EXPERIMENTAL STUDY

Exercise Preconditioning Protects against Acute Cardiac Injury Induced by Lipopolysaccharide Through General Control Nonderepressible 2 Kinase

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Summary

Exercise preconditioning may protect against cardiac injury induced by lipopolysaccharide (LPS), but the mechanism is unresolved. The aim of this study is to explore whether the general control nonderepressible 2 (GCN2) kinase gene is associated with the protective effect of exercise preconditioning. Eight-week-old male C57BL/6J (n = 40) and GCN2 knockout (KO) (n = 40) mice were divided into four groups: control, LPS (L), exercise preconditioning (E), and exercise preconditioning LPS (EL). Mice in the exercise groups performed exercise for eight weeks. After exercise, all mice were given an equal volume of LPS or saline (10 μg/g). We measured the cardiac function using echocardiography and then collected heart tissue. Exercise preconditioning improved cardiac inflammation (interleukin-6, tumor necrosis factor α) and cardiac dysfunction (ejection fraction, fraction shortening) in C57 mice induced by LPS and also decreased the expression levels of GCN2, phosphorylation of eukaryotic translation initiation factor 2α (p-eIF2α), and activating transcription factor 4 (ATF4). Moreover, GCN2 KO decreased inflammation and cardiac dysfunction induced by LPS in sedentary mice. The inflammation and cardiac dysfunction in the GCN2 KO EL group were lower than in the C57 EL group, and the expression of GCN2, p-eIF2α, and ATF4 in the GCN2 KO EL group was lower than in the C57 EL group. Exercise preconditioning alleviated cardiac injury induced by LPS. GCN2 KO also improved cardiac injury. Exercise preconditioning promoted the effect of GCN2 KO in alleviating cardiac injury, and the GCN2 and eIF2α/ATF4 pathways play an important role in the process.

Key words: Eukaryotic initiation factor 2α, Inflammation

The “2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease” indicates that exercise has many beneficial effects on attenuating cardiovascular disease risk factors in humans1 and reduces the incidence and mortality of cardiovascular disease.2 Exercise preconditioning has physiological benefits in many organs, including the heart, and can also increase tolerance to cardiac ischemia-reperfusion injury.3 In addition, exercise preconditioning can partially attenuate experimental cardiac infarction in rats and mice.4 Although the mechanism of these beneficial effects has not yet been fully established, recent evidence suggests that exercise may prevent these diseases by inhibiting chronic inflammation and may affect many important metabolic organs.5,6

General control nonderepressible 2 (GCN2) is a eukaryotic initiation factor 2α (eIF2α) kinase and is also an amino-acid-sensing kinase. Under conditions of amino acid deficiency, uncharged cellular horizontal transporter RNA is increased, which can be sensed and bound by GCN2. This binding leads to the activation of its protein kinase catalytic domain and subsequent phosphorylation of eukaryotic initiation factor 2α (p-eIF2α). It can also specifically promote the translation of certain mRNAs, such as GCN4, and activate the activating transcription factor 4 (ATF4). Finally, it can inhibit protein synthesis in the body.6,7 The GCN2/eIF2α/ATF4 pathway can increase the expression level of transcription factor CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) to help cells respond to external stimuli.8,9 GCN2 knockout (KO) reduced inflammatory responses in a mouse model of septicemia, with decreased interleukin-6 (IL-6) expression correlating with a significant reduction in animal mortality.10 Reduced GCN2 activity may protect against heart inflammation caused by a high-fat diet and cardiac damage induced by doxorubicin through the inhibition of the eIF2α/ATF4 pathway and then inhibit the expression of inflammatory or apoptotic factors.11 Although GCN2 is involved in inflammation and cardiac injury, its role in the exercise-preconditioning-mediated alleviation...
of inflammation and cardiac injury induced by lipopoly-
saccharide (LPS) remains unclear.

In this study, we investigated the role of GCN2 in the process of exercise preconditioning to prevent cardiac injury induced by LPS. LPS is a component of the cell wall of Gram-negative bacteria that is used to mimic sepsis, an acute systemic inflammatory response to infectious stimuli. First, we explored the expression of GCN2 protein during exercise preconditioning to prevent cardiac injury. Then, we used GCN2 gene deficiency mice for further verification. We hypothesized that exercise preconditioning could alleviate cardiac inflammation and that its mechanism is related to the expression of GCN2 and the eIF2α/ATF4 pathway.

Methods

Animals: GCN2 KO mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA)\(^{10}\) and then crossed with the C57BL/6J strain (obtained from Shanghai Model Organisms Center, China). Adult (eight-week-old) male C57BL/6J mice (n = 40) were randomly divided into four groups (n = 10 per group): C57 control, C57 LPS (C57 L), C57 exercise preconditioning (C57 E), and C57 exercise preconditioning + LPS (C57 EL). GCN2 KO mice (n = 40) were also randomly divided into four groups (n = 10 per group): GCN2 KO control, GCN2 KO LPS (GCN2 KO L), GCN2 KO exercise preconditioning (GCN2 KO E), and GCN2 KO exercise preconditioning + LPS (GCN2 KO EL). Mice in the exercise groups performed exercise for eight weeks. All animal studies were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Shanghai University of Sport. All animals in the study were humanely treated in accordance with the “Guide for the Care and Use of Laboratory Animals”.

Exercise protocol: An endurance swimming training program was conducted for five days/week for eight weeks. The mice were acclimated to swimming for 10 minutes the first day, 20 minutes the second day, and so forth, until they were swimming for 60 minutes per day. Swimming was performed in a plastic tank (45 x 60 x 40 cm) filled to a depth of 30 cm with water, which was maintained at a temperature of 32°C-36°C. Sedentary control animals were handled and immersed for a few minutes in the warm water (a depth of 3 cm) to subject them to stress similar to that of the trained mice.\(^{15,16}\)

LPS administration: Escherichia coli LPS (026: B6, 200 μg; Sigma-Aldrich, St. Louis, MO, USA) was diluted in prepared sterile saline at a concentration of 4 mg/mL. As shown in Figure 1, 24 hours after the last swimming training, we immediately started injecting LPS at a dose of 10 μg/g in the abdominal cavity of the mice. The control group was injected with an equal volume of physiological saline.\(^{17}\)

Echocardiography: Six hours after the injection of LPS, we performed echocardiography on all mice. All the mice were anesthetized (2% isoflurane inhalation) for echocardiography using a Vevo 1100 imaging system (VisualSonics, Toronto, Canada). Heart 2D, M-mode, and Doppler images were collected in the short axis view. The left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were measured. The left ventricular fraction shortening (FS, %) and the ejection fraction (EF, %) were then calculated using the following formulas: FS = (LVEDD – LVESD)/LVEDD and EF = (LVEDD – LVESD)/LVEDD.\(^{17}\)

Western blotting: We extracted proteins after collecting the heart samples, and a BCA protein assay was used to determine the protein concentration (Bradford Protein Concentration Assay Kit; Beyotime, Shanghai, China). The protein samples of each group were fractionated using SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Then, the PVDF membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1-2 hours at room temperature and incubated with primary antibodies against β-tubulin (1:1,000, CST), GCN2 (1:1,000, CST), eIF2α (1:500, CST), phosphorylation of eIF2α (1:500, CST), ATF4 (1:1,000, CST), atrial natriuretic peptide (ANP) (1:1,000, CST), IL-6 (1:1,000, CST), tumor necrosis factor α (TNF-α) (1:1,000, CST), and sirtuin deacetylase 1 (SIRT1) (1:1,000, CST) overnight at 4°C. Thereafter, the membranes were washed in TBST (5 minutes x 3 times) and exposed to the corresponding secondary antibodies (1:4,000) at room temperature for 1-2 hours. Then, we detected the fluorescent signal using a Bio-Rad imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The area and density of the protein bands were analyzed using ImageJ software.

Statistical analysis: All statistical analyses were performed using SPSS Statistics v.22.0 (IBM Corp., Armonk, NY, USA). All data are presented as the mean ± standard deviation (SD). Two-way analysis of variance (ANOVA)
was used to assess exercise preconditioning and gene type involved in the process of cardiac injury. If significant effects were found, Bonferroni’s post hoc test was used to determine the source of the difference. The significance level was set to $P < 0.05$.

## Results

**Exercise preconditioning alleviated cardiac injury induced by LPS in C57 mice:** In the Table, eight weeks of exercise preconditioning improved the body weight (BW) loss (4.58 ± 1.37% in C57 L versus 3.54 ± 1.30% C57 EL; $P < 0.05$) and heart weight (HW) loss (95.42 ± 4.4 mg in C57 L versus 98.9 ± 10.6 mg in C57 EL; $P < 0.05$) in C57 mice induced by LPS. Exercise preconditioning also increased the HW-to-BW ratio in C57 mice (4.18 ± 0.10 in C57 L versus 5.21 ± 0.23 in C57 EL; $P < 0.05$). Moreover, exercise preconditioning improved the EF (50.34 ± 6.94 in C57 L versus 59.32 ± 3.63 in C57 EL; $P < 0.05$) and FS (20.95 ± 3.65 in C57 L versus 25.96 ± 2.26 in C57 EL; $P < 0.05$) values in C57 mice.

Eight weeks of exercise decreased the level of ANP expression and increased the level of SIRT1 expression induced by LPS in C57 mice (Figure 2, $P < 0.05$). These results suggested that exercise preconditioning improved cardiac dysfunction. Exercise preconditioning also decreased the LPS-induced expression levels of IL-6 and TNF-α in C57 mice (Figure 2, $P < 0.05$), suggesting that exercise preconditioning improved inflammation.

**Exercise preconditioning inhibited the expression of GCN2 protein and the eIF2α/ATF4 pathway:** As shown in Figure 3, the expression of GCN2 protein in the C57 EL group was lower than that in the C57 L group, suggesting that exercise preconditioning decreased the expression of GCN2. Furthermore, the phosphorylation of eIF2α and expression of ATF4 proteins in the C57 EL group were lower than in the C57 L group (Figure 3, $P < 0.05$), suggesting that exercise preconditioning also decreased the p-eIF2α and expression of ATF4.

**GCN2 gene deficiency prevented cardiac injury induced by LPS:** The BW loss of the GCN2 KO L group was significantly lower than that of the C57 L group (2.89 ± 1.52% in GCN2 KO L versus 4.58 ± 1.37% in C57 L; see the Table; $P < 0.05$). The HW value of the GCN2 KO

### Table. Parameters and Echocardiographic Results of Mice

| Parameters          | C57 control | C57 L   | C57 E   | C57 EL   | GCN2 KO control | GCN2 KO L   | GCN2 KO E   | GCN2 KO EL   |
|---------------------|-------------|---------|---------|---------|-----------------|-------------|-------------|-------------|
| Post-BW (g)         | 26.70 ± 2.92| 22.83 ± 0.80* | 20.82 ± 0.44* | 18.97 ± 2.29* | 25.85 ± 0.64 | 23.47 ± 1.95* | 22.11 ± 0.74* | 22.29 ± 1.01* |
| BW loss (%)         | 1.25 ± 0.56 | 4.58 ± 1.37* | 1.03 ± 0.21 | 3.54 ± 1.30* | 1.09 ± 0.68 | 2.89 ± 1.52** | 1.12 ± 0.44 | 1.45 ± 0.83** |
| HW (mg)             | 106.8 ± 6.2 | 95.42 ± 4.4* | 98.0 ± 2.6* | 98.9 ± 10.6* | 105.7 ± 5.6 | 100.65 ± 7.6* | 104.4 ± 5.7* | 104.4 ± 5.7* |
| HW/BW (mg/g)        | 3.99 ± 0.43 | 4.18 ± 0.10 | 4.71 ± 0.07* | 5.21 ± 0.23* | 4.09 ± 0.11 | 4.29 ± 0.14 | 4.63 ± 0.15* | 4.68 ± 0.12** |
| LVEDD (mm)          | 5.33 ± 0.32 | 3.77 ± 0.29 | 3.66 ± 0.33 | 3.45 ± 0.11 | 3.48 ± 0.46 | 3.43 ± 0.29 | 3.59 ± 0.29 | 3.46 ± 0.23 |
| LVESD (mm)          | 2.32 ± 0.27 | 2.75 ± 0.31 | 2.33 ± 0.27 | 2.56 ± 0.19 | 2.25 ± 0.43 | 2.56 ± 0.28 | 2.28 ± 0.26 | 2.43 ± 0.22 |
| EF (%)              | 71.34 ± 5.61 | 74.29 ± 3.47 | 59.32 ± 3.63* | 73.10 ± 6.25 | 74.15 ± 5.15 | 63.98 ± 2.85** | 63.98 ± 2.85** |
| FS (%)              | 4.18 ± 0.10 | 4.71 ± 0.07* | 5.21 ± 0.23* | 5.21 ± 0.23* | 3.99 ± 0.43 | 4.18 ± 0.10 | 4.71 ± 0.07* | 5.21 ± 0.23* |

Values are presented as the mean ± SD. LPS indicates lipopolysaccharide; BW, body weight; HW, heart weight; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; EF, ejection fraction; and FS, fraction shortening. *$P < 0.05$, control versus L; **$P < 0.05$, control versus E, L versus EL; †$P < 0.05$, control versus E, L versus EL; ‡$P < 0.05$, control versus L; #$P < 0.05$, C57 L versus GCN2 KO L, C57 EL versus GCN2 KO EL; †$P < 0.05$, C57 L versus GCN2 KO L, C57 EL versus GCN2 KO EL.
Exercise preconditioning also improved BW loss (1.45 ± 0.83% in GCN2 KO EL; see the Table; P < 0.05). Exercise preconditioning also increased the HW-to-BW ratio (58.73 ± 3.14 in C57 L; see the Table; P < 0.05) values of the GCN2 KO L group were higher than those of the C57 L group. In mice induced by LPS, GCN2 gene deficiency decreased the expression of ANP, which exercise improves inflammation and cardiac dysfunction, the mechanism by which exercise improves inflammation and cardiac dysfunction was related to a decreased expression of the GCN2 protein. GCN2 KO also improved cardiac inflammation and cardiac dysfunction induced by LPS, producing a protective effect similar to that of exercise. Importantly, exercise preconditioning promoted the effect of exercise preconditioning protection against cardiac injury induced by LPS from the perspective of the GCN2 gene. We found that eight weeks of swimming training improved inflammation and cardiac dysfunction, the mechanism by which exercise improves inflammation and cardiac dysfunction was related to a decreased expression of the GCN2 protein. GCN2 KO also improved cardiac inflammation and cardiac dysfunction induced by LPS, producing a protective effect similar to that of exercise. Importantly, exercise preconditioning promoted the effect of exercise preconditioning protection against cardiac injury induced by LPS from the perspective of the GCN2 gene. We found that eight weeks of swimming training improved inflammation and cardiac dysfunction, the mechanism by which exercise improves inflammation and cardiac dysfunction was related to a decreased expression of the GCN2 protein. GCN2 KO also improved cardiac inflammation and cardiac dysfunction induced by LPS, producing a protective effect similar to that of exercise. 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GCN2 KO in alleviating inflammation and cardiac dysfunction. Therefore, the expression of GCN2 and the eIF2α/ATF4 pathway play an important role in this process.

Similar to ischemic preconditioning, exercise preconditioning can produce myocardial protection. Exercise preconditioning means that exercise training before ischemia can enhance the tolerance of the heart to ischemia-reperfusion stimulation.14) The reason for the myocardial protective effect may be that exercise can induce endogenous protective factors in the body, and the effect is the result of exercise itself causing ischemia or relative ischemia in the myocardium and, thus, causing ischemic preconditioning.

In our study, exercise preconditioning alleviated cardiac injury induced by LPS, including changes in cardiac injury indicators (ANP, SIRT1) and cardiac dysfunction. ANP is a marker of myocardial damage. SIRT1 is involved in survival regulation under stress conditions in cardiomyocytes; when upregulated, it reduces the progression of heart failure and aging in mice.15) IL-6 and TNF-α are pleiotropic cytokines that regulate host immune responses and inflammation. Exercise can prevent inflammation and cardiac dysfunction induced by LPS, via a mechanism of inflammatory cytokine expression regulation. Exercise preconditioning also improves cardiac dysfunction (EF, FS) in mice induced by LPS. LPS induced a decrease in cardiac output, and exercise preconditioning increased the sympathetic tone, thereby increasing the heart rate to compensate for cardiac output.20,21) Exercise preconditioning improved the change in BW induced by LPS, and a mild preconditioning exercise program could prevent acute sepsis from causing atrophy and maintain muscle mass.22)

In our study, the exercise protocol followed that of Kesherwani Varun and Jeong-sun Ju.23) Kesherwani Varun found that the EF value of the swimming group was slightly higher than that of the sedentary group, but there was no significant difference.24) We think that when the exercise intensity is relatively low, this will not cause changes in the structure and function of the heart but may affect the cardiac reserve function. In the physiological state, the EF value did not fully reflect the cardiac reserve function, so there was no difference in the EF values between the exercise group and the sedentary group; however, in the pathological stress state, the improved cardiac reserve function induced by exercise may prevent heart damage. In addition, for technical reasons, we did not measure the cardiac reserve function in this experiment. We may consider measuring the hemodynamic parameters of the heart through a catheter in future studies.

Moderate-intensity chronic exercise caused an exaggerated serum inflammatory response to endotoxins and an elevation in a serum marker of LPS-induced tissue damage, and constitutive NO seems to participate in this process.25) Cardiopulmonary adaptations promoted by exercise seem to be beneficial, counteracting the cardiovascular abnormalities and pulmonary edema seen in septicemia induced by LPS, and constitutive NO seems to participate in this process as well.26)

Exercise may increase the expression of HSP72 in the heart, protect against high mortality rates, and attenuate cardiovascular dysfunction in diabetic rats during endotoxemia.27) In our study, LPS injection leads to inflammation and induces a comprehensive emergency response manifested by increased phosphorylation of eIF2α. GCN2 has the ability to modulate immune responses, especially inflammation.28) eIF2α is the only downstream target of the GCN2 gene, and ATF4 and CHOP are downstream proteins of GCN2 and eIF2α.29) Phosphorylation of eIF2α reduces protein synthesis but increases the transcription of specific genes in response to stressors.22-26) Insufficient amino acids activate GCN2 and then induce eIF2α phosphorylation, which increases the translation of ATF4, thereby increasing the expression of CHOP and other stress response genes.30) LPS caused the body to enter an immune stress state, thus activating the GCN2 and eIF2α/ATF4 pathways to combat immune stress. We also found that exercise can reduce the expression of GCN2 and the eIF2α/ATF4 pathway. Therefore, the mechanism of exercise protection against cardiac injury is related to the decreased expression of the GCN2 gene, thereby inhibiting the eIF2α/ATF4 pathway and ultimately improving cardiac inflammation and cardiac dysfunction.

We then used GCN2 KO mice for further verification and found that GCN2 gene deficiency also improved cardiac inflammation and cardiac dysfunction and produced a protective effect similar to that of exercise because GCN2 gene deficiency inhibits the expression of the eIF2α/ATF4 pathway. These results were similar to those of a previous study; Liu et al. found that GCN2 gene deficiency reduced inflammatory responses, with decreased IL-6 expression correlating with a significant reduction in animal mortality.31) Reducing the GCN2 activity in the heart tissue may be a new way to reduce the development of congestive heart failure. GCN2 gene deficiency could reduce Bcl-2 expression and increase cardiomyocyte susceptibility to apoptotic stimuli.32) GCN2 gene deficiency can alleviate heart inflammation caused by a high-fat diet, thereby alleviating cardiac damage.33) Moreover, GCN2 gene deficiency can alleviate doxorubicin-induced cardiac damage, and eIF2α gene deficiency showed similar results.34) Therefore, inhibiting the GCN2 activity was an effective strategy for treating cardiac injury and was the mechanism underlying the relief of cardiac injury by exercise.

In our study, exercise preconditioning promoted the effect of GCN2 gene deficiency to prevent cardiac injury, and the effect was better than exercise or GCN2 gene deficiency alone. When GCN2 was not knocked out, exercise could prevent myocardial damage through many mechanisms, including eIF2α/ATF4 and the oxidative stress pathway.19) The decline in GCN2 activity was indeed effective in relieving the body’s inflammatory response in GCN2 KO mice. When GCN2 is knocked out, GCN2 gene deficiency itself was effective for improving myocardial injury. Exercise may inhibit other pathways, such as double-stranded RNA-activated protein kinase R-like endoplasmic reticulum kinase (PERK) and endoplasmic reticulum stress pathway. PERK is a major transducer of the endoplasmic reticulum stress response and directly phosphorylates eIF2α, resulting in translational attenuation.20) Exercise may inhibit the PERK and eIF2α/ATF4...
pathways, hence inhibiting myocardial injury and inflammation, producing a protective effect similar to that of exercise. For example, treadmill exercise can improve cardiac function in rats with myocardial infarction, whose mechanism is related to the attenuation of expression of GRP78, DERLIN-1, PERK phosphorylation, eIF2α phosphorylation, ATF4/6, XBP1, CHOP, and caspase-3.33,34 In addition, swimming training also reduced the obesity and insulin resistance in diabetic rats and reduced proinflammatory molecules (JNK, IκB, and NF-κB) in fat and liver tissue, and exercise can reduce ER stress and eIF2α phosphorylation in these tissues by reducing PERK.35 Their mechanisms are related to the content of GCN2. Hence, gene deficiency of GCN2 and inhibition of the expression of GCN2 protein can protect against heart damage and inflammation. Therefore, the protective effect of exercise will not be weakened when the GCN2 gene is knocked out.

Conclusion

In summary, eight weeks of swimming exercise preconditioning alleviated inflammation and cardiac dysfunction. GCN2 gene deficiency also improved inflammation and cardiac dysfunction. Importantly, exercise preconditioning can promote the protective effect of GCN2 gene deficiency on inflammation and cardiac dysfunction, and the GCN2 and eIF2α/ATF4 pathways play an important role in the process.

Disclosure

Conflicts of interest: No conflicts of interest, financial or otherwise, are declared by the authors.

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