effect of DRRSAb. Myocytes were preincubated with PD98059 (a selective ERK1/2 inhibitor, 30 μM, 30 min) or herbimycin A (a selective Src inhibitor, 1 μM, 2 h) before treatment with DRRSAb. It was observed that both PD98059 and herbimycin A abolished the enhanced rat myocyte contractility caused by DRRSAb (Figure 3A). A similar result was also found in mouse cardiac myocytes (data not shown). Western blot analysis shows that DRRSAb activated ERK1/2 (Figure 3B) was abolished by PD98059 and herbimycin A, suggesting that the stimulation of ERK1/2 by DRRSAb was secondary to the
activation of Src. Taken together, these data indicate that Src/ERK1/2 pathway may mediate the positive inotropic effect of DRRSAb.

3.4 Cardioprotection induced by DRRSAb in normal isolated ventricular cardiomyocytes

Cardiomyocytes are highly susceptible to damage during the isolation process and cannot be cultured for a long period. Since DRRSAb stimulated ERK1/2, a pro-survival protein kinase, we continued to study whether DRRSAb can prolong cardiomyocyte survival time. Trypan Blue exclusion was used as an index of myocyte viability. The cardiomyocyte viability was examined at different time points (0, 3, 6, 12, or 24 h) after incubating with DRRSAb (Figure 4A). It was found that DRRSAb-treated cardiomyocytes had higher viability when compared with the control cells treated with NRS. This cardioprotective effects lasted at least for 24 h.

To detect if DRRSAb-induced cardioprotection is mediated by the activation of ERK1/2 and the PI3K/Akt pathway, we measured the time course for the activation of these two kinases. As the results shown in Figure 4B and C indicate, both Akt and ERK1/2 were activated starting from 0.5 h and lasted for at least 12 h by DRRSAb but not A277.

3.5 Cardioprotection induced by DRRSAb in isolated ventricular myocytes subjected to ischaemia/reperfusion

To investigate if treatment with DRRSAb also confers cardioprotection against cellular injury caused by ischaemic/reperfusion, cells were treated with simulated ischaemic buffer for 10 min followed by reperfusion with normal culture medium for another 10 min. The experimental protocol was shown in Figure 5A and described in Section 2. Cell viability was determined 10 min after reperfusion. As shown in Figure 5B, cell viability was significantly decreased after ischaemia/reperfusion. DRRSAb obviously increased cell viability when compared with that in the NRS group. This finding suggested that DRRSAb may also protect heart against ischaemic/reperfusion-induced injury.

PKC is one of the well-known pro-survival protein kinases. We examined if PKC is involved in the cardioprotection offered by NKA activation. Unfortunately, all three PKC blockers, chelerythrine (a PKC general inhibitor), Go6976 (a PKCa inhibitor), and EAVSLKPT (a PKCa-selective peptide translocation inhibitor), failed to reverse the cardioprotection induced by DRRSAb (Figure 5B). These data suggest that the cardioprotection offered by DRRSAb was not dependent on PKC.

We further investigated if ERK1/2 and PI3K/Akt were playing a role in DRRSAb-induced cardioprotection. Cardiac myocytes were treated with PD98059 (30 μM, 30 min) or LY 294002 (15 μM, 15 min) before treatment with DRRSAb. As shown Figure 5C, both inhibitors abolished the protective effects of DRRSAb on myocyte viability, suggesting that the protective effects are mediated by ERK1/2 and PI3K/Akt pathways.

We continued to examine the signalling sequence of the activation of these kinases by determining ERK1/2 and Akt phosphorylation in the presence and absence of their selective inhibitors. As shown in Figure 5D and E, LY294002 abolished the phosphorylation of ERK1/2, whereas PD98059 failed to affect the effect of DRRSAb on
Akt activation. These observations indicated that PI3K/Akt may be upstream of the activation of ERK1/2 in our experimental condition.

3.6 Cardioprotective effects of DRRSAb in the isolated heart subjected to ischaemia/reperfusion

The protective effect of DRRSAb was also examined in the isolated heart. DRRSAb was also able to produce cardioprotective effects in the isolated heart. The experiment protocol was shown in Figure 6A and described in Section 2. As shown in Figure 6B–E, DRRSAb significantly increased LVDP (Figure 6B) and +dP/dt (Figure 6D), but decreased LVdP and −dP/dt at 15, 30, and 60 min after reperfusion (Figure 6C and E).

To confirm the protection effects of DRRSAb are mediated by Akt and ERK1/2, we also examined phosphorylation Akt and ERK1/2 at the end of 1 h reperfusion. As shown in Figure 6F and G, similar to that in cardiac myocytes, DRRSAb also stimulated Akt and ERK1/2 phosphorylation. These data confirm that DRRSAb protects hearts against ischaemic injury via activation of Akt and ERK1/2.

4. Discussion

NKA is a member of the P-type ATPase superfamily. In addition to the classic function of pumping ions, NKA is identified to engage in the assembly of multiple protein complexes that transmit signals into the cell through regulation of protein tyrosine phosphorylation. Binding of ouabain to the NKA activates the cytoplasmic tyrosine kinase Src which phosphorylates and assembles other proteins into different signalling modules. The activated protein kinase cascades include MAPK, PI3K/Akt, and PKC. Oubain also increases mitochondrial production of reactive oxygen species and regulates intracellular calcium concentration.

4.1 DRRSAb induces positive inotropic and cardioprotective effects

Extracellular DR region (897DVEDSYGQQWTYEQR911) of H7–H8 domain in alpha-subunit is an important activation site of NKA that is capable of promoting the catalytic function of the enzyme and regulating cardiac contractility. Antibodies like SSA412 against this region activate NKA and its subsequent signalling pathway. Although the
binding sites for the peptide antibody and ouabain are different. Binding of SSA412 also induces a conformational change of the pump, which will be sufficient to activate Src via autophosphorylation of the tyrosine residue. Src may further phosphorylate other effectors such as the epidermal growth factor receptor. Consistent with previous reports, we also found that DRRSAb, which is also against the DR region, stimulated NKA and produced positive inotropic effects in both rat and mouse cardiac myocytes. More importantly, we found in the present study that treatment with DRRSAb produced cardioprotective effects in both isolated cardiac myocytes and hearts. DRRSAb significantly prolonged the survival time of isolated cardiac myocytes and protected cardiac myocytes against ischaemic reperfusion-induced injury. Moreover, DRRSAb markedly improved heart contractile function in isolated hearts subjected to ischaemia/reperfusion. Our findings indicate that activation of NKA with DRRSAb may produce both positive inotropic and cardioprotective effects.

4.2 ERK1/2 is involved in the positive inotropic and cardioprotective effects of DRRSAb

We further examined the signalling mechanism underlying the positive inotropic and cardioprotective effects of DRRSAb. Mitogen-activated protein kinase (MAPK) signalling cascade pathways are among the most widespread mechanisms of cellular regulation. In mammals, there
are more than a dozen of MAPK genes.\textsuperscript{21,22} The ERK1/2 pathway is the prototypic MAPK and a key signalling pathway that is involved in the regulation of normal cell proliferation, survival, growth, and differentiation. It is commonly accepted that phosphorylation of ERK1/2 in the cardiac myocytes during early reperfusion serves as a defence mechanism against ischaemic stress stimuli.\textsuperscript{23} In the present study, we found that DRRSAb stimulated ERK1/2 starting from 0.5 h and lasting for at least 12 h. Blockade of ERK1/2 with PD98059 abolished DRRSAb-induced ERK1/2 phosphorylation and the subsequent positive inotropic and cardioprotective effects. These data suggest that DRRSAb may produce cardiac effects via stimulation of ERK1/2 pathway.

4.3 PI3K/Akt also mediates DRRSAb-induced cardioprotection

PI3K also plays a crucial role in effecting alterations in a broad range of cellular functions in response to extracellular signals. A key downstream effector of PI3K is the serine–threonine kinase Akt which in response to PI3K activation phosphorylates and regulates the activity of a number of targets including kinases, transcription factors and other regulatory molecules. After activation, Akt phosphorylates a variety of substrates involved in the regulation of cell growth, proliferation, and anti-apoptotic mechanisms to promote cell cycle and

Figure 5 Role of protein kinases in DRRSAb-induced cardioprotection in cardiac myocytes subjected to ischaemia reperfusion. (A) Experimental protocols. Vehicle, Krebs solution; Ischaemia, simulated ischaemia buffer; RE, reperfusion with normal culture medium. Ventricular myocytes were treated with DRRSAb for 3 h. Protein kinase inhibitors were given 15 min (for LY294002) or 30 min (for all other inhibitors) before DRRSAb treatment. Cell viability was measured 10 min after reperfusion. (B) PKC blockers [chelerythrine (Che), Go6976 and EAVSLKPT] failed to affect the cardioprotection induced by DRRSAb. n = 12. (C) Blockade of ERK1/2 with PD98059 or PI3K/Akt with LY294002 abolished the cardioprotection of DRRSAb. n = 12. (D and E) Western blot analysis showing DRRSAb-induced activation of Akt (D) and ERK1/2 (E). LY294002 inhibited the activation of both Akt and ERK1/2, whereas PD98059 failed to block the phosphorylation of Akt. n = 3. Values were mean ± SEM, *P < 0.05 vs. control (without ischaemia), #P < 0.05 vs. NRS; &P < 0.05 vs. vehicle (DRRSAb alone).
24,25 Akt inhibits formation of the pro-apoptotic proteins like Bad, Bax, and caspase26 and increases the formation of nitric oxide.27 Therefore, the PI3K signalling cascade may contribute to the recruitment of multiple endogenous cardioprotective pathways to reduce myocardial damage after ischaemia and reperfusion. We found in the present study that blockade of PI3K/Akt pathway with LY294002 attenuated DRRSAb-induced Akt phosphorylation and cardioprotection. These data suggest that apart from ERK1/2, PI3K/Akt may also serve as an important mediator for DRRSAb-induced cardioprotection. In addition, we found that there were two windows for DRRSAb-induced Akt phosphorylation. The first window was at 3 h and the second peak was observed at 12 h. These interesting data imply that there may be two or more upstream triggers to activate Akt and the cardioprotection caused by DRRSAb may be long-term.

The signalling sequence of DRRSAb was also studied. We found that either blockade of Src with herbimycin A or inhibition of PI3K with LY294002 attenuated DRRSAb-induced Akt phosphorylation and cardioprotection. These data suggest that apart from ERK1/2, PI3K/Akt may also serve as an important mediator for DRRSAb-induced cardioprotection. In addition, we found that there were two windows for DRRSAb-induced Akt phosphorylation. The first window was at 3 h and the second peak was observed at 12 h. These interesting data imply that there may be two or more upstream triggers to activate Akt and the cardioprotection caused by DRRSAb may be long-term.

The signalling sequence of DRRSAb was also studied. We found that either blockade of Src with herbimycin A or inhibition of PI3K with LY294002 attenuated DRRSAb-induced phosphorylation of ERK1/2. However, blockade of ERK1/2 with PD98059 failed to attenuate the phosphorylation of PI3K/Akt. These data suggest that both Src and PI3K/Akt may be upstream to ERK1/2.

4.4 The cardioprotection induced by DRRSAb was not mediated by PKC

PKC is another important protein survival kinase. The PKC family of serine/threonine kinases is a central component signalling molecules which play a significant role in mediating various cellular functions including apoptosis, proliferation, migration, motility, chemoresistance, and differentiation.28–31 PKC also correlates closely with cardioprotection.32–34 Numerous studies have documented a central role of PKC in the cardioprotection caused by ischaemic preconditioning.35–37 In the present study, we also tested the involvement of PKC and its isoforms in DRRSAb-induced cardioprotection. Since PKCa and PKCe were recently reported as essential pro-proliferative and survival molecules in different cell lines,38–40 we examined the cardioprotection of DRRSAb in the presence of chelerythrine, a general PKC inhibitor, Go6976, a selective PKCa inhibitor and EAVSLKPT, a selective PKCe peptide translocation inhibitor. All of them failed to attenuate the cardioprotection induced by DRRSAb. These data suggest that the cardioprotection induced by DRRSAb was not mediated by PKC.

In conclusion, we provide evidence for the first time that activation of NKA with DRRSAb may produce both positive inotropic and cardioprotective effects via activating the ERK1/2 and PI3K/Akt pathway. This unique property is of paramount clinical significance, due to the fact that most of, if not all, current available clinical drugs for heart failure treatment are potentially cardiotoxic.

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