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Original Article

Allicin prevents rat cardiomyocytes from oxidative injury and apoptosis during repeated exhaustive exercise via Akt and sirtuin 1 pathways

Yongyan Fan[1], Ran Zhang[2], Yang Li[2], Caiyi Lu[2], Feng Xu[3], Chao Zhu[2], Yutang Wang[1,4], Qiao Xue[2]

[1]College of Medicine, Nankai University, Tianjin, China; Departments of [2]Cardiology, [4]Geriatric Cardiology, Chinese PLA General Hospital, Beijing, China; [3]Department of Bone and Joint, Shijingshan Teaching Hospital of Capital Medical University, Beijing Shijingshan Hospital, Beijing, China

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Abstract: Repeated exhaustive exercise could cause obvious oxidative stress-related injury in cardiomyocytes. This study was designed to investigate effects of allicin on rat myocardial oxidative injury during repeated exhaustive exercise, and its molecular mechanisms. Forty Sprague-Dawley rats (6-7 wk old) were randomly divided into five groups: control (Control group); rats undergoing seven consecutive daily sessions of exhaustive swimming (SW group); and three groups of SW rats also receiving allicin at 4.2, 6.3 or 12.6 mg/kg for 7 d (SW+Allicin group). The effects of repeated exhaustive exercise on structural, functional and molecular biological changes in heart have been examined. Haematoxylin-eosin staining showed that treatment with allicin relieved myocardial cell swelling and inflammatory cell infiltration induced by repeated exhaustive exercise. Myocardial superoxide dismutase (SOD) activity was significantly reduced in the SW group compared with the Control group (P<0.01), while the levels of cardiac troponin T (cTnT), heart fatty acid binding protein (H-FABP), cortisol (COR), high sensitivity C-reactive protein (hs-CRP), malondialdehyde (MDA), and the apoptotic index were significantly higher in the SW group than in the Control group (P<0.01). In addition, the protein levels of phosphorylated (p-) Akt, p-FOXO3a, Bcl-xL and nuclear sirtuin 1 (SIRT1) were significantly lower in the SW than in the Control group (P<0.01). All these effects were reversed by allicin (P<0.01). Allicin treatment significantly increased the activity of SOD, and inhibited the activities of cTnT, H-FABP, COR, hs-CRP and MDA, especially in the SW+Allicin (6.3 or 12.6 mg/kg) groups. Moreover, allicin treatment (4.2, 6.3 or 12.6 mg/kg) significantly increased the protein levels of p-Akt, p-FOXO3a, SIRT1, Bcl-xL compared with those in the SW group, while the protein levels of FOXO3a, Bax, cytochrome c and cleaved caspase-3 were changed in the opposite direction. These data suggested that allicin protected rat cardiomyocytes from oxidative stress-induced injury and apoptosis during repeated exhaustive exercise by enhancing FOXO3a phosphorylation via Akt and SIRT1 activation.

Keywords: Allicin, apoptosis, Akt, FOXO3a, sirtuin 1, repeated exhaustive exercise

Introduction

The role of exercise in prevention, management and treatment of cardiovascular diseases has been well described [1-3]. Although regular exercise training can decrease risk of cardiovascular disease, repeated exhaustive exercise, a strong source of stress, caused oxidative stress-related injury in cardiomyocytes [4, 5]. Oxidative stress is promoted by excessive production of reactive oxygen species (ROS), which are chemically modified proteins, nucleic acids and even intracellular membranes. Such effects induce inflammation, cellular damage and cell death under a variety of pathological conditions and are associated with mitochondrial dysfunction and apoptosis [6, 7]. Furthermore, oxidative stress induced by repeated exhaustive exercise is implicated in impairment of myocardial structure and function [8, 9]. Therefore, understanding the intracellular mechanisms regulating ROS formation and apoptosis is important to develop methods to protect cardiomyocytes from exercise-induced myocardial oxidative stress. However, the mechanisms of cardiomyocyte dysfunction induced by

repeated exhaustive exercise are still unclear, with no effective protective measures identified [10, 11].

The FOX transcription factors, belonging to the other (O) subfamily (FOXO) include four members (FOXO1, FOXO3a, FOXO4 and FOXO6) in mammals [12]. FOXOs are important for diverse cellular functions, through their transcriptional regulation of downstream target genes [13]. Subcellular localization and transcriptional functions of FOXOs are often regulated by post-translational modifications, including phosphorylation, acetylation and ubiquitination [14]. Among the FOXOs family, FOXO3a can mediate stress responses by acting as a key sensor for cellular stresses, which is crucial for regulating cell survival, oxidative stress resistance and apoptosis [15, 16]. Akt is well known as an upstream negative mediator of its kinase substrate, FOXO3a. FOXO3a phosphorylation enables its export into the cytoplasm, where it binds to 14-3-3 protein and is eventually degraded in the proteasome [17]. The transcriptional activity of FOXO3a can be modulated not only by Akt, but also by nuclear longevity protein sirtuin 1 (SIRT1) [18]. In the nucleus, SIRT1 can interact with FOXO3a and deacetylate it, subsequently inhibiting its transcriptional activity [19]. It has been reported that FOXO3a activity strengthens the antioxidant defence systems and increases the longevity in several experimental organisms [20]. Previous research also shows that FOXO3a promotes cardiomyocyte's survival under oxidative stress conditions through the induction of antioxidant and cell survival pathways [21]. However, little is known about the effects of FOXO3a on ROS formation and apoptosis in cardiomyocytes subjected to conditions of repeated exhaustive exercise. Therefore, our study was designed to investigate the mechanisms and potential roles of FOXO3a during rat myocardial oxidative stress-induced injury caused by repeated exhaustive exercise.

Allicin, the main biologically active compound in garlic, has been extensively studied for its numerous cardiovascular benefits, including antioxidant effects, antiarrhythmic activity, cardiac hypertrophy prevention, reduction of serum lipid levels and inhibition of platelet aggregation [22]. A previous study reports that garlic extracts effectively prevents norepinephrine-induced cardiomyocyte hypertrophy and apoptosis and, thus, have cardioprotective properties. This suggests that suppression of myocardial oxidative stress is important for the anti-hypertrophic and anti-apoptotic properties of garlic extracts [23]. Moreover, allicin improves cardiac function by protecting against apoptosis in a rat myocardial infarction model, and these protective effects are partially dependent on activation of Bcl-2 and the resulting anti-apoptotic activity [24]. Altogether, allicin appears to be a highly safe antioxidant and anti-inflammatory preparation, with potential value for preventing oxidative stress and apoptosis induced by repeated exhaustive exercise. However, whether allicin is indeed effective and, if so, the molecular signalling mechanisms involved are still unclear. Therefore, in this study, we aimed to investigate whether allicin administration would confer cardioprotection against myocardial oxidative stress-induced injury during repeated exhaustive exercise, as well as its potential mechanisms.

Material and methods

Animal preparation

All animals used in this study received humane care in compliance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences and approved by the Chinese guidelines for experimental animals. The protocols and care of experimental rats were supervised and approved by the Animal Ethical Committee of Chinese People's Liberation Army General Hospital. Rats were maintained in the animal facility under controlled temperature (22±2°C) and a 12 h light/dark cycle. Food and water were provided ad libitum throughout the 7 d experimental period.

Experimental design

Forty adult male Sprague-Dawley rats (180-200 g), 6-7 wk of age, were from the animal centre of Chinese People's Liberation Army General Hospital. Rats were randomly divided into five groups (n = 8 per group), as follows: control (Control group); rats undergoing seven consecutive daily sessions of exhaustive swimming (SW group); and three groups of SW rats also receiving allicin at 4.2, 6.3 or 12.6 mg/kg for 7 d (SW+Allicin group). Allicin (15 mg/ml) (Ryen Pharma Co., Ltd., Xuzhou, China)

in distilled water was administered daily by intraperitoneal injection (IP). The allicin doses were comparable to human doses used for anti-infective therapy, pharmacologically scaled for rats. Rats in the Control and SW groups received equal volumes of vehicle (distilled water) IP.

Swimming sessions were performed with five rats per plastic barrel. The barrel was 70 cm high and 50 cm at its maximum diameter, filled with water to a depth of 55 cm. Water temperature was maintained between 31 and 35°C during swimming. A weight (2% of each rat's body weight) was attached to one foreleg during swimming, such that rats more rapidly reached the criteria for exhaustion. On the day before beginning formal exercise sessions, rats in the exercising groups each performed a 10 min warm-up swimming session to become acclimated to swimming. Exhaustive criteria were defined as spending more than 10 s below the surface and lacking a "righting reflex" when placed on a flat surface [8].

Sample preparation

After exhaustive exercise, the rats in exercise groups were anesthetized with pentobarbital sodium (40 mg/kg, IP) and killed immediately. The control rats were sacrificed in a similar manner. Blood samples were collected from the abdominal aorta and hearts were excised and divided into several portions. Blood samples were centrifuged at 3,000 g at 4°C for 15 min and supernatants collected for measuring serum parameters. To prepare myocardial homogenates for further biochemical analysis, cardiac tissues were collected, weighed and homogenized in phosphate-buffered saline (PBS, pH 7.4; 1 g tissue per 9 mL PBS) using a tissue homogenizer (Ultra-Turrax® T25 basic, IKA®-Werke, Staufen, Germany). After centrifugation at 15,000 g for 10 min at 4°C, the supernatants were collected and stored at -80°C until analysis. One portion of the left ventricular myocardium from each animal was fixed in 10% phosphate-buffered formalin and embedded in paraffin for morphologic and immunohistochemical analyses, by light microscopy. The remainder of each myocardial sample was snap-frozen in liquid nitrogen and stored at -80°C for subsequent RNA and protein extraction.

Serum parameter measurements

Serum cortisol (COR) levels were determined using the COR assay kit for the ADVIA Centaur CP immunoassay system (Siemens Medical Solutions Diagnostics, Shanghai, China). Serumcardiac troponin T (cTnT) was determined using the Roche cTnT electrochemiluminescence immunoassay on acobas® 8000 analyser (Roche Diagnostics, Indianapolis, IN, USA). Serum high sensitivity C-reactive protein (hs-CRP) and heart fatty acid binding protein (H-FABP) concentrations were determined with sandwich ELISA kits (eBioscience, San Diego, CA, USA and Hycult Biotech, Uden, Netherlands, respectively).

Analysis of oxidative stress in myocardial homogenates

Myocardial homogenates were used to estimate malondialdehyde (MDA) levels and superoxide dismutase (SOD) activities in tissues, using commercial assay kits (Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions. The method for MDA determination was based on its reaction with 2-thiobarbituric acid to form a pink complex with an absorption maximum at 532 nm [25]. The SOD assay was based on the ability of the sample to inhibit superoxide anion (O_2^-) dependent reactions, with O_2^- generated by xanthine oxidase [26].

Haematoxylin-eosin staining

The left ventricle of each rat heart was fixed in 10% phosphate-buffered formalin and embedded in paraffin. Paraffin blocks were sliced into 4 μm serial sections and stained with haematoxylin-eosin (H&E) solution. Photomicrographs were obtained under the Olympus BX53 microscope (Olympus, Tokyo, Japan).

TUNEL assay

The 4 μm paraffin sections were deparaffinised with a graded series of Histoclear and ethanol solutions. The TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling) assay was used to assess cardiomyocyte apoptosis, according to the instructions in the reagent kit (Roche Applied Science, Mannheim, Germany). TUNEL-positive cardiomyocytes were identified under double-blinded conditions and

Table 1. Sequences of the primers used for quantitative real-time PCR

Genes	Forward	Reverse
Akt	5'-CCTCAAGAAGGAGGTCATCG-3'	5'-GTCGTGGGTCTGGAATGAGT-3'
FOXO3a	5'-CCCTCTCGGACTCTCTCTCA-3'	5'-GTTCGCTACGGATGATGGAC-3'
Bax	5'-TTGCTACAGGGTTTCATCCAG-3'	5'-ATGTTGTTGTCCAGTTCATCG-3'
Bcl-xL	5'-TCATTTCCCATTCTGCTGTG-3'	5'-GGTCCTTGCCTCGTTATCC-3'
SIRT1	5'-AGGGAACCTCTGCCTCATCT-3'	5'-TGGCATACTCGCCACCTAAC-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'

TUNEL-positive cardiomyocyte nuclei were counted in high power fields (5 at 400× magnification per section), under the Olympus BX53 microscope. The proportion of apoptotic cell nuclei (Apoptotic Index, AI) was defined as apoptotic positive cell nuclei/total cell nuclei.

Western blotting for Akt, phosphorylated Akt, FOXO3a, phosphorylated FOXO3a, Bax, Bcl-xL and nuclear SIRT1

Briefly, proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), typically using 8% gels for Akt, phosphorylated (p-) Akt, FOXO3a, p-FOXO3a and SIRT1 and 10% gels for Bax and Bcl-xL. The primary antibodies for Akt (1:1000), p-FOXO3a (specific for the phosphorylation site at threonine-32, 1:1000), SIRT1 (1:1000) and Bcl-xL (1:1000) were from Cell Signaling Technology (Danvers, MA, USA). Those for p-Akt (specific for the phosphorylation site at serine-473, 1:5000), FOXO3a (1:1000) and Bax (1:1000) were from Abcam (Cambridge, MA, USA). The fractionated proteins were electrophoretically transferred to nitrocellulose membranes (Amersham, Piscataway, NJ, USA). The membranes were incubated with the appropriate primary antibodies for each target protein at 4°C overnight. The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Abcam), diluted 1:5000. Stained bands were visualized, after incubation with HRP-conjugated secondary antibody, with enhanced chemiluminescence (ECL) detection reagents (Amersham). The anti-GAPDH antibody (1:1000) and anti-histone H3 (1:1000) (Abcam) were used as controls to normalize for loading variabilities.

Quantitative real-time PCR assay for Akt, FOXO3a, Bax, Bcl-xL and SIRT1

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol, and cDNA was synthesized with a cDNA Synthesis Kit (Fermentas International Inc., Burlington, Ontario, Canada), also following the manufacturer's instructions. After reverse-transcription, cDNA samples were subjected to polymerase chain reaction (PCR) amplification. For real-time assays, PCR reactions were prepared in SYBR Green Supermix (Toyobo, Ltd., Tokyo, Japan). Each PCR was performed in triplicate in a final volume of 20 μL, containing 10 μL SYBR Green dye, 1 μL diluted cDNA products, 0.2 μM of each paired primer and 8.6 μL deionized water. The protocol was initial denaturation for 15 min at 95°C, followed by 40 cycles denaturation for 15 s at 95°C and extension for 30 s at 60°C. Specific primers were designed for each mRNA sequence of interest. Threshold cycle (Ct) values for Akt, Bax, Bcl-xL and SIRT1 were measured, values normalized to those for GAPDH, then each expressed as relative ratios. A $2^{-\Delta\Delta Ct}$ was used for analysing the data. Primer sequences are listed in **Table 1**.

Immunohistochemistry for cytochrome c and cleaved caspase-3

Specimens fixed in 10% phosphate-buffered formalin were embedded in paraffin and sectioned. For pretreatment, the 4 μm sections were deparaffinized with a graded series of Histoclear and ethanol solutions. After preincubation with 2% bovine serum albumin to block nonspecific antigens, the sections were incubated with a rabbit monoclonal antibody against rat cytochrome c (1:300, Abcam) or cleaved caspase-3 (1:2000, Cell Signaling Technology) at 4°C overnight in a moist chamber. Sections were washed and then incubated with a biotinylated secondary antibody (1:700, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBS at room temperature for 1 h. Following another wash with PBS, avidin-biotin peroxidase complex (1:500, Santa Cruz Biotechnology) in PBS was applied at room temperature for 30 min. Finally, the HRP was detected with 3,3'-diaminobenzidine (DAB) (Roche Applied Science) as the chromogenic substrate. The sections were washed, dehydrated in ethanol, cleared in xylene and mounted.

Table 2. Effects of allicin on levels of cTnT, H-FABP, COR, hs-CRP, MDA and SOD

Index	Control	SW	SW+Allicin (4.2 mg/kg)	SW+Allicin (6.3 mg/kg)	SW+Allicin (12.6 mg/kg)
cTnT (ng/ml)	0.003±0.001	0.061±0.008**	0.029±0.003$^{**,\#\#}$	0.016±0.004$^{**,\#\#,\Delta\Delta}$	0.019 ±0.003$^{**,\#\#,\Delta}$
H-FABP (ng/ml)	3.386±0.745	20.348±2.096**	13.980±1.371$^{**,\#\#}$	8.365±1.416$^{**,\#\#,\Delta\Delta}$	10.113±1.487$^{**,\#\#,\Delta}$
COR (μg/dl)	0.880±0.074	1.693±0.118**	1.363±0.071$^{**,\#\#}$	1.173±0.056$^{**,\#\#,\Delta}$	1.250±0.042$^{**,\#\#}$
hs-CRP (mg/dl)	16.113±0.267	19.400±0.416**	18.405±0.141$^{**,\#}$	17.590±0.436$^{**,\#\#,\Delta}$	17.853±0.458$^{**,\#\#}$
MDA (nmol/mg)	1.161±0.186	8.798±0.647**	6.250±0.387$^{**,\#\#}$	4.550±0.557$^{**,\#\#,\Delta\Delta}$	5.875±0.695$^{**,\#\#,\blacktriangle}$
SOD (U/mg)	27.375±2.635	13.125±1.228**	16.975±0.650$^{**,\#}$	20.870±0.987$^{**,\#\#,\Delta}$	19.110±0.961$^{**,\#\#}$

Values are means ± SEM (n=8/group); $^{*}P$<0.05 and $^{**}P$<0.01, *vs.* Control; $^{\#}P$<0.05 and $^{\#\#}P$<0.01, *vs.* SW; $^{\Delta}P$<0.05 and $^{\Delta\Delta}P$<0.01, *vs.* SW+Allicin (4.2 mg/kg); $^{\blacktriangle}P$<0.05, *vs.* SW+Allicin (6.3 mg/kg); SW, rats undergoing seven consecutive daily sessions of exhaustive swimming; SW+Allicin, three groups of SW rats receiving allicin at 4.2, 6.3 or 12.6 mg/kg for 7 d; cTnT, cardiac troponin T; H-FABP, heart fatty acid binding protein; COR, cortisol; hs-CRP, high sensitivity C-reactive protein; MDA, malondialdehyde; SOD, superoxide dismutase.

Statistical analysis

Statistical evaluation was performed with SPSS 19.0 (Statistical Packages for Social Sciences; SPSS Inc., Chicago, Illinois, USA). Results are expressed as means ± SEM. Prism 5.0 (GraphPad, San Diego, CA, USA) was used for statistical analyses and to generate figures. Statistical significance was assessed using one-way ANOVA, with post hoc analysis by Tukey's test for multiple comparisons. A value of P<0.05 was considered statistically significant.

Results

Effects of allicin on myocardial oxidative injury induced by repeated exhaustive exercise

As shown in **Table 2**, myocardial SOD activity was significantly reduced in the SW group than in the Control group (13.125±1.228 *vs.* 27.375±2.635, P<0.01). In contrast, the Control group myocardial MDA levels were significantly higher in the SW group than in the Control group (8.798±0.323 *vs.* 1.161±0.093, P<0.01). Moreover, cTnT and H-FABP levels were clearly higher in the SW group than those in the Control group (P<0.01). The same was true of COR and hs-CRP levels, confirming the reliability of repeated exhaustive exercise as an animal model for myocardial injury. Furthermore, allicin treatment (4.2, 6.3 or 12.6 mg/kg) significantly reversed these indexes of injury (P< 0.05), especially in the SW+Allicin (6.3 or 12.6 mg/kg) groups. This indicated that allicin inhibited inflammation and protected the rats from myocardial oxidative injury.

H&E staining was performed to observe changes, by light microscopy, in myocardial histomorphology (**Figure 1**). This analysis showed swollen myocardial fibres, disappearance of horizontal stripes, granular degeneration, fatty degeneration and even cardiomyocyte necrosis in rats from the SW group. In these animals, there were also inflammatory cell infiltration and strong acidophilic staining compared with the Control group. However, these myocardial histomorphological changes were attenuated in the groups treated with allicin (4.2, 6.3 or 12.6 mg/kg), especially at doses of 6.3 and 12.6 mg/kg.

Effects of allicin on FOXO3a activation after repeated exhaustive exercise

To explore the molecular mechanisms regulating FOXO3a activation in this experimental system, we examined the activation of Akt pathway. As shown in **Figure 2A** and **2B**, representative Western blots and quantitative analyses showed no differences in total Akt protein levels among groups (P>0.05). There were also no significant differences in Akt mRNA levels (P>0.05). To investigate whether allicin affected Akt activation, p-Akt protein levels were determined by Western blotting. Compared with the Control group, the p-Akt/Akt ratio was dramatically reduced in the SW group (P<0.01). In contrast, the ratio of p-Akt/Akt in SW rats treated with allicin at 4.2 (0.76±0.03), 6.3 (0.96±0.04) or 12.6 (0.75±0.02) mg/kg was significantly higher than those in the SW group (0.39±0.03).

Furthermore, to assess whether allicin affected FOXO3a activation, protein levels of FOXO-

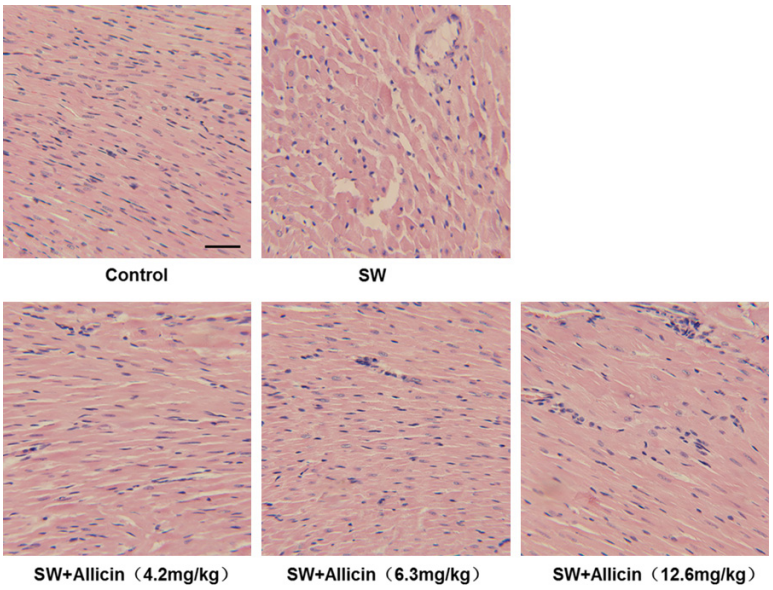

Figure 1. Effects of allicin on myocardial histomorphology, evaluated by H&E staining (400×). SW, rats undergoing seven consecutive daily sessions of exhaustive swimming; SW+Allicin, three groups of SW rats receiving allicin at 4.2, 6.3 or 12.6 mg/kg for 7 d. Bar = 100 μm.

3a and p-FOXO3a were determined by Western blotting and their mRNA levels by qRT-PCR. As shown in **Figure 2A** and **2C**, FOXO3a protein levels were higher in the SW group than in the Control group (P<0.01). Allicin treatment (4.2, 6.3 or 12.6 mg/kg) clearly decreased FOXO3a protein and mRNA levels compared with the SW group (P<0.01). In addition, there was a substantial decrease in the ratio of p-FOXO3a/FOXO3a in the SW group compared with the Control group (P<0.01). Allicin treatment (4.2, 6.3 or 12.6 mg/kg) significantly increased the p-FOXO3a/FOXO3a ratio, compared with the SW group (0.27±0.04 *vs*. 0.04±0.01, P<0.01; 0.93±0.05 *vs*. 0.04±0.01, P<0.01; 0.71±0.04 *vs*. 0.04±0.01, P<0.01, for the three allicin doses, respectively).

We measured SIRT1 expression in cardiomyocytes by Western blotting and qRT-PCR to assess whether allicin affected SIRT1 activation. When SIRT1 was activated, it should promote FOXO3a deacetylation and block its accumulation in the nucleus, thus attenuating transcriptional effects of FOXO3a [19]. As shown in **Figure 2A** and **2D**, SIRT1 expression was significantly decreased in the SW group compared with the Control group (P<0.01). However, allicin treatment (4.2, 6.3 or 12.6 mg/kg) significantly upregulated nuclear SIRT1, at both protein and mRNA levels, compared with the SW group. This was especially true with 6.3 and 12.6 mg/kg allicin.

Effects of allicin on cardiomyocyte apoptosis, induced by repeated exhaustive exercise

To confirm that allicin caused functional improvements in myocardial apoptosis induced by repeated exhaustive exercise, we used TUNEL staining to locate apoptotic nuclei (**Figure 3**). There was a significant increase in the number of apoptotic cell nuclei in the SW group compared with the Control group (36.85±1.85 *vs*. 12.53±1.04, P<0.01). In contrast, the AI values in allicin-treated groups (4.2, 6.3 or 12.6 mg/kg) were substantially lower than those in the SW group (27.04±1.46 *vs*. 36.85±1.85, P<0.01; 18.50±0.96 *vs*. 36.85±1.85, P<0.01; 20.60±2.26 *vs*. 36.85±1.85, P<0.01, for the three allicin doses, respectively). This indicated that apoptosis caused by repeated exhaustive exercise was attenuated, in a dose-dependent manner, by allicin.

To further elucidate the molecular mechanism underlying the protective anti-apoptotic effects of allicin, we used Western blotting and qRT-PCR to quantitatively determine protein and mRNA levels of members of the Bcl-2 protein family and to investigate whether the status of FOXO3a phosphorylation affected downstream signalling. As shown in **Figure 2A** and **2E**, repeated exhaustive exercise led to a significantly decreased expression of the anti-apoptotic protein Bcl-xL in the SW group compared with the Control group (P<0.01). In contrast, expression of the pro-apoptotic protein Bax was changed in the opposite direction. Allicin treatment (4.2, 6.3 or 12.6 mg/kg) clearly increased protein and mRNA levels of Bcl-xL compared with the SW group (P<0.01). In contrast, the protein and mRNA levels of Bax were lower in allicin-treated groups (4.2, 6.3 and 12.6 mg/kg) than those in the SW group (P<0.01).

We next examined whether allicin affected protein levels of cleaved caspase-3, an activation

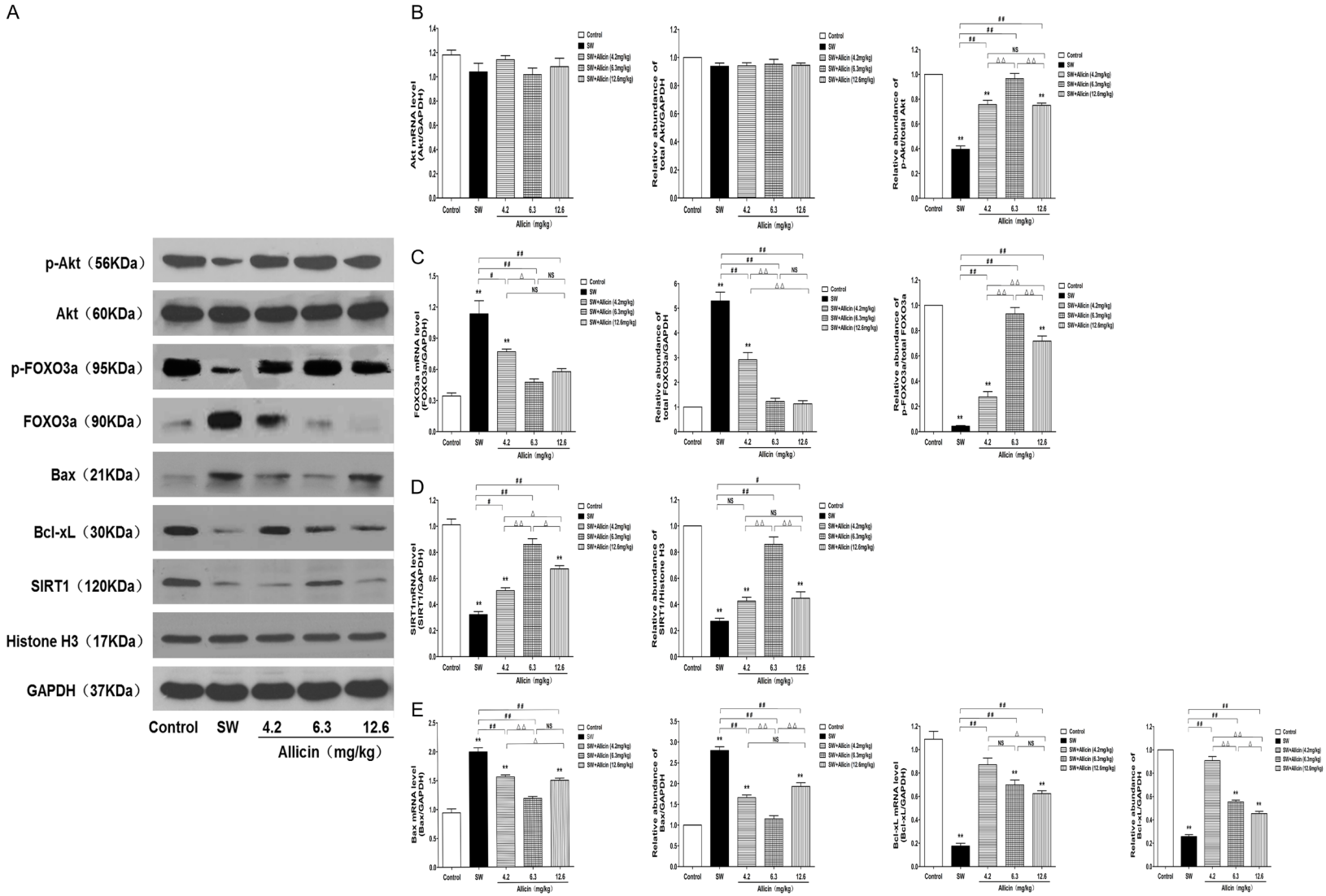

Figure 2. Effects of allicin on expression of p-Akt, Akt, p-FOXO3a, FOXO3a, nuclear SIRT1, Bax and Bcl-xL in cardiomyocytes. A: Western blots for p-Akt, Akt, p-FOXO3a, FOXO3a, Bax, Bcl-xL and SIRT1 in samples from Control, SW and SW+Allicin (4.2, 6.3 and 12.6 mg/kg) groups, respectively. B: Quantitative analysis of the mRNA of Akt, and the proteins of Akt and p-Akt. C: Quantitative analysis of the mRNA of FOXO3a, and the proteins of FOXO3a and p-FOXO3a. D: Quantitative analysis of the mRNA and protein of nuclear SIRT1. E: Quantitative analysis of the mRNA and proteins of Bax and Bcl-xL. Values are means ± SEM (n = 8/group); NS, not significant; $^{*}P<0.05$ and $^{**}P<0.01$, *vs.* Control; $^{\#}P<0.05$ and $^{\#\#}P<0.01$, *vs.* SW; $^{\Delta}P<0.05$ and $^{\Delta\Delta}P<0.01$, between two groups, as indicated, with different allicin treatment doses; SW, rats undergoing seven consecutive daily sessions of exhaustive swimming; SW+Allicin, three groups of SW rats receiving allicin at 4.2, 6.3 or 12.6 mg/kg for 7 d.

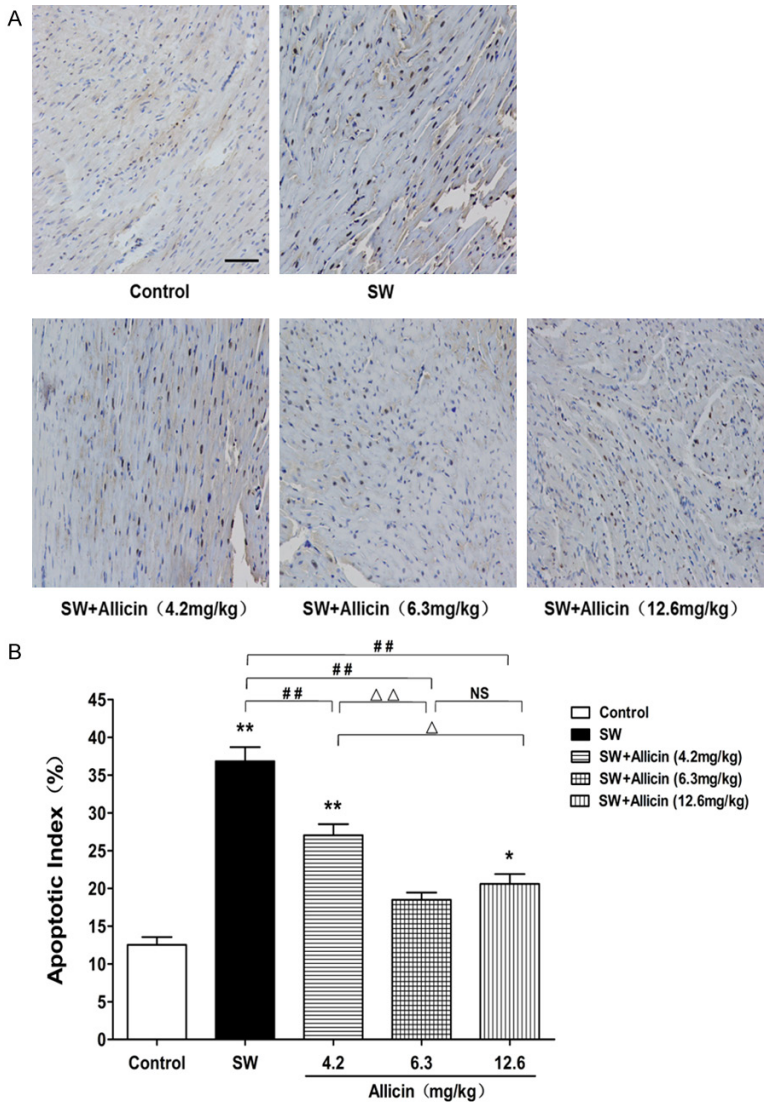

Figure 3. Effects of allicin on cardiomyocyte apoptosis, evaluated by TUNEL assay (200×). A: Representative images showing TUNEL staining in samples from Control, SW and SW+Allicin (4.2, 6.3 and 12.6 mg/kg) groups. B: Effect of allicin on the apoptotic index. Values are means ± SEM (n = 8/group); NS, not significant; $^{*}P<0.05$ and $^{**}P<0.01$, *vs.* Control; $^{\#\#}P<0.01$, *vs.* SW; $^{\Delta}P<0.05$ and $^{\Delta\Delta}P<0.01$, between two groups, as indicated, with different allicin treatment doses; SW, rats undergoing seven consecutive daily sessions of exhaustive swimming; SW+Allicin, three groups of SW rats receiving allicin at 4.2, 6.3 or 12.6 mg/kg for 7 d. Bar = 50 μm.

step very critical for execution of apoptosis, mediated by cytochrome c release from the mitochondria [27]. Therefore, to assess the mechanism underlying the anti-apoptotic action of allicin, immunohistochemical staining was performed for cytochrome c and cleaved caspase-3 proteins. Changes in cytochrome c and cleaved caspase-3 protein levels are shown in **Figure 4A** and **4B**. Compared with the Control group, cytochrome c and cleaved caspase-3 protein levels were dramatically increased in the SW group ($P<0.01$). However, allicin treatment (4.2, 6.3 or 12.6 mg/kg) clearly decreased levels of both proteins, compared with the SW group ($P<0.01$). Thus, allicin partially reversed the effects of repeated exhaustive exercise, leading to inhibition of both cytochrome c release and caspase-3 activation.

Discussion

Ours is the first report, to the best of our knowledge, of two important findings. First, repeated exhaustive exercise caused myocardial oxidative injury in the rat, subsequently inducing inflammatory responses, impaired cellular function and cardiomyocyte apoptosis, via the Akt/FOXO3a/SIRT1 mediated signalling pathway. Second, allicin protected cardiomyocytes in this model from both oxidative injury and apoptosis induced by repeated exhaustive exercise, through enhancing FOXO3a phosphorylation by activating both Akt and SIRT1 pathways (**Figure 5**).

SOD and MDA are important oxidative stress indicators. Based on our findings, repeated exhaustive exercise increased ROS levels and lipid peroxidation. cTnT and H-FABP are highly sensitive and specific serum markers for myocardial damage [28, 29]. Shave *et al*. suggested that exercise-induced increases in myocardial sarcolemmal permeability and cell necrosis facilitated release of cytosolic cTnT and H-FABP [30]. In our study, we found that cTnT and H-FABP levels were clearly elevated over Control values in the SW group, indicating that repeated exhaustive exercise led to myocardial microdamage. COR is the main component of the glucocorticoid and has been termed"the stress

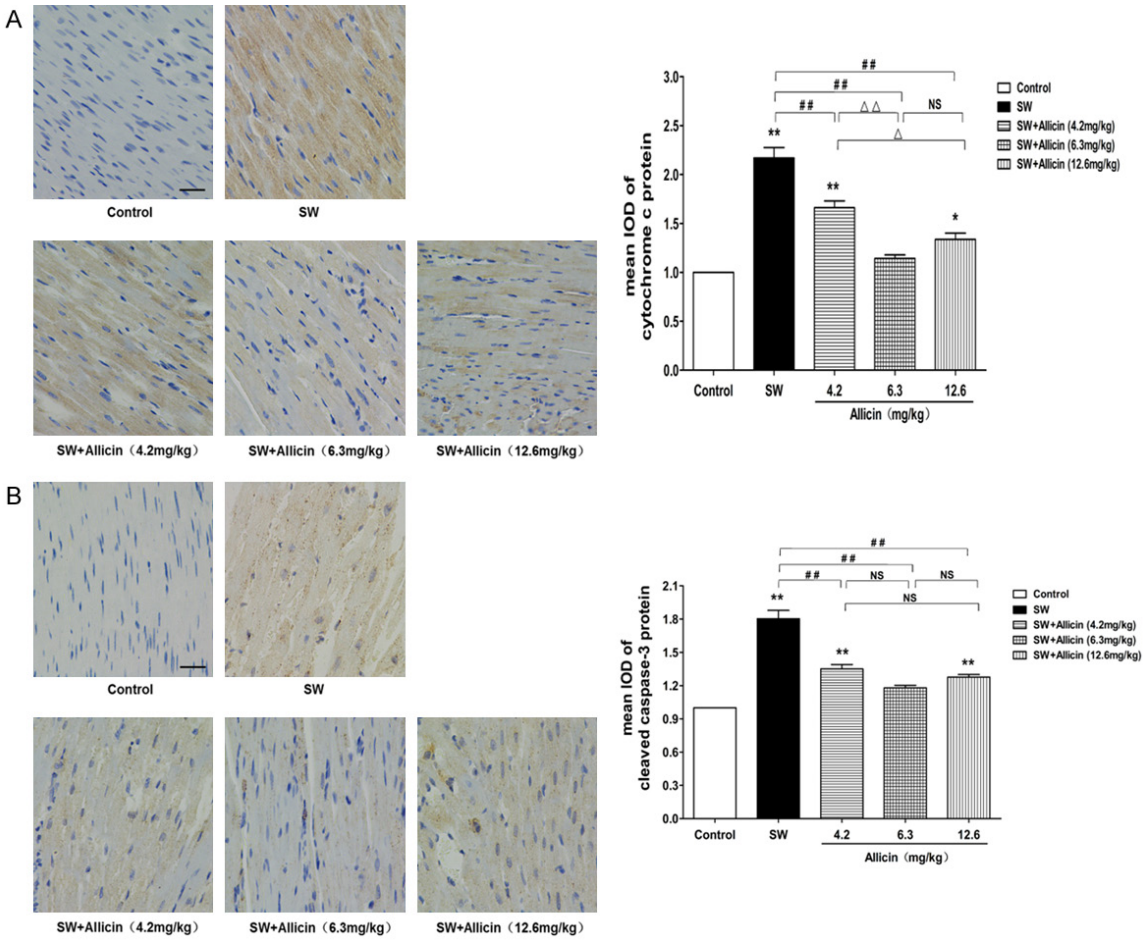

Figure 4. Effect of allicin on cytochrome c and cleaved caspase-3 levels (400×). A: Representative photomicrographs of immunohistochemistry and mean integrated optical density (IOD) values for cytochrome c. B: Representative photomicrographs of immunohistochemistry and mean IOD values for cleaved caspase-3. Values are means ± SEM (n = 8/group); NS, not significant; $^{*}P<0.05$ and $^{**}P<0.01$, *vs.* Control; $^{\#\#}P<0.01$, *vs.* SW; $^{\Delta}P<0.05$ and $^{\Delta\Delta}P<0.01$, between two groups, as indicated, with different allicin treatment doses; SW, rats undergoing seven consecutive daily sessions of exhaustive swimming; SW+Allicin, three groups of SW rats receiving allicin at 4.2, 6.3 or 12.6 mg/kg for 7 d. Bar = 100 μm.

hormone", which is secreted by the adrenal glands during the activity of hypothalamus-pituitary-adrenal cortical axis [31]. hs-CRP is a hepatically derived pentraxin that plays a key role in the acute phase immune response and has been demonstrated to be a strong predictor of future cardiovascular events, as well as the most common inflammatory marker [32]. Elevation of COR and hs-CRP levels in serum further confirmed that our animal model of repeated exhaustive exercise was reliable and successful, consistent with previous reports [33, 34]. However, treatment with allicin, especially at 6.3 or 12.6 mg/kg, significantly enhanced SOD activity and decreased levels of cTnT, H-FABP, COR, hs-CRP and MDA, indicating attenuation of both lipid peroxidation and myocardial oxidative injury induced by repeated exhaustive exercise.

The results of H&E and TUNEL staining also showed that repeated exhaustive exercise led to myocardial injury, indicated by structural changes in the myocardial tissue and acceleration of cardiomyocyte apoptosis. Allicin treatment significantly decreased the extent of cardiomyocyte apoptosis and improved myocardial histomorphology and function.

Akt is considered a vital regulatory factor for signal transduction [35]. Akt activation not only decreases cardiomyocyte apoptosis in an *in vivo* model, but also improves overall myocardial function [36]. Akt is well known as an upstream negative mediator of FOXO3a, promot-

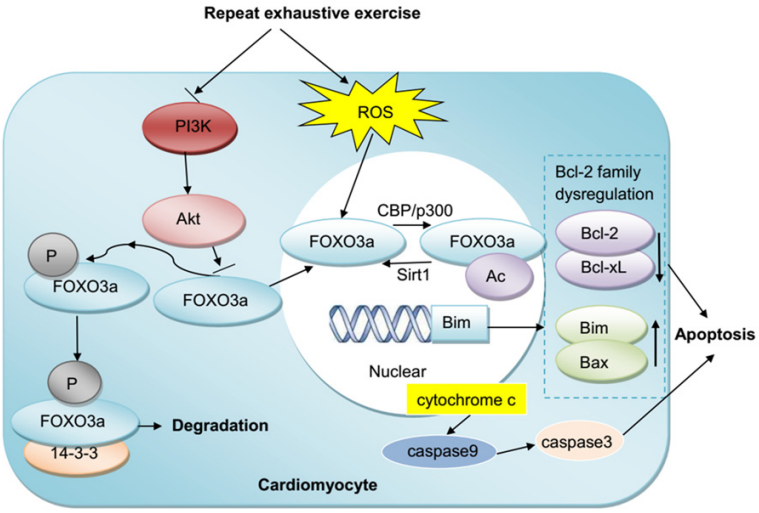

Figure 5. Mechanism of allicin-mediated cardioprotection against myocardial oxidative injury and apoptosis, induced by repeated exhaustive exercise. FOXO3a is a downstream protein phosphorylated through activation of the Akt signaling pathway and dysfunction of SIRT1 deacetylase. Repeated exhaustive exercise inhibits Akt and SIRT1 activation, resulting in decreased phosphorylation and degradation of FOXO3a. Dephosphorylated FOXO3a translocates to the nucleus and promotes its own transcription, resulting in Bcl-2 protein family dysregulation. The increased Bax/Bcl-xL ratio induces cytochrome c release from the mitochondria and caspase-3 activation, which trigger cardiomyocyte apoptosis. ROS, reactive oxygen species; CBP/p300, cAMP response element binding protein/p300 histone; 14-3-3: 14-3-3 protein; Ac, acetylation.

ing its phosphorylation and, thereby, its exclusion from the nucleus, cytoplasmic retention and inactivation, suppressing FOXO3a transcriptional activity [17]. SIRT1 is known to localize to the nucleus, regardless of cellular stress status, while cytoplasmic FOXO3a relocates to the nucleus in response to various stress stimuli [37]. Previous studies reported that the red wine polyphenol, resveratrol, and the white wine component, tyrosol, both caused increased SIRT1 activity, favouring cell survival [38]. Giannakou *et al*. demonstrated that SIRT1 might increase longevity by shifting FOXO-dependent responses from cell death towards cell survival [19]. Furthermore, SIRT1 was shown to regulate stress responses and cell survival by modulating FOXO3a transcription factor [18]. FOXO3a is an upstream regulator of the Bcl-2 family and these family members are downstream regulators of FOXO3a. Thus, these proteins modulate the process of cell apoptosis and proliferation by balancing levels of pro- and anti-apoptotic proteins in the mitochondria [39]. Cytochrome c associates with apoptotic protease activating factor 1 (Apaf-1) and procaspase-9 to form the apoptosome, which activates caspase-9 and, as an essential hallmark of apoptosis, caspase-3, eventually resulting in apoptosis [27]. Levels of Bcl-xL, a pro-survival factor, determine whether the cell initiates apoptosis or re-enters the cell cycle. In contrast, Bax is an important pro-apoptotic protein, facilitating cytochrome c release from the mitochondria. Cytochrome c release is, furthermore, inhibited by the anti-apoptotic protein Bcl-xL. Therefore, the ratio of Bax/Bcl-xL determines whether there will be cell survival or cell death in response to apoptotic stimulation [40].

In our study, we found that repeated exhaustive exercise impaired activation of Akt and SIRT1, as indicated by decreased FOXO3a phosphorylation, subsequently causing translocation of the dephosphorylated FOXO3a from the cytoplasm to nucleus. The dephosphorylated FOXO3a transcriptionally activated downstream target genes, resulting in Bcl-2 protein family dysregulation. The upregulated pro-apoptotic protein Bax and the increased Bax/Bcl-xL ratio induced cytochrome c release from the mitochondria to the cytosol, activating caspase-9 and caspase-3, thus triggering the mitochondrial cascade leading to cardiomyocyte apoptosis.

However, treatment with allicin (especially at 6.3 or 12.6 mg/kg) activated both the Akt and SIRT1 pathways, promoting synergistic cardioprotective effects. Allicin stimulated Akt signalling, causing nuclear export of FOX3a, inducing its phosphorylation and retention in the cytoplasm and inhibiting its transcriptional effects, eventually decreasing apoptosis. Our results suggested that allicin treatment activated SIRT1 and increased the interaction of SIRT1 with FOXO3a, promoting FOXO3a deacetylation and blocking its accumulation in the nucleus, thus attenuating FOXO3a induced apoptosis and potentiating its effects on cell survival.

Accordingly, FOXO3a plays a central role in the myocardial oxidative injury and apoptosis induced by repeated exhaustive exercise.

In conclusion, our findings demonstrated that allicin protected cardiomyocytes from the myocardial oxidative injury and apoptosis induced by repeated exhaustive exercise. Its cardioprotective effects may be linked to activation of both Akt and SIRT1 pathways. Though further studies must be conducted to better understand the detailed mechanisms, including how allicin is metabolized, our findings support allicin as a promising effective treatment for diseases related to oxidative stress and inflammation.

Our animal study had certain limitations. Allicin was not previously investigated as a treatment for repeated exhaustive exercise, so its doses were selected based on clinical studies for anti-infective therapy. The frequency and duration of allicin treatments were similar to those used in previous animal experiments and clinical trials. Further animal experiments and clinical trials will be needed to determine the required dosage and frequency of allicin treatments.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qiao Xue, Department of Cardiology, Chinese PLA General Hospital, 28 Fuxing Rd., Beijing 100853, China. Tel: +86-13810634170; E-mail: xueqiao301301@sina.com; Dr. Yutang Wang, College of Medicine, Nankai University, 94 Weijin Rd., Tianjin 300071, China; Department of Geriatric Cardiology, Chinese PLA General Hospital, 28 Fuxing Rd., Beijing 100853, China. Tel: +86-13901163874; E-mail: wangyutang2017@163.com

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