

Full-length article

Insulin improves cardiomyocyte contractile function through enhancement of SERCA2a activity in simulated ischemia/reperfusion¹Jie YU², Hai-feng ZHANG², Feng WU², Qiu-xia LI², Heng MA², Wen-yi GUO³, Hai-chang WANG³, Feng GAO^{2,3,4,5}²Department of Physiology and ³Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China;⁴Institute of Molecular Medicine, Peking University, Beijing 100871, China**Key words**

insulin; ischemia/reperfusion; cardiomyocyte; contraction; calcium transients; SERCA2a; Akt

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Abstract

Aim: Insulin exerts anti-apoptotic effects in both cardiomyocytes and coronary endothelial cells following ischemia/reperfusion (I/R) via the Akt-endothelial nitric oxide synthase survival signal pathway. This important insulin signaling might further contribute to the improvement of cardiac function after reperfusion. In this study, we tested the hypothesis that sarcoplasmic reticulum calcium-ATPase (SERCA2a) is involved in the insulin-induced improvement of cardiac contractile function following I/R. **Methods:** Ventricular myocytes were enzymatically isolated from adult SD rats. Simulated I/R was induced by perfusing cells with chemical anoxic solution for 15 min followed by reperfusion with Tyrode's solution with or without insulin for 30 min. Myocyte shortening and intracellular calcium transients were assessed and underlying mechanisms were investigated. **Results:** Reperfusion with insulin (10^{-7} mol/L) significantly improved the recovery of contractile function ($n=15-20$ myocytes from 6–8 hearts, $P<0.05$), and increased calcium transients, as evidenced by the increased calcium (Ca^{2+}) fluorescence ratio, shortened time to peak Ca^{2+} and time to 50% diastolic Ca^{2+} , compared with those in cells reperfused with vehicle ($P<0.05$). In addition, Akt phosphorylation and SERCA2a activity were both increased in insulin-treated I/R cardiomyocytes, which were markedly inhibited by pretreatment of cells with a specific Akt inhibitor. Moreover, inhibition of Akt activity abolished insulin-induced positive contractile and calcium transients responses in I/R cardiomyocytes. **Conclusion:** These data demonstrated for the first time that insulin improves the recovery of contractile function in simulated I/R cardiomyocytes in an Akt-dependent and SERCA2a-mediated fashion.

Introduction

Our previous study showed that *in vivo* treatment with insulin reduced postischemic apoptotic death in both cardiomyocytes and coronary endothelial cells by activating the phosphatidylinositol 3-kinase-Akt-endothelial nitric oxide synthase (PI3-kinase-Akt-eNOS) pathway, that is, the survival signaling pathway, which might further contribute to the prolonged improvement of cardiac performance following ischemia/reperfusion (I/R)^[1–3]. In a recent study, we have demonstrated that insulin exerts direct positive inotropic effect by increasing calcium (Ca^{2+}) transients in I/R

cardiomyocytes. This insulin-induced improvement of contractile function in I/R myocytes shares the same signaling cascade for protection as previously described for myocardial tissue^[4]. These observations therefore indicate that this survival pathway is also active to directly improve cardiac myocyte function recovery following I/R *in vitro* experiments that guarantee constant conditions and exclude other factors, such as stress, hormone secretion, or inflammatory mediators, rendering insulin even more important as a protective agent applicable to reperfused myocardial tissue. However, the mechanisms underlying the insulin-induced improvement of I/R myocyte functional recovery through activation of the

survival pathway *in vitro* remain largely elusive.

Cardiac contraction and relaxation are closely regulated by intrinsic machineries governing sequential rise and fall of cytosolic Ca^{2+} . Ca^{2+} enters the cytosolic space through voltage-dependent Ca^{2+} channels after membrane depolarization and triggers release of Ca^{2+} from sarcoplasmic reticulum (SR), which initiates activation of cardiac contractile proteins and actin–myosin cross-bridge linking. Termination of contraction and cardiac relaxation are initiated with the removal of cytosolic Ca^{2+} mainly through sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) and, to a lesser extent, Na^+ - Ca^{2+} exchanger. It was shown that in rat ventricular myocardium, 92% of calcium removal occurs by SR calcium uptake^[5]. SERCA2a, the isoform of SERCA in myocardium and slow twitch skeletal muscle, is responsible for not only Ca^{2+} extrusion but also ventricular contractility because of their role in Ca^{2+} extrusion and SR Ca^{2+} loading for the next cardiac cycle^[5,6]. Defects in SERCA2a lead to decreased peak myocyte contractility, reduced intracellular Ca^{2+} removal, and prolonged duration of the cardiac cycle. Heart failure (HF) is a common outcome of myocardial infarction (MI) and is associated with a poor prognosis. Because of the loss of contractile function in the infarcted area, MI results in increased mechanical load on the intact myocardium, which undergoes molecular, cellular, morphological, and functional remodeling. Decreased SR Ca^{2+} uptake and decreased expression of SERCA2a are key features of cardiac myocyte dysfunction in both experimental and human HF^[7,8]. Hypertrophic cardiomyocytes with reduced contractility and relaxation have been found in the failing rat heart after MI, accompanied by a reduced peak in Ca^{2+} transients and prolonged Ca^{2+} transients decay^[9]. SERCA mRNA and protein levels decreased with increasing severity of HF after left coronary artery ligation^[7]. Indeed, in cardiomyocytes isolated from the left ventricle of patients with end-stage HF, SERCA2a overexpression can restore normal Ca^{2+} cycling^[6]. Previous studies in adult myocytes isolated from rat hearts 3 weeks after MI demonstrated abnormal contractility, intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) homeostasis and decreased SERCA2a expression and activity^[10]. However, no data are available so far on the effect of insulin on SERCA2a activity in I/R cardiomyocytes.

Last, but not least, activation of Akt and its upstream signal PI3-kinase might dramatically improve cardiac function and protect against apoptosis^[1,2,4], establishing an important role for Akt in the maintenance of heart morphology and function. Transgenic overexpression of protein kinase Akt enhances myocyte contractility and relaxation through acceleration of intracellular Ca^{2+} transients. The underlying

cellular mechanism(s) include potentiation of L-type Ca^{2+} channel function and upregulation of SERCA2a^[11]. However, to date, a direct link between the effects of insulin on SERCA2a activity in I/R myocardium and the insulin-induced survival pathway and subsequent improvement of I/R myocyte functional recovery has not been established.

The aims of this study, therefore, were to determine the role of SERCA2a in the insulin-induced improvement of I/R cardiomyocyte functional recovery, and to further investigate the mechanism involved.

Materials and methods

Preparation of isolated cardiomyocytes The experiments were carried out in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care. Calcium-tolerant ventricular myocytes were isolated from adult male Sprague-Dawley rats (body weight, 220–250 g) hearts by a standard enzymatic technique^[4]. Briefly, rats were anesthetized and anticoagulated with heparin sodium (1000 U/kg, ip). The hearts were rapidly excised and mounted on a Langendorff perfusion apparatus, and immediately perfused with Ca^{2+} -free Tyrode's solution containing (in mmol/L) 143.0 NaCl, 5.4 KCl, 0.5 MgCl_2 , 0.3 NaH_2PO_4 , 5.0 HEPES, 5.0 glucose (pH 7.4, 36 °C, equilibrated with O_2 , until spontaneous contraction of the heart ceased. The hearts were then perfused with Ca^{2+} -free Tyrode's solution containing 0.4 g/L collagenase II (283 U/mg; Worthington Biochemical, Lakewood, USA) and 0.7 g/L bovine serum albumin for approximately 20 min until the heart became soft. After perfusion with Ca^{2+} -free Tyrode's solution for 5 min to remove enzymes, the atria and aorta were removed and the ventricles were minced and incubated with Krebs solution containing (in mmol/L) 70.0 L-glutamic acid, 25.0 KCl, 20.0 Taurine, 10.0 KH_2PO_4 , 3.0 MgCl_2 , 0.5 EGTA, 10.0 HEPES, 10.0 glucose (pH 7.4) supplemented with 2% bovine serum albumin before being filtered through a nylon mesh (200 mesh). The cells were subsequently separated by sedimentation for 10 min twice. Cardiac myocytes were then re-suspended in the Ca^{2+} -free Tyrode's solution, and Ca^{2+} was slowly added to the cell suspension until Ca^{2+} reached a final concentration of 1.8 mmol/L. Approximately 70%–80% rod-shaped cardiomyocytes were obtained.

Measurement of myocyte contractile and relaxation function The mechanical contraction of ventricular myocytes was assessed by a video-based motion edge-detection system (IonOptix, Milton, USA)^[12]. In brief, myocytes were transferred to a cell chamber on the stage of an inverted

microscope (Olympus, Tokyo, Japan) and continuously perfused with Tyrode's solution (1 mL/min, 35 °C). Myocyte contraction was induced at a frequency of 0.5 Hz by platinum electrodes connected to an electrical stimulator. Peak twitch amplitude (PTA), time to peak shortening (TPS), time to 70% relengthening (TR70), and the maximal velocities of shortening/relengthening ($\pm dL/dt$) were automatically calculated from the cell length data by a computer. Criteria for choosing myocytes for the experiment included: (1) a rod shape with clear edges; (2) clearly defined sarcomeric striations; (3) steady contraction in response to electrical stimulation but without spontaneous contractions; and (4) stable steady-state contraction amplitude for at least 5 min before drugs were given.

Measurement of myocyte Ca^{2+} transients Myocytes were loaded with Fura-2/AM (0.5 μ mol/L; Alexis Biochemicals, San Diego, USA) for 30 min. The myocytes were excited by light emitted by a 75 W lamp and passed through either a 360 or 380 nm filter, and the Fura emission wavelength (510 nm) was synchronously monitored. Intracellular free Ca^{2+} in loaded myocytes was measured as the fluorescence ratio (360/380 nm)^[4].

Experimental protocols Ischemia conditions were simulated by chemical anoxia solution according to the method described by Esumi *et al*^[13], which contained (in mmol/L): 137.0 NaCl, 15.8 KCl, 0.49 MgCl₂, 0.9 CaCl₂·2H₂O, 4.0 HEPES, 10.0 deoxyglucose, 0.75 sodium dithionate and 20.0 lactate, pH 6.5. Ventricular myocytes from rats were perfused with Tyrode's solution and field stimulated at a frequency of 0.5 Hz, 5 ms duration. After 10–15 min of equilibration, the myocytes were exposed to one of the following treatments (15–20 myocytes from 6–8 rats/group): (1) control (Con), myocytes were perfused with Tyrode's solution for 45 min; (2) control plus insulin (Con+Ins), myocytes were perfused with 10⁻⁷ mol/L insulin for 45 min; (3) (I/R), after ischemia was simulated by perfusion of myocytes with chemical anoxia solution for 15 min, the cells were reperfused with Tyrode's solution for 30 min; (4) I/R plus insulin (I/R+Ins), after undergoing the same ischemia procedure as the I/R group, the cardiomyocytes were reperfused with insulin for 30 min; (5) I/R plus insulin plus Akt inhibitor (I/R+Ins+AI), the isolated myocytes were subjected to the same I/R procedure as the I/R plus insulin group, and pretreated with a selective Akt inhibitor, AI (1L-6-Hydroxy-methyl-*chiro*-inositol 2-(R)-2-*O*-methyl-3-*O*-octadecyl-carbonate, 5.0 μ mol/L; Calbiochem, Darmstadt, Germany)^[14,15] for 2 h before the I/R procedure and were treated with AI in the same concentration throughout the whole I/R duration. Myocyte shortening and intracellular Ca^{2+} transients were

assessed as described above.

Western blot analysis To determine the mechanism of the protective effect of insulin on I/R cardiomyocytes, an additional experiment was carried out to examine Akt expression and activation by Western blot analysis as described^[1]. Isolated cells were randomly divided into 5 groups to receive the treatments according to the experimental protocols. Ventricular myocytes were washed with phosphate-buffered saline and centrifuged at 1000 \times g. Cells were collected, washed and homogenized in lysis buffer containing (in mmol/L) 20.0 Tris, 150.0 NaCl, 1.0 EDTA, 1.0 EGTA, 1% Triton X-100, 2.5 sodium pyrophosphate, 1.0 β -glycerolphosphate, 1.0 Na₃VO₄, 1.0 g/L leupeptin, and 1.0 phenylmethylsulphonyl fluoride (pH 7.5). After sonication, the lysate was centrifuged at 4 °C (12 000 \times g, 10 min). The protein of the lysate was quantified and separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride-plus membrane. After being blocked with 5% milk, the immunoblots were probed with anti-pAkt (1:1000) antibodies overnight at 4 °C, followed by incubation with the corresponding secondary antibodies at room temperature for 1 h. The blots were visualized with ECL-plus reagent (Amersham Pharmacia Biotech, USA). pAkt immunoblots were then stripped with stripping buffer at 50 °C for 30 min and reblotted for total Akt (1:1000). Anti-Akt and anti-pAkt antibodies (#9272 and #9271, respectively) were obtained from Cell Signaling Technology (Beverly, USA).

Preparation of SR from rat heart SR was prepared according to the methods of Jones as modified by Kodavanti *et al*^[16–18]. The myocytes were put in homogenizing medium, containing (in mmol/L) 50.0 Na₂HPO₄, 10.0 Na₂EDTA, and 25.0 NaF (pH 7.4). The minced ventricle tissue was placed in 10 mL of ice-cold homogenizing medium and homogenated 3 times. An additional 5 mL of homogenizing medium was added and the homogenate was sedimented twice for 20 min at 14 000 \times g at 4 °C. The supernatant was recentrifuged at 45 000 \times g for 30 min. The pellet obtained after this centrifugation, consisting of crude membrane vesicles (SR), was suspended in storage buffer containing (in mmol/L) 30.0 histidine, 0.25 mol/L sucrose, 10.0 EDTA, and 10.0 NaF (pH 7.4) to a final concentration of 30–40 mg/mL protein and stored at -80 °C until used.

Measurement of Ca^{2+} -ATPase activity The activity of Ca^{2+} -ATPase was determined with a kit (Jiancheng, Nanjing, China) by measuring the inorganic phosphate (Pi) liberated from ATP hydrolysis^[17]. Ca^{2+} -ATPase activity was assayed in a medium containing (in mmol/L) 50.0 histidine, 3.0 MgCl₂, 100.0 KCl, 5.0 sodium azide, 3.0 ATP, and 50.0 μ mol/L CaCl₂

(pH 7.0)^[18]. Cardiac SR membranes were added to the reaction mixture at a final concentration of 20–25 µg of protein per milliliter, preincubated for 10 min at 37 °C and the reaction was initiated by the addition of ATP. The ATP hydrolysis that occurred in the absence of Ca²⁺ (1 mmol/L EGTA) was subtracted to determine the activity of Ca²⁺-stimulated ATPase. Ouabain was added fresh to a final concentration of 1 mmol/L in the media, which remained unchanged throughout the incubation. Mitochondrial contamination was assessed by determining the activity of azide-sensitive ATPase, that is, that activity inhibited by 5 mmol/L sodium azide^[19].

Statistical analysis All values in text, tables and figures are presented as mean±SEM. Differences were compared by Student's *t*-test or ANOVA, where appropriate. Probabilities of <0.05 were considered to be statistically significant. All of the statistical tests were carried out with GraphPad Prism software version 4.0 (GraphPad Software, San Diego, USA).

Results

Insulin improved cardiomyocyte contractile functional recovery in simulated I/R A simulated I/R model of myocytes was developed according to Esumi *et al*^[13], and the changes in cell contractile activity and intracellular Ca²⁺ transients during I/R were determined. Ventricular myocytes were perfused with Tyrode's solution for 10–15 min while the field was stimulated at a frequency of 0.5 Hz, 5 ms duration. Following this equilibration period the myocytes were exposed to chemical anoxia solution for 15 min. PTA of myocytes undergoing ischemia was gradually decreased to 38%±8% of the pre-ischemic level ($P<0.01$, $n=20$ myocytes from 8 hearts). On commencement of reperfusion, PTA rapidly re-

turned to near or transiently above pre-ischemia, then gradually declined to 87%±10% of the pre-ischemic level ($P>0.05$) with continued reperfusion (Figure 1A). Consistent with the contractile amplitude, $\pm dL/dt$ also had similar changes under conditions of simulated I/R (Figure 1B).

Insulin (10^{-7} mol/L, the concentration used in most *in vitro* studies)^[20,21] exerted significant inotropic action in normal myocytes (21.8%±0.5% of Con+Ins group *vs* 17.9%±0.8% of Con group; $P<0.05$, $n=20$ myocytes from 8 hearts) (Table 1). Although the contractile function of simulated I/R myocytes (undergoing ischemia for 15 min and reperfusion with Tyrode's solution for 30 min) could not completely recover to the level of the control group, in which the cells were perfused with Tyrode's solution for 45 min, there was no significant difference in contraction of the cells in the two groups (17.9%±0.8% of Con group *vs* 14.5%±0.7% of I/R group; $P>0.05$, $n=20$ myocytes from 8 hearts). However, consistent with our previous results, treatment with insulin at the beginning of reperfusion increased myocyte shortening in a concentration-dependent manner^[4]. As summarized in Table 1, insulin (10^{-7} mol/L) significantly increased PTA (% baseline) and $\pm dL/dt$, and markedly reduced time to peak shortening (TPS) and time to 70% relengthening (TR70) (20 myocytes from 7 rats, $P<0.05$ *vs* I/R group). The insulin-induced effect on myocyte shortening appeared rapidly with the maximal response, which occurred within 5 min of exposure to insulin, and cells recovered almost completely after washout. These data showed that insulin improved cardiomyocyte contractile functional recovery in simulated I/R.

Insulin increased intracellular calcium transients in simulated I/R cardiomyocytes The time-course of the fluo-

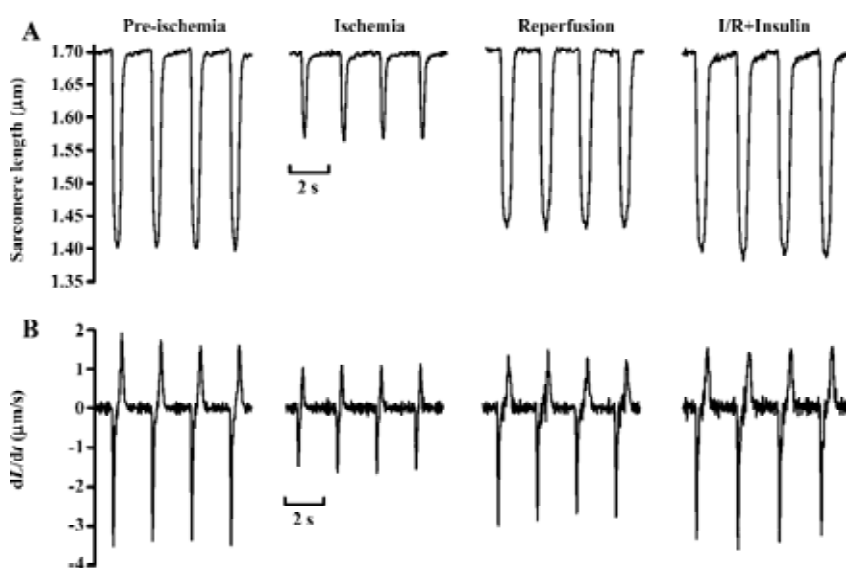


Figure 1. Representative traces showing the contractile function of rat cardiomyocytes in simulated I/R. After perfused with Tyrode's solution and stimulated at a frequency of 0.5 Hz, 5 ms duration for 10–15 min, the myocytes were exposed to chemical anoxia solution for 15 min and then reperused with or without insulin for 30 min. (A) Contraction amplitude of myocytes; (B) maximal velocity of shortening/relengthening of myocytes (dL/dt).

Table 1. Parameters of contractile function in rat cardiomyocytes with different treatments. *n*=15 to 20 myocytes from 6–8 hearts. Mean±SEM. ^b*P*<0.05, ^c*P*<0.01 vs Con. ^f*P*<0.01 vs I. ^h*P*<0.05 vs I/R. ^k*P*<0.05 vs I/R+Ins.

Group	PTA % baseline	TPS (ms)	TR70 (ms)	+dL/dt (μm·s ⁻¹)	-dL/dt (μm·s ⁻¹)
Con	17.9±0.8	230±14	139±6	298±14	252±17
Con+Ins	21.8±0.5 ^b	217±9 ^b	129±8	333±10 ^b	288±12 ^b
I	4.4±0.9 ^c	173±11 ^c	107±7 ^c	189±11 ^c	176±6 ^c
I/R	14.5±0.7 ^f	247±8 ^f	150±8 ^f	279±29 ^f	241±10 ^f
I/R+Ins	20.7±0.7 ^h	218±10 ^h	122±7 ^h	322±12 ^h	302±7 ^h
I/R+Ins+AI	16.3±0.6 ^k	245±6 ^k	142±10 ^k	282±9 ^k	254±10 ^k

I, ischemia; baseline, resting sarcomeric length.

rescence signal augment [time to peak Ca²⁺ (TTPCa)] and decay [time to 50% diastolic Ca²⁺ (T₅₀Dca)] were evaluated to assess the rate of intracellular Ca²⁺ release and clearing, respectively. Simulated ischemia by perfusion with chemical anoxia solution for 15 min significantly decreased calcium transients amplitude (ΔFFI, shown as twitch of fluorescence 360/380 ratio) (34%±9% of pre-ischemic level, *P*<0.01, *n*=20 myocytes from 8 hearts). Reperfusion with vehicle (Tyrode's solution) for 30 min almost recovered the twitch of fluorescent ratio to the pre-ischemic level, although not completely (90%±8% of pre-ischemic level, *P*>0.05) (Figure 2).

Consistent with our previous results, insulin significantly increased the calcium transients amplitude in normal myocytes (0.39±0.03 of Con+Ins group vs 0.35±0.04 of Con group, *P*<0.05, *n*=20 myocytes from 8 hearts) (Table 2). Although vehicle reperfusion had no effect on the intracellular Ca²⁺ transients, it was observed that insulin treatment at the onset of reperfusion significantly enhanced the ΔFFI from 0.32±0.03 of the I/R group to 0.36±0.04 (*P*<0.05) 5 min after reperfusion with insulin. Significantly shorter TTPCa and T₅₀Dca were seen after exposure to insulin in I/R myocytes (both *P*<0.05), whereas the diastolic Ca²⁺ level (resting FFI) had no significant change compared with the Con or I/R groups (Table 2). These data suggested that there was

Table 2. Parameters of Ca²⁺ transients in rat cardiomyocytes with different treatments. *n*=15 to 20 myocytes from 6–8 hearts. Mean±SEM. ^b*P*<0.05, ^c*P*<0.01 vs Con. ^f*P*<0.01 vs I. ^h*P*<0.05, ⁱ*P*<0.01 vs I/R. ^k*P*<0.05, ^l*P*<0.01 vs I/R+Ins.

Group	Resting FFI	ΔFFI	TTPCa/ms	T ₅₀ Dca/ms
Con	0.86±0.05	0.35±0.04	69.7±2.4	254±11
Con+Ins	0.87±0.07	0.39±0.03 ^b	58.3±3.3 ^b	219±7 ^b
I	0.85±0.03	0.20±0.03 ^c	86.6±5.1 ^c	284±9 ^c
I/R	0.88±0.04	0.32±0.03 ^f	77.5±3.5 ^f	278±8 ^f
I/R+Ins	0.87±0.03	0.36±0.04 ^h	61.2±2.0 ⁱ	206±9 ⁱ
I/R+Ins+AI	0.88±0.07	0.31±0.05 ^k	73.7±2.4 ^k	268±10 ^l

enhanced Ca²⁺ handling elicited by insulin in I/R myocytes.

Mechanisms involved in improvement of contractile function Defects in SERCA2a lead to decreased peak myocyte contractility, reduced intracellular Ca²⁺ removal, and prolonged duration of the cardiac cycle^[10]. To study whether SERCA2a is also involved in the insulin-induced improvement of contractile function following I/R, the present study examined Ca²⁺-ATPase activity by an optical assay in crude SR extracted from control and simulated I/R myocytes. As

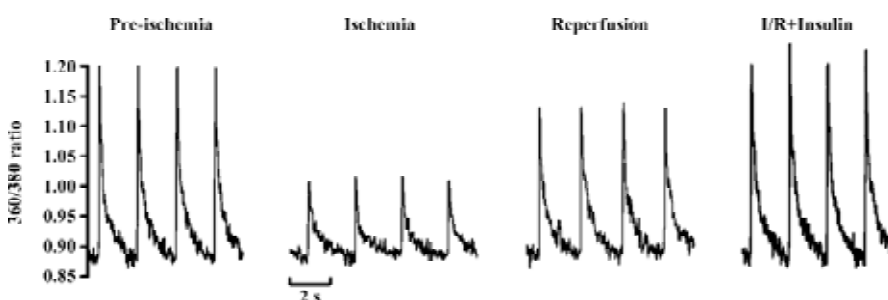


Figure 2. Representative traces showing the electrically stimulated intracellular Ca²⁺ transients of rat cardiomyocytes in simulated I/R. Myocytes were loaded with Fura-2/AM (0.5 μmol/L) for 30 min, then excited by light emitted by a 75 W lamp and passed through either a 360 or 380 nm filter. The Fura emission wavelength (510 nm) was synchronously monitored. The trace shows the twitch of fluorescence ratio (360/380 nm) in myocytes.

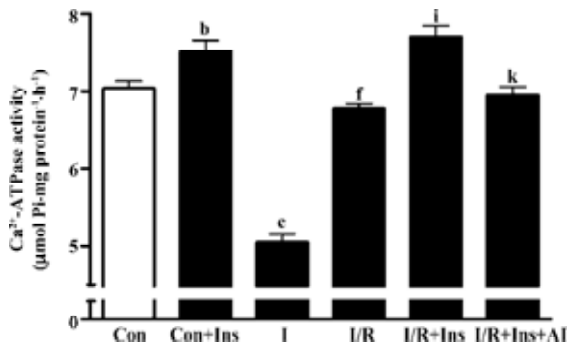


Figure 3. Effect of insulin (10^{-7} mol/L) on the activity of SR Ca^{2+} -ATPase in ischemic/reperfused rat myocytes. I, ischemia. $n=8$. Mean \pm SEM. ^b $P<0.05$, ^c $P<0.01$ vs Con. ^f $P<0.01$ vs I. ⁱ $P<0.01$ vs I/R. ^k $P<0.05$ vs I/R+Ins.

shown in Figure 3, insulin increased the SR Ca^{2+} -ATPase activity in normal myocytes. It was seen that the SR Ca^{2+} -ATPase activity was significantly decreased at the end of ischemia, whereas the vehicle reperfusion almost recovered the SR Ca^{2+} -ATPase activity in I/R myocytes compared with that of control group. Interestingly, treatment with insulin at the onset of reperfusion significantly increased the SR Ca^{2+} -ATPase activity in I/R myocytes (7.8 ± 0.4 $\mu\text{mol Pi}\cdot\text{mg protein}^{-1}\cdot\text{h}^{-1}$ vs 6.8 ± 0.6 $\mu\text{mol Pi}\cdot\text{mg protein}^{-1}\cdot\text{h}^{-1}$ of I/R group, $n=8$, $P<0.05$), suggesting that increased SERCA2a activity might be involved in the insulin-induced improvement of I/R myocyte contraction.

A previous study demonstrated that transgenic over-expression of Akt increases myocyte contractility through acceleration of intracellular Ca^{2+} transients^[11]. To study the role of Akt in the insulin-induced positive inotropic effects on I/R myocytes, an additional experiment was carried out to determine the insulin-induced Akt activation in isolated cardiomyocytes by Western blot. As shown in Figure 4, insulin markedly activated Akt activity in normal myocytes. Although the I/R procedure made no change in Akt activation, treatment with insulin resulted in a 2.9-fold increase in Akt phosphorylation ($n=5$, $P<0.01$ vs I/R group). There was no difference in total Akt among the groups studied (Figure 4). These results showed that the insulin-induced inotropic effect on I/R myocytes might be, at least in part, Akt-dependent.

To further establish a cause-effect relationship between increased SERCA2a activity and the improved contractile response to insulin in simulated I/R myocytes through Akt activation, we pre-treated the myocytes with a selective AI (5.0 $\mu\text{mol/L}$) before insulin stimulation and assessed the changes in SERCA2a activity and myocyte contractile

function. Pre-treatment with AI not only markedly decreased the Akt phosphorylation by insulin ($P<0.01$ vs I/R plus insulin group, Figure 4), but also inhibited the augmentation of SERCA2a activity in the insulin-treated I/R myocytes ($n=8$, $P<0.05$, Figure 3). In addition, AI treatment also almost completely abolished the contractile effect and the augmented intracellular calcium transients induced by insulin synchronously, whereas the same concentration of AI alone had no effect on either myocyte contraction or calcium transients (Tables 1,2). Previous studies and our preliminary experiment showed that AI, at the concentration of 5.0 $\mu\text{mol/L}$, selectively blocked Akt phosphorylation induced by insulin but did not affect PI-3 kinase activity^[22,23]. These results provided direct evidence demonstrating that increased SERCA2a activity, which is at least partly by enhanced Akt activation, plays a critical role in cardiac contractile response to insulin in I/R myocytes.

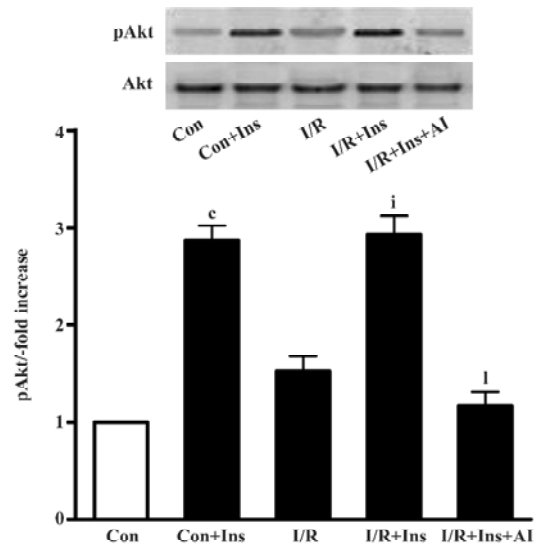


Figure 4. Phosphorylation of Akt by insulin treatment and its modification by the pretreatment of AI in ischemic/reperfused rat cardiomyocytes. Insets are representative results of Western blots showing signals of phosphor-Akt (pAkt) and total Akt. There was no difference in total Akt among the groups studied. The bar graph shows pAkt signal intensity obtained from quantitative densitometry analysis of 5 independent experiments. Mean \pm SEM. ^c $P<0.01$ vs Con. ⁱ $P<0.01$ vs I/R. ^l $P<0.01$ vs I/R+Ins.

Discussion

In the present study we have demonstrated, for the first time, that insulin increases the SERCA2a activity and subsequently improves functional recovery in simulated I/R myocytes. This enhancement of SERCA2a activity by insu-

lin is at least partly due to Akt activation. This finding suggests that insulin plays a more important role in the cardioprotection of ischemic/reperfused myocardium by modulating the intracellular Ca^{2+} concentration homeostasis.

Increasing evidence indicates that, in addition to its inimitable function in glucose metabolism, insulin plays critical roles in a variety of other physiological and pathological modulations, such as regulation of inflammatory response and nitric oxide production^[1,24,25]. We have also previously demonstrated that insulin improves myocardial functions following I/R in an *in vivo* model^[3]. However, the mechanism underlying these insulin-induced effects is unclear. In a recent study, we provided evidence that insulin exerts direct positive inotropic action on cardiomyocytes^[4]. To further determine the effect of insulin on myocardium in I/R conditions *in vitro*, in our present study, ventricular myocytes isolated from adult rat were subjected to 15 min ischemia and subsequent 30 min reperfusion, during which the cells were field-stimulated and myocyte shortening/relengthening and intracellular Ca^{2+} transients were simultaneously observed.

Considering the cardiac myocyte is composed of bundles of myofibrils that contain sarcomeres representing the basic contractile units of the myocyte and related to the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), we reflected the cardiomyocyte contractile function with the change of the myocyte sarcomere length. It was seen that simulated ischemia by chemical anoxia solution decreased the amplitude of contraction to approximately 40% of the pre-ischemic level, which was restored, though not completely, to the pre-ischemic level by reperfusion with Tyrode's solution. Interestingly, treatment with insulin (10^{-7} mol/L) at the onset of reperfusion significantly enhanced the recovery of contractile function as evidenced by increased PTA and $\pm dL/dt$ and shortened TPS and TR70 in I/R cardiomyocytes, compared with those in cells reperfused with vehicle (Table 1). Concomitantly, the intracellular Ca^{2+} transients (Ca^{2+} fluorescence ratio, ΔFFI) induced by field-stimulation was restored to the pre-ischemic level by reperfusion with vehicle in simulated I/R myocytes. As the amplitude of Ca^{2+} transients was increased, and TTPCa and $T_{50}\text{DCa}$ were overtly reduced by insulin treatment in I/R myocytes (Table 2), the alteration of myocyte Ca^{2+} handling is largely responsible for the enhanced contractile response of myocytes to insulin. Taken together, these data indicate that insulin exerts direct positive inotropic effects on myocytes in I/R conditions by modulating intracellular Ca^{2+} .

The Ca^{2+} -ATPase of SR catalyzes the most important step in relaxation by coupling cleavage of ATP to transport two

Ca^{2+} into the SR lumen^[26,27]. A dysfunction of SERCA2a has been proposed as a contributing factor to the development of cardiovascular diseases in which cytokines are involved, such as genetic hypertension, I/R injury, myocardial stunning, and heart failure^[28]. However, SERCA2a overexpression in cardiomyocytes from the left ventricle of patients with HF can restore normal Ca^{2+} cycling^[6]. In the present study, to determine the role of SERCA2a in the insulin-induced alteration of Ca^{2+} transients, we measured SERCA2a activity in myocytes. No remarkable change was observed in myocytes undergoing simulated I/R procedure, which is different from some previous data that SERCA2a expression and activity decreased with increasing severity of HF after MI^[10]. The difference might be due to the shorter time-course of I/R *in vitro* in this study. In contrast, we found that treatment with insulin significantly increased SERCA2a activity compared with vehicle reperfusion, suggesting that increased SERCA2a activity might contribute to the improvement of intracellular Ca^{2+} transients and subsequent contraction by insulin in I/R myocytes.

We have demonstrated that insulin exerts anti-apoptotic effects in both cardiomyocytes and coronary endothelial cells following I/R by Akt-eNOS signal *in vivo*, that is, the survival signaling pathway, which might further contribute to the prolonged improvement of cardiac function after reperfusion^[1-3]. In addition, it was also shown in our previous study that insulin has a direct positive inotropic effect on isolated cardiomyocytes by activating Akt. This is consistent with the notion that transgenic overexpression of Akt enhances myocyte contractility and relaxation through acceleration of intracellular Ca^{2+} transients in mice^[11]. However, the direct link between Akt activation and improvement of myocyte contraction by insulin remains largely unclear. In the present study, we found that insulin resulted in a 2.9-fold increase in phosphorylation of Akt in I/R myocytes, which was inhibited by pretreatment of the cells with a specific AI. Most importantly, inhibition of Akt activity with AI also significantly abolished increased SERCA activity, Ca^{2+} transients and contractile activity by insulin simultaneously in I/R myocytes. These data suggest that Akt is probably involved in the effects of insulin on SERCA2a, intracellular Ca^{2+} handling and subsequent contractile function in I/R cardiomyocytes.

In summary, the present study demonstrated that insulin improves the recovery of contractile function in simulated I/R cardiomyocytes in an Akt-dependent and SERCA2a-mediated fashion. This finding suggests that the insulin-activated Akt survival signaling not only contributes to the previously observed cardiac protective effects, but also plays a

causative role in the direct inotropic action induced by insulin in I/R myocardium.

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