Ginsenoside Rg3 improves cardiac mitochondrial population quality: Mimetic exercise training

Mengwei Sun, Chenglin Huang, Cheng Wang, Jianheng Zheng, Peng Zhang, Yangshu Xu, Hong Chen, Weili Shen

Abstract

Emerging evidence indicates exercise training could mediate mitochondrial quality control through the improvement of mitochondrial dynamics. Ginsenoside Rg3 (Rg3), one of the active ingredients in Panax ginseng, is well known in herbal medicine as a tonic and restorative agent. However, the molecular mechanism underlying the beneficial effects of Rg3 has been elusive. In the present study, we compared the effects of Rg3 administration with aerobic exercise on mitochondrial adaptation in cardiac muscle tissue of Sprague–Dawley (SD) rats. Three groups of SD rats were studied: (1) sedentary control, (2) Rg3-treated and (3) aerobic exercise trained. Both aerobic exercise training and Rg3 supplementation enhanced peroxisome proliferator-activated receptor coactivator 1 alpha (PGC-1α) and nuclear factor-E2-related factor 2 (Nrf2) protein levels in cardiac muscle. The activation of PGC-1α led to increased mRNA levels of mitochondrial transcription factor A (Tfam) and nuclear related factor 1 (Nrf1), these changes were accompanied by increases in mitochondrial DNA copy number and complex protein levels, while activation of Nrf2 increased levels of phase II detoxifying enzymes, including nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase 1 (NQO1), superoxide dismutase (MnSOD) and catalase. Aerobic exercise also enhanced mitochondrial autophagy pathway activity, including increased conversion of LC3-I to LC3-II and greater expression of beclin1 and autophagy-related protein 7 (ATG7), these effects of aerobic exercise are comparable to that of Rg3. These results demonstrate that Rg3 mimics improved cardiac adaptations to exercise by regulating mitochondria dynamic remodeling and enhancing the quantity and quality of mitochondria.

1. Introduction

Emerging evidence indicates regular physical activity may promote health and reduce the risk of cardiovascular disease [1]. Mitochondria represent approximately one-third of the mass of the heart and play a critical role in maintaining cellular function. It is well established that mitochondrial adaptations are likely to be important in exercise-induced cardio-protection [2]. Exercise training results in a reduction in mitochondrial oxidant production [3] and enhanced mitochondrial antioxidant capacity [4]. Nrf2 is a redox-sensitive, basic leucine zipper protein that regulates the transcription of several antioxidant genes. Some studies have reported that exercise may modulate Nrf2/ARE (antioxidant response element) signaling and subsequent enhancement of antioxidant defense pathways in cardiac muscle [5].

More recent work has demonstrated aerobic exercise could ameliorate cardiovascular dysfunction and enhance longevity, possibly through improving mitochondrial quality control [6]. The critical role of mitochondria in cardiac contraction involves specific regulation and adaptations of the mitochondrial network structure and function [7]. This plasticity results from the mitochondrial dynamic interplay of fusion, fission, autophagy and mitochondrial biogenesis, which ensures proper organization of the mitochondrial network [8]. Proteins controlling fission include mitofusin-1 (MFN1), MFN2, and optic atrophy-1 (OPA1), and fission is regulated by dynamic related protein-1 (DRP1) and fission-1 (Fis1). Recently studies have indicated that mitochondrial mass was significantly decreased in a variety of cardiac conditions such as dilated cardiomyopathy and inhibiting mitochondrial fission could protect the heart against ischemia/reperfusion injury [9], post-myocardial infarction heart failure [10] and myocardial...
tion is critical for maintaining proper mitochondrial function. As mentioned above, a continuous balance between mitochondrial fission and fusion is critical for maintaining proper mitochondrial function.

Rg3, one of the active ingredients in Panax ginseng, is well known in herbal medicine as a tonic and restorative agent. A number of studies have recently described the beneficial effect of Rg3 on cardiovascular diseases. Rg3 may inhibit tumor necrosis factor-α-induced expression of cell adhesion molecules in human endothelial cells and promote human endothelial cell proliferation, migration and tube formation in vitro. Recently, Kim et al. reported that a mixture of Rg3 and Compound K increased cardiac resistance to ischemia/reperfusion injury. Intranasal administration of Rg3 shows an anti-fatigue effect, which could promote exercise performance. We and others have previously demonstrated that Rg3 can significantly improve a swimmer’s maximal oxygen uptake and the respiratory quotient, maximal lactate and lactic acid clearance rate 1 min after a maximal oxygen uptake test. These beneficial effects suggested that Rg3 has similar effects to endurance exercise training. Identifying natural compounds and nutrients that mimic or potentiate the beneficial effects of aerobic exercise may be an effective strategy for preventing and treating cardiovascular disease. We and others have previously demonstrated that resveratrol, hydroxytyrosol or mitochondrial nutrients could enhance exercise performance by increasing mitochondrial number and function and mitochondrial fusion both in skeletal and cardiac muscle. These beneficial effects suggested that Rg3 has similar effects to endurance exercise training. Identifying natural compounds and nutrients that mimic or potentiate the beneficial effects of aerobic exercise may be an effective strategy for preventing and treating cardiovascular disease. We and others have previously demonstrated that resveratrol, hydroxytyrosol or mitochondrial nutrients could enhance exercise performance by increasing mitochondrial number and function and mitochondrial fusion both in skeletal and cardiac muscle. Of particular interest, the same effects have been shown by several drugs such as peroxisome proliferator activated receptor (PPAR) β/δ agonist 5-aminomimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) or 2-methyl-4-4-methyl-2-[4-(trifluoromethyl) phenyl]-5-thiazolyl methylthio phenoxy acetic acid (GW1516).

Based on our findings, it seems reasonable to speculate that as Rg3 improves exercise performance this is presumably associated with mitochondrial dynamic remodeling. In this study, we characterized the effects of aerobic exercise on cardiac mitochondrial activities by primarily assessing dynamics, biogenesis and autophagy. Then, we tested whether treatment of rats with Rg3 would improve cardiac mitochondrial quality control.

2. Materials and methods

2.1. Materials

Anti-autophagy-related protein 7 (ATG7), NQO-1, phosphomammalian target of rapamycin (p-mTOR), phospho-5′ adenosine monophosphate-activated protein kinase (p-AMPK), phospho-acetyl CoA carboxylase (p-ACC) and anti-beclin-1 were purchased from Cell Signaling Technology (Danvers, MA, USA); antibodies against PGC-1α, Nrf2, catalase, OPA1, DRP-1 and MnSOD were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and microtubule-associated protein-1 light chain-3B (LC3B) were from Sigma (St. Louis, MO, USA); anti-OXPHOS Complex I (1:5000), p-mTOR (1:1000), catalase (1:1000), PGC-1α (1:1000), OPA1 (1:1000), LC3B (1:1000), beclin-1 (1:1000), ATG-7 (1:1000), MnSOD (1:5000), OxPhos Complex I (1:2000), and OxPhos Complex V (1:2000) in 5% (w/v) milk/TBST. Fresh cardiac tissue were fixed in 2.5% (v/v) glutaraldehyde in phosphate-buffered saline, postfixed in 4% (w/v) osmium tetroxide, and embedded in Epon resin. Ultrathin sections (50–80 nm thick) were prepared, stained with lead citrate and uranyl acetate, and observed with a transmission electron microscope (CM 10; Philips, Eindhoven, the Netherlands). Soluble lysates (20 µg/lane) were subjected to 10% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the proteins were subsequently transferred to nitrocellulose membranes and blocked with 5% (w/v) nonfat milk/TBST. Membranes were incubated overnight at 4°C with primary antibodies directed against GAPDH (1:5000), p-mTOR (1:1000), catalase (1:1000), PGC-1α (1:1000), OPA1 (1:1000), LC3B (1:1000), beclin-1 (1:1000), ATG-7 (1:1000), MnSOD (1:5000), OxPhos Complex I (1:2000), and OxPhos Complex V (1:2000) in 5% (w/v) milk/TBST. The membranes were washed three times with TBST and then incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed using electrochemiluminescence and quantified by scanning densitometry.

2.2. Methods

2.2.1. Animals

Eight-week-old male Sprague–Dawley (SD) rats were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and were housed in a temperature-controlled room with a 12:12-h light–dark cycle. Water and rodent chow were provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Research Institute of Sports Science (IACUC #10-09011). All experiments involving the animals were conducted in conformance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th edition, 2011).

A total of 36 rats were randomly divided into three groups of 12 rats each: sedentary control, sedentary control with ginsenoside Rg3 and trained group. The Rg3 group received Rg3 (5 mg/kg/d) by gavage for 8 weeks.

2.2.2. Training protocol

The aerobic training protocol has been described previously. Briefly, rats were exercise-trained for the entire 8-week experimental period on a treadmill at room temperature 6 days/week. After acclimatizing to a motor-driven rodent treadmill (Hangzhou, China) for 2 days, animals ran on treadmill at 10 m/min, 0% slope, for 10 min/day during the first week. Exercise training intensity and duration were progressively increased until week 4, when the animals were running at 20 m/minute, 5% slope, for 60 min/day. This training intensity was maintained for the last 4 week.

At the end of the drug treatment and/or training protocol, the animals in each group were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg), and if no response occurred following cornea stimulation and incision, the chest was opened quickly and the heart was excised.

2.2.3. Transmission electron microscopy (TEM)

Fresh cardiac tissue were fixed in 2.5% (v/v) glutaraldehyde in phosphate-buffered saline, postfixed in 4% (w/v) osmium tetroxide, and embedded in Epon resin. Ultrathin sections (50–80 nm thick) were prepared, stained with lead citrate and uranyl acetate, and observed with a transmission electron microscope (CM 10; Philips, Eindhoven, the Netherlands).

2.2.4. Western blotting

Cardiac tissue were finely minced and homogenized in isolation buffer (in mmol/l): 70 sucrose, 210 mannitol, 1.0 EDTA-Na2, 50 Tris–HCL, pH 7.4 using a Potter–Elvejem homogenizer. The homogenate was transferred into a 2.0 ml Eppendorf tube and centrifuged at 13000×g for 3 min. The supernatant was carefully transferred into a clean 1.5 ml Eppendorf tube and centrifuged at 10,000×g for 10 min. The pellet containing crude mitochondria was
resuspended in isolation buffer [23]. NADH–CoQ oxidoreductase (complex I) activity was assayed by Kumar’s method [24]. Complex V activity was measured as oligomycin-sensitive Mg²⁺-ATPase activity [25].

2.2.6. RNA isolation and reverse transcription PCR

Total RNA was extracted from 30 mg of tissue using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. Samples of 2.0 μg total RNA were reverse transcribed into cDNA. Quantitative real-time PCR was performed on the MX 3000P™ PCR Instrument (Stratagene, La Jolla, USA) using SYBR Premix EX Taq™ (TaKaRa, Dalian, China). The primers were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Nrf1</td>
<td>5′-TTACAGGGCGGTGAATGAC-3′</td>
<td>5′-GTTAAGGCGCATGTGACAG-3′</td>
</tr>
<tr>
<td>Tfam</td>
<td>5′-CCCTGGAAACCTTCTGATAAG-3′</td>
<td>5′-AATTTGACAGGATGAGG-3′</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5′-CGAACGTCTGCCCTATCAACTT-3′</td>
<td>5′-CTTGGATGTGGTAGGCTT-3′</td>
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The rat 18S ribosomal RNA (rRNA) gene served as the endogenous reference gene. The evaluation of relative changes of PCR product among the treatment groups was carried out using the ΔΔCT method. The reciprocal of 2ΔCT (used CT as an exponent for the base 2) for each target gene was normalized by that for 18S rRNA. Results are presented as the fold of the sedentary group [26].

2.2.7. Total DNA isolation and real-time PCR

Total DNA was extracted using the QIAamp DNA Mini kit, and quantitative PCR was performed using mitochondrial DNA and genomic DNA-specific primers. The following primers were used:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>mitochondrial D-loop</td>
<td>5′-AATCATCCATCTCTCCGTG-3′</td>
<td>5′-GACTAATGATCCCTTACG-3′</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5′-CGAACGTCTGCCCTATCAACTT-3′</td>
<td>5′-CTTGGATGTGGTAGGCCCT-3′</td>
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The rat 18S rRNA gene served as the endogenous reference gene. A melting curve was performed to ensure specific amplification. The standard curve method was used for relative quantification. The ratio of mitochondrial D-loop to 18S rRNA was then calculated. Results are presented as the fold of the sedentary group [26].

3. Statistical analysis

Statistical differences were determined by two-way analysis of variance (ANOVA) followed by Tukey’s tests. Differences were considered significant at \( p < 0.05 \). The results are presented as mean ± SEM.

4. Results

4.1. Effect of ginsenoside Rg3 on endogenous antioxidants

We investigated the impact of exercise and Rg3 on Nrf2 and phase II detoxifying enzyme expression. Aerobic training enhanced nuclear Nrf2 protein levels, while antioxidant enzyme levels including catalase, MnSOD and NQO-1 were significantly higher in trained animals than in the sedentary group. After 8 weeks of Rg3 administration, we observed that Rg3 also increased catalase and MnSOD expression in cardiac muscle tissue. Together, these results indicate that long-term Rg3 treatment elevated antioxidant enzyme expression, which may lead to cardiac protection (Fig. 1).

4.2. Effect of ginsenoside Rg3 on mitochondrial biogenesis

As shown in Fig. 2A, the mitochondria were typically intact in the left ventricles from sedentary control rats. The mitochondria are regular in shape, and compact with a high electron dense matrix. Electron microscopy on cardiac sections revealed that aerobic exercise hearts had bigger mitochondria compared to sedentary control hearts. The number of grid intersections superimposed with mitochondria was higher in the aerobic exercise group compared to the sedentary group, indicating that mitochondria were not only bigger but also more numerous. In addition, morphometric analysis revealed that Rg3 treatment displayed a mitochondrial population similar to aerobic exercised hearts. The ratio of mtDNA D-loop/18SrRNA in the exercise or the Rg3 administration group was significantly higher than in the sedentary control group (Fig. 2B). Meanwhile, mitochondrial complex V protein level was increased by exercise training or Rg3 treatment; however, Rg3 treatment did not significantly increase complex I expression. As shown in Fig. 2C and D, exercise training significantly enhanced the activity of mitochondrial complexes I and V; Rg3 treatment only significantly increased complex V activity. Exercise training significantly increased PGC-1α expression. Similarly, Rg3 treatment also increased PGC-1α protein levels (Fig. 2E). Transcription factors Nrf1 and Tfam are involved in regulating expression of nuclear genes and encoding major mitochondrial proteins that regulate mtDNA transcription and replication. Both exercise and Rg3 supplementation resulted in a significant increase in Nrf1 and Tfam mRNA expression (Fig. 2F).
4.3. Effect of ginsenoside Rg3 on mitochondrial dynamic remodeling

We investigated the impact of exercise and Rg3 on mitochondrial dynamic remodeling in cardiac muscle tissue. It was reported that mitochondrial morphology, protein tyrosine phosphatase (PTP) function, and cardiac adaptation to pressure overload were altered by down-regulation of OPA1 [27]. We observed that both aerobic exercise and Rg3 increased OPA1 protein levels compared to the sedentary controls. Exercise training increased expression of the mitochondrial fission-related protein DRP1, but the difference was not significant. Rg3 administration had no effect on DRP1 protein levels compared to the sedentary control group (Fig. 3A).

4.4. Rg3-induced mitochondrial dynamics are mediated via the AMPKα signaling pathway

We next tested whether exercise would impact AMPK and other related factors like ACC in cardiac muscle. As shown in Fig. 4, exercise training significantly induced AMPKα activation, we further measured its downstream effector, ACC, consistent with higher levels of p-AMPKα, and we detected elevated levels of p-ACC in cardiac tissue upon exercise. To address the question of whether activation of AMPKα further led to the inhibition of mTOR, we utilized immunoblot analysis to assess the functional status of the mTOR pathway. We observed a reduction in p-mTOR levels upon both aerobic exercise and Rg3 treatment (Fig. 4).

5. Discussion

Rg3 is a naturally occurring ginsenoside that has been demonstrated to have a wide range of benefits for the cardiovascular system. In this study, we report that Rg3 enhanced Nrf2 and PGC-1α protein levels in cardiac muscle. Nrf2 is a key component of cellular redox homeostasis in the attenuation of oxidative stress-associated pathological processes. It has been reported that sedentary older humans exhibit Nrf2-Keap1 dysfunction, but an active lifestyle increases Nrf2 function and thereby maintains redox homeostasis in the skeletal muscles of older humans [28]. Aerobic training might produce higher levels of antioxidant defenses as an adaptive mechanism, especially in mitochondria, in an attempt to overcome mitochondrial superoxide derived from metabolic demand. In the present study, Rg3 increased protein levels of Nrf2; furthermore this activation of Nrf2 can be attributed to...
a marked increase in antioxidant enzymes such as catalase and MnSOD in the myocardium. This result is consistent with the findings that 20(S)-Rg3, but not ginsenosides Rg1 or Re, could protect hepatocytes against oxidative stress through Nrf2 activation [29].

Fusion/fission balance plays an important role in determining the fate of depolarized mitochondria. We previously confirmed that aerobic physical training improved acetylcholine (ACh)-induced vascular relaxation; these beneficial effects were associated with the induction of mitochondrial biogenesis, fusion, and autophagy in aorta. In the present study, we demonstrated that cardiac tissue from treadmill-trained rats also exhibited significantly increased mitochondrial mass and greater protein levels of mitochondrial complexes, and PGC-1α, Nrf-1, and Tfam. This result is consistent with evidence from mitochondrial mutator (Polgm/m) mice, which have shown that exercise induces mitochondrial biogenesis and restores mitochondrial morphology [30]. Although cardiac mitochondria biogenesis is stimulated in the context of diabetes and appears to be maladaptive for cardiac energy production [31], emerging evidence suggests that impaired myocardial mitochondrial biogenesis may lead to diminished cardiac substrate flexibility, decreased cardiac energetic efficiency, and diastolic dysfunction [32].

Mitochondrial autophagy governs mitochondrial quality control and eliminates damaged mitochondria. Impaired cardiac autophagy would cause abnormal proteins and organelles to accumulate, leading to cardiac dysfunction. On the other hand, excessive induction autophagy may lead to autophagic cell death [33]. In this study, we found that autophagy-related proteins ATG7, beclin-1, and LC3II/I were induced by training and Rg3 treatment, accompanied with improvement in mitochondrial complex activities. This is consistent with the recent report that improvement of ventricular function in the infarcted heart by exercise was due to changes in autophagic function and fatty acid utilization [34].

Rg3 has been reported to influence various biological activities, including anti-inflammatory, anti-allergy, anti-tumor, and vascular relaxation. A recent study reported Rg3 could inhibit cholesterol biosynthesis and triglyceride accumulation in HepG2 cells by...
activation of sterol regulatory element binding protein-2 (SREBP-2) and AMPK [35]. Activation of AMPK can influence cardiac metabolism by regulating uptake and oxidative phosphorylation of fatty acids, the primary source of ATP in a normal myocardium. In the present study, we demonstrated that both aerobic exercise and Rg3 induced AMPK activation. It is now clear that AMPK activation promotes mitochondrial biogenesis and expression of nuclear-encoded mitochondrial genes by up-regulating PGC-1α. Long-term supplement with AICAR sustained the activation of AMPK, led to the up-regulation of nuclear-encoded mitochondrial genes and improved exercise performance in mice [21]. In addition, AMPKα2 now appears to play an important role in the disposal of dysfunctional mitochondria. We found activated AMPKα2 inhibition of the mammalian target of mTOR kinase via phosphorylation at Ser2448. Our previous study demonstrated that the AMPK/mTOR pathway is involved in autophagy-mediated endothelial cell angiogenesis [36]. It is presumed that both aerobic training and Rg3 promote autophagy through the AMPK/mTOR pathway.

Based on these findings, we conclude that Rg3 is an ergogenic aid that improves mitochondrial antioxidant capacity and regulates mitochondrial dynamic remodeling; these effects mimic improved cardiac adaptations to exercise by training. Nevertheless, even though Rg3 alone matches some of the benefits of traditional exercise, we feel that the potential of a pill could never truly replace exercise.

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References