Swim training sensitizes myocardial response to insulin: Role of Akt-dependent eNOS activation

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Abstract

Objectives: Physical activity has been well known to benefit heart function. The improved autonomic nervous activity is considered to be mainly responsible for this beneficial effect. However, the precise mechanism behind the intrinsic myocardial responsiveness to exercise is still unclear. This study was designed to examine the effect of swim training on myocardial response to insulin with a special focus on the endogenous endothelial nitric oxide synthase (eNOS)–nitric oxide (NO) cascade.

Methods: Adult male Sprague–Dawley (SD) rats were subjected to a 10-week free-loading swim training (3 h/day, 5 days/week). Contractile response to insulin at the levels of cardiomyocytes and isolated perfused heart, myocardial glucose uptake and post-insulin receptor signaling cascades were evaluated.

Results: Swim training enhanced cardiac contractile response to insulin in cardiomyocytes and isolated perfused heart, respectively. The improved cardiac response was accompanied by facilitated insulin-stimulated glucose uptake, GLUT4 translocation and upregulation of Akt and eNOS expression (p<0.01). Treatment with insulin resulted in a 3.6- and 2.2-fold increase of eNOS phosphorylation (p<0.01), as well as a 3.0- and 1.9-fold increase of Akt phosphorylation in exercise and sedentary groups, respectively (p<0.01). In addition, exercise significantly facilitated insulin-induced myocardial NO production (p<0.01 vs. sedentary). Moreover, pretreatment with either LY294002, a phosphatidylinositol-3 kinase (PI-3K) inhibitor or L-NAME, a NOS inhibitor, abolished the exercise-induced sensitization of myocardial contractile response to insulin, insulin-induced NO production and phosphorylation of Akt and eNOS.

Conclusion: These results demonstrate that swim training is capable of sensitizing myocardial contractile response to insulin via upregulation of Akt- and eNOS signaling cascades.

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Keywords: Swim training; Myocardial contraction; Insulin; Nitric oxide

1. Introduction

Insulin facilitates insulin receptor substrates (IRSs)-mediated activation of phosphatidylinositol-3 kinase (PI-3K) through binding to its cell surface receptor, the effect of which mainly responsible for metabolic properties of insulin. The serine/threonine protein kinase Akt has been shown to participate in the insulin-induced peripheral glucose metabolism [1]. Impaired insulin signaling transduction is believed to underscore systemic insulin resistance [2]. Recent evidence has demonstrated that improved myocardial insulin sensitivity using rosiglitazone protects against ischemia/reperfusion-induced myocardial injury in Zucker diabetic fatty rats [3]. Therefore, it is conceivable...
that adequate myocardial insulin sensitivity is essential to the maintenance of physiological cardiovascular function.

Physical activity is known to improve insulin sensitivity in peripheral insulin-targeted organs such as skeletal muscle in both human and experimental animals [4,5]. However, little knowledge is available regarding the impact of physical activity on myocardial insulin sensitivity, given that exercise can significantly improve cardiorespiratory fitness and insulin sensitivity in insulin-targeted peripheral organs. Accumulating evidence has suggested that exercise-induced insulin sensitization is related to Akt activation in peripheral insulin-targeted organs [4,5]. Recently, Huisamen and Lochner [6] reported that short-term swimming exercise significantly increased insulin-stimulated Akt phosphorylation and GLUT4 expression in diabetic hearts, suggesting that Akt may be crucial for myocardial insulin sensitization. However, the exact nature of the insulin sensitizing molecule downstream of Akt in response to exercise has not been elucidated. Our previous studies revealed that insulin exerts cardioprotective effect against ischemic/reperfused myocardial injury through an Akt–eNOS–NO-dependent mechanism [7], suggesting that the eNOS–NO system may serve as a downstream player for Akt in the exercise-associated cardiac insulin sensitization.

NO is a ubiquitous signaling molecule involved in a wide variety of physiological processes ranging from muscle contraction to cell survival and death. In a recent study, we demonstrated that upregulation of eNOS via adenoviral gene contraction to cell survival and death. In a recent study, we demonstrated that upregulation of eNOS via adenoviral gene

2. Materials and methods

2.1. Materials

All chemicals and reagents including bovine insulin were purchased from Sigma (A.R.) unless indicated otherwise. L-NAME was from Calbiochem (San Diego, CA, USA). LY294002, anti-PI-3 kinase p85, Anti-Akt, anti-phosphorylated-Akt (Ser-473), anti-eNOS and anti-phosphorylated-eNOS antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-GLUT4 (H-61) and anti-IRS2 (H-205) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). D-[2-3H]-glucose and ECL-plus reagent were from Amer sham Pharmacia Biotech (Uppsala, SE, Sweden). Percoll and density-marker beads were from Pharmacia (Piscataway, NJ, USA). Collagenase II (270 U/mg) was purchased from Worthington Biochemical Co. (Lakewood, NJ, USA). Fura-2/AM was from Alexis Biochemicals (San Diego, CA, USA). Glycogen Assay Kit and NO Assay Kit were from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Bicinchoninic acid (BCA) protein assay Kit was from Pierce (Rockford, IL, USA).

2.2. Animals

Adult male Sprague–Dawley (SD) rats (8-week-old) were randomly divided into sedentary and 10-week-exercise groups. All animals were housed individually in a temperature-controlled animal room (22 °C–24 °C) under a 12-h light (7:30–19:30)/12-h dark (19:30–7:30) circadian cycle with free access to food and water. All biochemical and functional measurements were conducted with the investigators blinded to treatment. All animal procedures described in this study were performed in adherence 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), with approval from the Fourth Military Medical University Committee on Animal Care.

2.3. Exercise protocol

The swimming exercise training protocol was modified from a previously published procedure [13]. Rats in the exercise group were trained, free of loading, 5 days/week for 10 weeks in a 60×90-cm tank filled with approximately 50 cm depth of water at 33 °C–35 °C. Rats were allowed to swim in groups of three or four animals. Rats swam for 30 min on the first day; the swimming duration was then progressively increased from 30 min/day to 180 min/day in 2-week periods. All training sessions took place during the early morning hours (8:00–11:00). All functional measurements including hemodynamic parameters, cardiomyocyte contraction and insulin-stimulated glucose uptake were commenced 24 h after the last swimming bout during 12:00–19:00.

2.4. Oral glucose tolerance test (OGTT) and insulin sensitivity test (IST)

OGTT and IST were performed following a 9-h fasting (23:00–8:00). Rats were given an oral glucose (2 g/kg of body weight) challenge by intragastric administration using a delivery tube or an i.p. injection of insulin (0.5 unit/kg of
body weight). Whole blood glucose levels were determined at 0, 30, 60, 90 and 120 min after glucose challenge for OGGT, or at 0, 30, 60, 90, 120, and 240 min for IST using tail clipping. Value was normalized to the initial glucose levels prior to initiation of the IST test.

2.5. Preparation of isolated cardiomyocytes

Cardiomyocytes were isolated by a standard enzymatic technique [7] 24 h after the last swimming bout. The hearts were rapidly excised and mounted on a Langendorff perfusion apparatus. The hearts were perfused with Ca²⁺-free Tyrode’s solution containing (in mmol/L) 143 NaCl, 5.4 KCl, 0.5 MgCl₂, 0.3 NaH₂PO₄, 5.0 HEPES, 5.0 glucose (pH 7.4, 36 °C), equilibrated with oxygen, until spontaneous contraction of the heart was ceased. The hearts were then perfused with a Ca²⁺-free Tyrode’s solution containing 0.4 g/L Collagenase II and 0.7 g/L bovine serum albumin (BSA) for approximately 20 min until the heart became tender. After perfusion with Ca²⁺-free Tyrode’s solution for 5 min to remove the enzyme, the ventricles were minced and incubated with Krebs solution containing (in mmol/L, pH 7.4) 70 L-glutamic acid, 25 KCl, 20 Taurine, 10 KH₂PO₄, 3.0 MgCl₂, 0.5 EGTA, 10 HEPES, 10 glucose supplemented with 2% BSA before being filtered through a nylon mesh (200 mesh). The cells were subsequently separated by sedimentation. Cardiomyocytes were resuspended in the Ca²⁺-free Tyrode’s solution, and Ca²⁺ was slowly added to the cell suspension till Ca²⁺ reached the final concentration of 1.8 mmol/L. About 70%–80% rod-shaped cardiomyocytes were obtained.

2.6. Measurement of myocyte contractile function

Contraction of cardiomyocytes was assessed as described previously [14]. Cardiomyocytes were transferred to a cell chamber and were perfused with Tyrode’s solution (1 mL/min, 35 °C). Cardiomyocyte contraction was induced at a frequency of 0.5 Hz by a pair of platinum electrodes connected to a stimulator. Peak twitch amplitude (PTA), and positive and negative maximal velocities of shortening/relengthening (±dV/dt max) were automatically analyzed. After 10 min of stable perfusion, L-NAME (0.5 mmol/L) or LY294002 (20 μmol/L) was added to Krebs–Henseleit (KH) solution containing (in mmol/L) 117 NaCl, 25 NaHCO₃, 4.7 KCl, 1.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 1.2 sodium palmitate prebound to 3% fatty acid-free BSA, 11 glucose (pH 7.4, 36 °C) for an additional 15 min of repertusion, then insulin was added into KH with L-NAME or LY294002 for 40 min of perfusion. When all the above procedures were completed, the hearts were weighed immediately, and frozen in liquid nitrogen, and then stored at −80 °C for future analysis.

2.7. Measurement of cardiomyocytes Ca²⁺ transients

Cardiomyocytes were excited by light emitted by a 75-W lamp and passed through either a 360- or a 380-nm filter, and the fura emission wavelength (510 nm) was synchronously monitored. Intracellular free Ca²⁺ in fura-2 loaded cardiomyocytes was measured as the fluorescent ratio (360/380 nm).

2.8. Assessment of myocardial contractile function

Whole heart contractile function was assessed as described previously [15]. Briefly, a latex balloon was inserted into the left ventricular cavity and connected to a pressure transducer. The balloon was initially inflated with deionized water to produce an end diastolic pressure of 5 to 10 mm Hg. Left ventricular pressure was continually recorded via a data acquisition system. The left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP; LVDP=LVSP–LVEDP), and positive and negative maximal values of the instantaneous first derivative of left ventricular pressure (±dP/dt max) were automatically analyzed. After 10 min of stable perfusion, L-NAME (0.5 mmol/L) or LY294002 (20 μmol/L) was added to Krebs–Henseleit (KH) solution containing (in mmol/L) 117 NaCl, 25 NaHCO₃, 4.7 KCl, 1.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 1.2 sodium palmitate prebound to 3% fatty acid-free BSA, 11 glucose (pH 7.4, 36 °C) for an additional 15 min of repertusion, then insulin was added into KH with L-NAME or LY294002 for 40 min of perfusion. When all the above procedures were completed, the hearts were weighed immediately, and frozen in liquid nitrogen, and then stored at −80 °C for future analysis.

2.9. Glucose uptake

Hearts were perfused with 250 mL recirculating KH solution containing (in mmol/L) 11 glucose and 1.2 sodium palmitate prebound to 3% fatty acid-free BSA with 14.5 μCi/mmol D-[2-³H]-glucose. Insulin was added to the buffer reservoir after 30 min to a final concentration of 10⁻⁷ mol/L. Perfusion was continued for another 30 min and buffer samples were taken from the reservoir every 4 min throughout the protocol. The glucose used was plotted against time, and the rates of glucose uptake, with or without insulin, were calculated. Other groups of hearts were used to determine the effects of either the eNOS inhibition by L-NAME or Akt inhibition by LY294002 on insulin-stimulated glucose uptake.

2.10. Heart plasma membrane preparation

Heart plasma membrane (PM) fraction was performed as described previously [16]. Ventricular tissue was minced in ice-cold buffer A containing (in mmol/L, pH 7.0): 10 NaHCO₃, 5 NaN₃. The resultant slurry was homogenized with a Heidolph DIA900 tissue homogenizer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at a maximum rev for 10–15 s. The homogenate was transferred to a handheld glass/glass homogenizer and subjected to 10
pestle strokes. The homogenate was centrifuged at 7000 \( \times \) g for 20 min. The pellet was resuspended in buffer B (10 mmol/L Tris–HCl, pH 7.4), and centrifuged at 200 \( \times \) g for 20 min. The supernatant was gently layered on top of a 20% (vol/vol) Percoll gradient in buffer C (in mmol/L: 255 sucrose, 10 Tris–HCl (pH 7.4), 2 EDTA) and centrifuged at 55,000 \( \times \) g for 1 h. The band at density of 1.030 was aspirated and pelleted by centrifugation at 170,000 \( \times \) g for 1 h and resuspended in buffer C as the PM fraction. Protein concentration of the PM solution was determined with BCA protein assay. PM solution was used to assess GLUT4 translocation to PM by Western blot.

2.11. Western blot analysis

The expressions of IRS2, PI-3 kinase p85, Akt, eNOS, GLUT4 and the phosphorylations of Akt and eNOS were measured using Western blot. Briefly, ventricle tissue was homogenized in ice-cold lysis buffer containing (in mmol/L): 20 Tris–HCl, 50 NaCl, 50 NaF, 50 Na\(_4\)P\(_2\)O\(_7\), 250 sucrose, 2 Na\(_3\)VO\(_4\), 1 DTT and 1% protease inhibitor cocktail. The homogenate was centrifuged at 12,000 \( \times \) g for 10 min; the supernatant was collected. Protein concentration was determined with BCA protein assay. Proteins (50–120 \( \mu \)g protein per lane) were separated by electrophoresis on 8–12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane in a semidry blotting apparatus (Bio-Rad Laboratories, Hercules, CA, USA), blocked with 5% nonfat dry milk at room temperature for 1 h. The blots were visualized with primary 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 and the results were quantitated by LabImage version 2.7.1 (Kapelan GmbH, Halle, Germany). p-Akt or p-eNOS immunoblots were then stripped with strip buffer at 50 °C for 30 min and reprobed for total Akt or eNOS. Beta-actin was used as the internal loading control.

2.12. Measurement of NO content in myocardium

Myocardium was rinsed, homogenized in 0.9% NaCl solution (1:10, wt/vol), and centrifuged at 3000 \( \times \) g for 5 min. The pellet was discarded. NOx concentrations in the supernatant were quantified by a NO detection kit (Nitrate Reductase).

2.13. Determination of the glycogen content in myocardium

Myocardium was washed in 0.9% NaCl solution, absorbed by filter paper and then accurately weighed. Following the decomposition of myocardium in high basic solution at 100 °C for 20 min, the content of glycogen in myocardium was tested as described by a detection kit.

2.14. Statistical analysis

Data were presented as mean±S.D. Student’s t-test or ANOVA was used to assess significance of differences. When analysis of variance revealed significant differences, Bonferroni correction for post hoc t-tests was used to correct for multiple comparisons. Probability value of <0.05 was considered to be statistically significant.

3. Results

3.1. Effects of swim training on body weight, heart weight, left ventricular weight, fat weight and in vivo hemodynamic parameters in rats

As shown in Tables 1 and 2, swim training decreased heart rate, elevated the ratio of heart and left ventricular (LV) to body weight, +LVdP/dt max as well as LVEDP (p<0.05–0.01) without affecting blood pressure, LV systolic pressure, −LVdP/dt max, heart and LV weights (p>0.05). The elevation in the ratio of heart to body weight and LV to body weight was partially due to reduction in body weight, fat content and the ratio of fat to body weight (p<0.01). These data suggested that swim training induced LV hypertrophy and partially improved hemodynamic parameters.

3.2. Swim training improved whole body insulin sensitivity

As shown in Fig. 1A, following oral glucose challenge (2 g/kg of body weight), blood glucose levels in both exercise and sedentary groups started to decline after peaking at 30 min. While blood glucose levels in the exercise rats returned to nearly baseline value after 120 min, the post-challenge glucose levels remained at much higher levels between 90 and 120 min in the sedentary rats (p<0.01). Whole body insulin sensitivity
was assessed by relative blood glucose reduction in response to insulin (Fig. 1B). After insulin injection (0.50 unit/kg of body weight), reduction of blood glucose, relative to their initial level, was 18%, 14% and 11% less pronounced at 30, 60 and 90 min points, respectively, in control rats compared with those from exercised rats ($p < 0.05$). These results suggested that swim training significantly improves whole body insulin sensitivity.

### 3.3. Swim training improved myocardial insulin sensitivity

To determine whether swim training affects myocardial insulin sensitivity, GLUT4 expression, insulin-stimulated glucose uptake and GLUT4 translocation to PM were measured in isolated perfused hearts. Although swim training and insulin failed to upregulate GLUT4 expression, insulin...
significantly increased cardiac glucose uptake and GLUT4 translocation to PM, and such increases were further improved in isolated hearts following swim training \((p<0.05)\), indicating that swim training may significantly improve myocardial insulin sensitivity \((\text{Fig. 1C, E and F})\). Moreover, insulin-stimulated glycogen synthesis in myocardium was significantly improved following swim training \((p<0.05)\) \((\text{Fig. 1D})\).

3.4. Swim training promoted contractile and \(\text{Ca}^{2+}\) transient response to insulin in cardiomyocytes

To investigate whether contractile response to insulin may be potentiated along with the improvement in myocardial insulin sensitivity, cardiomyocytes from control and exercise rat hearts were exposed to insulin ranging from \(10^{-8}\) to
Table 3

<table>
<thead>
<tr>
<th></th>
<th>Resting FFI</th>
<th>ΔFFI</th>
<th>TTPCa (ms)</th>
<th>T50DCa (ms)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.82±0.03</td>
<td>0.18±0.04</td>
<td>63.03±4.46</td>
<td>177.08±21.28</td>
</tr>
<tr>
<td>Control + insulin</td>
<td>0.82±0.03</td>
<td>0.18±0.01</td>
<td>63.03±4.46</td>
<td>177.08±21.28</td>
</tr>
<tr>
<td>Exercise</td>
<td>0.81±0.06</td>
<td>0.14±0.04</td>
<td>57.56±3.18</td>
<td>138.79±19.01</td>
</tr>
<tr>
<td>Exercise + insulin</td>
<td>0.81±0.04</td>
<td>0.21±0.03</td>
<td>60.21±5.18</td>
<td>141.90±17.57***</td>
</tr>
</tbody>
</table>

Data are mean±S.D. ΔFFI, fura-2 fluorescence intensity change; TTPCa, time to peak Ca2+; T50Dca, time to 50% diastolic Ca2+. 6–8 myocytes per heart, n=6–8 hearts for each group; ††p<0.01 vs. vehicle in exercise.††p<0.01 vs. vehicle in control; §p<0.05, †p<0.01 between the two groups. 10−7 mol/L insulin. Dotted and solid traces indicate resting fura-2 fluorescence intensity (resting FFI) and T50Dca at 10−7 mol/L (6–8 myocytes per heart, n=6–7, p>0.05). PTA in response to electrical stimuli were 7.73±0.97% and 9.21±0.96% in cardiomyocytes from sedentary and exercise groups, respectively (p<0.05 between the two groups), indicating an enhanced basal myocyte contractile capacity following swimming exercise. Fig. 2A and B shows representative traces depicting the typical effects of insulin (10−7 mol/L) on cell shortening in cardiomyocytes from sedentary and exercise hearts. Following exposure to this concentration of insulin, PTA was increased by 47% in cardiomyocytes from exercise rats, but only by 31% in cardiomyocytes from sedentary rats (p<0.01 between the two groups). Insulin (10−8–10−6 mol/L) caused a concentration-dependent increase in PTA and maximal velocity of shortening (Fig. 2C and D). These results indicated that along with swim-improved myocardial insulin sensitization, cardiac contractile response to insulin was markedly potentiated.

In addition to the changes of cardiomyocytes contractile function, the time course of the fluorescence signal augment (time to peak Ca2+, TTPCa) and decay (time to 50% diastolic Ca2+, T50DCa) was evaluated to assess the rate of intracellular Ca2+ release and clearing, respectively. Consistent with its positive inotropic effect on cardiomyocytes, insulin significantly increased calcium transient amplitude (ΔFFI) and T50DCa at 10−7 mol/L (6–8 cells per heart, n=6–7, p<0.05 vs. vehicle). Following swim training, a change in intracellular calcium transient in response to insulin stimulation (as measured by ΔFFI and T50DCa) was markedly improved in cardiomyocytes from exercised rats compared with that from control rats (Table 3 and Fig. 3). Although insulin failed to shorten TTPCa in cardiomyocytes from control and exercised rats, cardiomyocytes from exercised rats exhibited shorter TTPCa compared with those from control rats. There was no difference in the resting fura-2 fluorescence intensity (resting FFI, representing diastolic intracellular Ca2+ level) between cardiomyocytes from control and exercised rats (Table 3).

3.5. Swim training promoted contractile response to insulin in isolated hearts

To further consolidate the exercise-elicited augmentation in insulin-induced positive contractile response, myocardial contractile response to insulin was further assessed in isolated perfused heart using the Langendorff heart perfusion system. As shown in Fig. 6A, B, C and D, swim training elevated left ventricular developed pressure (LVDP) (46±8 mm Hg in exercise, vs. 26±9 in control, n=6, p<0.05), and increased the maximal positive and negative values of the instantaneous first derivative of left ventricular pressure (LVP) (+dP/dt max) (p<0.05), indicating that swim training significantly sensitizes myocardial contractile response to insulin in whole hearts, similar to insulin-induced cardiomyocytes contractile response. These results favored the notion that swim training not only markedly improves insulin-stimulated glucose uptake in hearts, but also facilitates myocardial contractile response to insulin stimulation.

Fig. 3. Representative traces showing effects of swim training on insulin (10−7 mol/L)-induced response on the electrically stimulated increase of intracellular Ca2+ transient in cardiomyocytes from rats. Cardiomyocytes were loaded with 0.5 μmol/L of fura-2/AM for 30 min, then excited by light emitted by a 75 W lamp and passed through either a 360- or a 380-nm filter. The fura emission wavelength (510 nm) was synchronously monitored. Intracellular free Ca2+ was measured as the fluorescent ratio (360/380 nm). Panel A shows the representative traces of Ca2+ transient in cardiomyocytes from control rats by in vitro treatment with 10−7 mol/L insulin. Panel B indicates the representative traces of Ca2+ transient in cardiomyocytes from exercised rats by in vitro treatment with 10−7 mol/L insulin. Dotted and solid traces indicate the response of cardiomyocytes to 10−7 mol/L insulin, respectively.
Fig. 4. Effects of swim training on the expressions of IRS2, PI-3 kinase p85, Akt and eNOS, and the phosphorylation of insulin-stimulated Akt in myocardium. IRS2 (A), PI-3 kinase p85 (B), Akt (C), eNOS (D) and Akt phosphorylation (E) were determined using Western blots (50–120 μg protein per lane). Typical blots show IRS2, PI-3 kinase p85, Akt, eNOS expressions and Akt phosphorylation. The bar graphs below the blots show statistical results. Panel F shows the ratio of the p-Akt to Akt expression. Data are expressed as mean±S.D. (n=5 per group). I: insulin; p-Akt: phosphorylated Akt; LN: L-NAME, eNOS inhibitor; LY: LY294002, PI-3 kinase inhibitor; C: control; E: exercise. ⁎⁎p<0.01 vs. control. #p<0.05, ##p<0.01 vs. vehicle in control; §§p<0.01 vs. insulin alone in control; ††p<0.01 vs. vehicle in exercise; ‡‡p<0.01 vs. insulin alone in exercise.

Fig. 5. eNOS phosphorylation and NOx production in myocardium. A: eNOS phosphorylation. Typical blots show eNOS phosphorylation by Western blots from eight different groups, respectively. The bar graphs below the blots show statistical results. B: The ratio of p-eNOS to eNOS expression. C: NO production. Data are expressed as mean±S.D. (n=5 per group). I: insulin; p-eNOS: phosphorylated eNOS; NOx: NO production; C: control; E: exercise. #p<0.05, ##p<0.01 vs. vehicle in control; §p<0.05, §§p<0.01 vs. insulin alone in control; ††p<0.01 vs. vehicle in exercise; ‡‡p<0.01 vs. insulin alone in exercise.
3.6. Swim training upregulated the expression of IRS2, PI-3 kinase, Akt and eNOS

To determine the potential mechanisms of action behind the exercise-induced myocardial insulin sensitization, protein expression of key insulin signaling molecules including IRS-2, PI-3 kinase, Akt and eNOS were examined in myocardium from control and exercised rats. As depicted in Fig. 4, expressions of IRS-2, PI-3 kinase p85 subunit, Akt and eNOS were increased by 100%, 81%, 80% and 108%, respectively, following swim training \((p<0.01)\), indicating that swim training can markedly upregulate these signaling molecules pertinent to myocardial insulin sensitivity.

3.7. Swim training improved myocardial insulin sensitivity in an eNOS–NO-dependent manner

As illustrated in Fig. 5A, insulin \(10^{-7}\) mol/L triggered a 2.2- and 3.6-fold increase in eNOS phosphorylation in hearts from control and exercised rats, respectively \((p<0.01)\). Moreover, swim training significantly facilitated insulin-

Fig. 6. Effects of swim training on insulin-stimulated cardiac contractile functions, cardiac glucose uptake and GLUT4 translocation to PM in rats. Cardiomyocytes were pretreated with 20 \(\mu\)mol/L LY294002 or 5 mmol/L L-NAME for 15 min and then perfused with \(10^{-7}\) mol/L insulin alone, insulin plus 20 \(\mu\)mol/L LY294002 or insulin plus 5 mmol/L L-NAME. Cell shortening (A) of cardiomyocytes was measured. LVDP (B) and \(dP/dt\) max (C and D); glucose uptake (E) were measured using Langendorff system in isolated hearts, respectively. GLUT4 translocation to PM (F) was assessed using Western blot (50–120 \(\mu\)g protein per lane). Typical blots show GLUT4 on PM. The bar graphs below the blots show statistical results. Data are presented as mean±S.D. (more than 40 cardiomyocytes from 6 hearts for each group for cell shortening; \(n=6–8\) per group for LVDP and \(dP/dt\) max and GLUT4 translocation to PM, \(n=6–8\) per group for glucose uptake). GLUT4: Glucose uptake transporter 4; PM: plasma membrane; L-NAME, eNOS inhibitor; LY294002, PI-3 kinase inhibitor. \#p<0.05, \#\#p<0.01 vs. vehicle in control; \$p<0.05, \$\$p<0.01 vs. insulin alone in control; ††p<0.01 vs. vehicle in exercise; ‡p<0.05, ‡‡p<0.01 vs. insulin alone in exercise.
induced increase in the ratio of eNOS phosphorylation to eNOS expression (p < 0.05) (Fig. 5B). Basal NOx levels were significantly enhanced in hearts from exercised rats compared to those from the sedentary ones (p < 0.05). Insulin (10^{-7} \text{ mol/L}) elicited additional NOx production in both groups, and the increase in NO production by insulin in the exercised hearts was significantly higher than in sedentary hearts (p < 0.01) (Fig. 5C). Treatment with the NOS inhibitor L-NAME (0.5 mmol/L) exhibited no effect on eNOS phosphorylation although it significantly attenuated NOx production in response to insulin in exercised hearts (p < 0.01) (Fig. 5C). Foremost, L-NAME treatment abolished exercise-augmented insulin-stimulated cardiac contractile response, glucose uptake and GLUT4 translocation in exercised hearts or cardiomyocytes (Fig. 6). These results demonstrated that insulin-stimulated eNOS phosphorylation with a subsequent increase in NOx content plays a key role for exercise-induced myocardial insulin sensitization.

### 3.8. Swim training-induced activation of eNOS–NO via a PI3-kinase–Akt-dependent mechanism

Given that the facilitated eNOS–NO cascade may play an essential role in exercise-elicited myocardial insulin sensitization, we further examined the mechanisms through which cardiac eNOS–NO system was regulated following swim training. As shown in Figs. 4 and 5, swim training upregulated markedly the level of total Akt and eNOS as well as insulin-stimulated Akt and eNOS phosphorylation, and significantly increased the ratio of Akt phosphorylation to Akt expression (p < 0.05). Incubation of the PI-3 kinase inhibitor LY294002 (20 \mu\text{mol/L}) ablated insulin-stimulated phosphorylation of Akt and eNOS, as well as NOx production in exercised rats. These data are consistent with the findings that both LY294002 and L-NAME abolished the exercise-augmented insulin-stimulated cardiac contractile response, glucose uptake and GLUT4 translocation in exercised rat hearts or cardiomyocytes (Fig. 6). These results suggested that swim training-associated myocardial insulin sensitization is likely mediated through a PI-3 kinase–Akt-dependent eNOS–NO-dependent mechanism. In addition, neither LY294002 (20 \mu\text{mol/L}) nor L-NAME (0.5 mmol/L) alone exerted any overt effect on cardiac contractile function, glucose uptake and GLUT4 translocation (data not shown).

### 4. Discussion

The major findings from our present study are as follows. First, we demonstrated for the first time that swim training significantly potentiates insulin-induced positive inotropic effects on cardiac contractile function, myocardial insulin sensitivity manifested by insulin-stimulated glucose uptake and GLUT4 translocation; secondly, our data provided direct evidence that the eNOS–NO cascade plays a critical role in the exercise-induced myocardial insulin sensitization. To the best of our knowledge, this is the first piece of evidence demonstrating a critical role of eNOS–NO signaling in myocardial insulin sensitization following swim training; thirdly, this study revealed Akt as a key upstream activator of the eNOS–NO cascade in swim training-induced myocardial insulin sensitization.

Although exercise has been shown to markedly upregulate GLUT4 expression in the skeletal muscle [6], GLUT4 expression was found to be comparable between sedentary and exercised hearts in our study. No precise mechanism may be offered for this seemingly tissue-specific effect in GLUT4 expression following swim training. Our results indicated that insulin markedly increased GLUT4 translocation to heart PM, and such increase was further facilitated in isolated hearts following swim training. In addition, our results also showed that insulin alone does not affect GLUT4 expression. Therefore, our data favored a critical role of GLUT4 translocation to heart PM, but not a change of GLUT4 expression by either swim training or insulin, towards swim training-elicited myocardial insulin sensitization as evidenced by insulin-stimulated glucose uptake.

Insulin-stimulated glucose transport in insulin-targeted organs is closely related with insulin receptor (IR), IRSs, PI-3 kinase and Akt in skeletal muscle and liver [1]. Our present study demonstrated that swim training upregulated IRS-2, PI-3 kinase and Akt expression as well as insulin-stimulated Akt phosphorylation and the ratio of Akt phosphorylation to Akt expression in myocardium. Several rationales may be speculated for exercise-induced upregulation or activation of insulin signaling molecules. For example, exercise is capable of activating mitogen-activated protein kinase (MAPK) [17], which may in turn contribute to the exercise-induced upregulation of insulin signaling molecules. It has also been demonstrated that the exercise-induced insulin sensitization at the level of Akt may be relevant to its effects on cytokine profiles [18,19]. The upregulation in protein expression was paralleled by changes in myocardial function. However, molecule downstream of IRS-2–PI-3 kinase–Akt also sparsely identified for myocardial insulin sensitization. To date, more than 30 proteins have been identified as the direct Akt targets or substrates [20]. However, there has been little direct evidence indicating the utmost specific signaling molecule(s) responsible for exercise-induced myocardial insulin sensitization. Recent experimental results from our lab as well as others depicted a close association among insulin signaling, NOS and insulin-stimulated eNOS activation, which is significantly attenuated in heart tissues of type 2 diabetics [7,21,22]. However, no direct association between Akt activation and NO production has been unveiled in the exercise-induced myocardial insulin sensitization. Data from our present study demonstrated that in vitro administration of insulin resulted in eNOS phosphorylation and subsequent NO production via a PI-3 kinase–Akt-dependent mechanism. These data have provided a permissive role of eNOS phosphorylation and NO production in the exercise-induced myocardial insulin sensitization through Akt phosphorylation. In addition, our data indicated that following swim training, the activation of insulin.
on eNOS was significantly improved as evidenced by the ratio of eNOS phosphorylation to eNOS expression. In addition to Akt-induced eNOS phosphorylation, exercise-induced MAPK activation and upregulation of heat shock protein (HSP) 70 may also contribute to upregulated eNOS expression [17]. A combination of HSP70 and HSP90 has been shown to potently induce eNOS expression [23]. In addition, HSP90 can rapidly bind to the eNOS molecule in response to stimulation, thereby enhancing its catalytic activity and subsequently eNOS-derived NO production [23]. Although exercise has been demonstrated to upregulate HSP90 expression [24], further study is warranted to elucidate the contribution of HSP90 to the exercise-induced sensitization in eNOS in response to insulin stimulation.

It is possible that exercise-facilitated NO-regulated Ca$^{2+}$ transient may play a significant role in NO-elicited myocardial insulin sensitization. Several putative mechanisms have been postulated to explain the exercise-facilitated effect of NO on Ca$^{2+}$ transient. Sarcoplasmal Ca$^{2+}$ influx in excited myocardium activates sarcoplasmic reticulum (SR) Ca$^{2+}$ release and NOS. The subsequent increase in NO may poly-S-nitrosylate myocardial Ca$^{2+}$ release channel (ryanodine receptor), leading to a concerted activation of SR Ca$^{2+}$ channel. This effect may override the NO-initiated cGMP-induced inhibition of Ca$^{2+}$ channel [25,26]. In addition, NO has been shown to participate in insulin-stimulated glucose uptake in skeletal muscle, liver and adipose tissue [10–12]. These mechanisms are likely to contribute to insulin-induced positive inotropic effect and glucose uptake in exercised heart.

Apart from Akt-mediated activation of eNOS cascade, exercise may directly promote eNOS expression in myocardium, thus providing a possible scenario of an Akt-independent insulin sensitization avenue via the endogenous eNOS–NO system. However, our result using LY294002 did not favor this Akt-independent mechanism. Data from the Langendorff perfused hearts system revealed a causal relationship between exercise-associated insulin sensitization and insulin-induced positive inotropic response. Interestingly, both effects were blunted by inhibition of NOS with L-NAME, depicting a role of NOS–NO-dependent mechanism in insulin sensitization and improved myocardial contractile function. This notion of insulin sensitization-facilitated myocardial contractile function following swim training is further substantiated by our cardiomyocyte study where L-NAME significantly attenuated exercise-induced insulin sensitization. These data recapitulated that endogenous eNOS–NO-mediated myocardial insulin sensitization is responsible, at least in part, for the improved myocardial contractile capacity following swim training.

In summary, we have demonstrated that swim training improves insulin’s positive inotropic effect and insulin sensitivity in the hearts. The exercise-improved PI-3 kinase–Akt-dependent activation of insulin on eNOS and subsequently NO production may be responsible for exercise-induced myocardial insulin sensitization. However, despite of the improved insulin sensitivity following swim training, our data failed to identify any effect of swim training and insulin on GLUT4 expression. Collectively, these findings should shed some light on a better understanding of insulin-sensitizing effects following physical activity, which may help to identify new therapeutic regimen for insulin-resistance-related cardiovascular diseases.

4.1. Limitations

In the present study, we did not observe the effect of a single bout of swimming on myocardial insulin sensitivity. It is therefore unclear whether a single bout of exercise may influence insulin signaling cascade and myocardial insulin sensitivity. It was reported that a single bout of intermittent exercise ameliorated whole glucose tolerance for a short duration (<48 h), while insulin signaling cascade had no change in skeletal muscle [27]. Thus the time course of exercise-enhanced myocardial insulin sensitivity remains to be elucidated. In addition, it has been reported that the addition of fatty acid, i.e., 1.2 mM palmitate, to the isolated heart perfusate had no effect on cardiac function in normal rat heart [28,29]. However, fatty acid has been indicated to negatively affect cardiac function under pathologic conditions, such as diabetes [29], hypothermia in isolated perfused heart [30]. Thus, the effect of such high concentration of palmitate on insulin-stimulated inotropic effect and glucose uptake cannot be excluded in the present study.

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References


