Biochemical and Biophysical Research Communications 461 (2015) 354–360

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Strenuous exercise induces mitochondrial damage in skeletal muscle of old mice

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ARTICLE INFO

Article history:
Received 31 March 2015
Available online 15 April 2015

Keywords:
Mitochondria
Inflammation
Exercise
Redox signals
Skeletal muscle

ABSTRACT

Strenuous exercise is known to cause excessive ROS generation and inflammation. However, the mechanisms responsible for the regulation of mitochondrial integrity in the senescent muscle during high-intensity exercise (HE) are not well studied. Here, we show that HE suppresses up-regulation of mitochondrial function despite increase in mitochondrial copy number, following excessive ROS production, proinflammatory cytokines and NFκB activation. Moreover, HE in the old group resulted in the decreasing of both fusion (Mfn2) and fission (Drp1) proteins that may contribute to alteration of mitochondrial morphology. This study suggests that strenuous exercise does not reverse age-related mitochondrial damage and dysfunction by the increased ROS and inflammation.

1. Introduction

Skeletal muscle aging is associated with a decline in muscle mass and strength, which can be exacerbated by mitochondrial dysfunction [1]. Due to its vital role in the regulation of energy metabolism, intracellular signaling and apoptosis [2–4], maintaining mitochondrial function has been a key strategy to preserve muscle integrity and function during aging. However, mitochondrial morphological alterations and dysfunction increase with age [5], following which several adverse incidents such as a higher level of mitochondrial DNA (mtDNA) mutation, apoptosis and reactive oxygen species (ROS) induced oxidative damage take place [6–8]. Signaling cascades affecting the phenotypical changes of mitochondria are driven by some key pathways of mitochondrial biogenesis, dynamics and autophagy, thereby maintaining or changing mitochondrial function [9,10]. Upon the above multiple mechanisms, calorie restriction (CR), resveratrol or antioxidant supplementation and exercise have been introduced as therapeutic applications to ameliorate mitochondrial dysfunction in skeletal muscles [11–14], although there is still a controversy as to whether the above mentioned treatments always have beneficial effects [15,16]. Nevertheless, it has been generally believed that exercise is securely linked to muscle metabolic adaptations including enhanced mitochondrial function [17,18]. Indeed, most studies performed with non-senescent muscles have reported that increases in mitochondrial biogenesis signals and functions occur widely in response to various types, durations and intensities of exercise, importantly, not limited to any specific forms of exercise [19–21]. However, since excess inflammatory cytokines and oxidative stress may trigger mitochondrial deterioration and malfunction [22–24], it has been questioned whether these beneficial effects of exercise on mitochondria can also be observed in senescent muscles, which are more vulnerable to inflammation and ROS-induced oxidative damage. Moreover, strenuous exercise can exacerbate disruption of the cellular environment by increased oxidative damage and inflammatory process [25–27], which may have a negative synergic impact on the senescent muscle. Thus, upon the pathophysiological traits of skeletal muscle aging, we hypothesized there would be an adverse effect of exercise on mitochondrial function in the aged muscle due to the disturbance of cellular homeostasis by strenuous exercise, which may hamper pathways for maintaining mitochondrial integrity. In the current study, we aimed to investigate the link between strenuous exercise

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http://dx.doi.org/10.1016/j.bbrc.2015.04.038
0006-291X/

and mitochondrial function in mouse skeletal muscle following treadmill exercise.

2. Material and methods

2.1. Animals, experimental design

All experimental protocols were carried out in accordance with the National Institutes of Health Guidelines for animal research (Guide for the Care and Use of Laboratory Animal) and approved by the Animal Care and Use Committee at Hankuk University of Foreign Studies, South Korea. Male C57BL/6 mice at age 2 months (young, Y) and 24 months (old, O) were housed in temperature-controlled rooms (22 °C), on a reverse 12-h light/dark cycle. After a 1-week acclimation, mice were randomly assigned to six groups: young and non-exercise (Y-NE, $N = 10$), young and low intensity treadmill exercise (Y-LE, $N = 10$), young and high intensity treadmill exercise (Y-HE, $N = 10$), old and non-exercise (O-NE, $N = 10$), old and low intensity treadmill exercise (O-LE, $N = 10$) or old and high intensity treadmill exercise (O-HE, $N = 10$).

2.2. Treadmill exercise training

Animals from the exercise groups were subjected to 5 days of exercise regimen on a treadmill, while control animals were exposed to daily handling and spent the same time on a treadmill. Exercise intensity and duration were gradually increased during the first week of exercise training from 5 min at a speed of 4 m/min for aged mice and 6 m/min for young animals to a regular regimen and then increasing the speed 1 m/min per minute until exhaustion. Starting from the second week, for 5 days at 50 min a day, mice of the LE and HE groups ran on a motor-driven treadmill at 35% and 70%, respectively, of the speed at which the mice reached exhaustion. Thus, the Y-LE and Y-HE mice were run at 11.9 and 23.8 m/min, respectively and the O-LE and O-HE were run at 8.8 and 17.45 m/min, respectively.

2.3. Western blot analysis

Western blot was performed as described previously [28] using the following antibodies: anti-p-IκBα (Ser^{32} and Ser^{36}, #9246), IκBα (#9242) and Beclin-1 (#3495) (Cell Signaling Technology); anti-α-tubulin (loading control, ab18251), Tfam (#ab131607), histone H2B (nuclear loading control, ab1790), LC3B (#ab48394), p62 (#ab56416), Mfn1 (#ab126575) and Mfn2 (#ab50843) (Abcam); anti-Drp1 (#sc-21804) (Santa Cruz Biotechnology) and anti-PGC-1α (ST1202) (Calbiochem).

2.4. RNA extraction and RT-qPCR

Quantification of monocyte chemoattractant protein (MCP)-1 mRNA, which was normalized to the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was carried out using the following primers; MCP-1 forward primer, 5′-ATTGGGATCATCTTGCTGGT-3′, reverse primer: 5′-CCTGCTGTTCACAGTTGCC-3′; GAPDH forward primer, 5′-CGTCCCGTAGACAAAATGGT-3′, reverse primer, 5′-TTGATGGCAACAATCTCCAC-3′.

2.5. Mitochondrial isolation, mitochondrial ATP production rate and ROS generation

Mitochondria were isolated according to the manufacturer's instructions (Mitochondria Isolation Kit for Tissue, Thermo Scientific) and measurements of the mitochondrial ATP production rate and mitochondrial ROS production were carried out as previously described [28].

2.6. Quantitative analysis of mitochondrial DNA (mtDNA)

Mitochondria were lysed in the presence of 0.5% SDS and 0.2 mg/ml proteinase K in 10 mM Tris–HCl, 0.15 M NaCl, and 0.005 M EDTA. mtDNA was then purified by DNA purification kit (Qiagen). Quantification of relative copy number differences was carried out as previously described [28].

2.7. Pro-inflammatory cytokines and NFκB DNA binding

TNF-α, IL-6 (BD bioscience), IL-1β (Abcam) and NFκB DNA binding (Thermo Scientific) were measured according to the manufacturer's instructions in muscle homogenate.

2.8. Measurement of reduced and oxidized glutathione levels and enzyme activities

Glutathione assay kit (Cayman Chemical Company) was used to measure the reduced glutathione (GSH) and oxidized glutathione (GSSG) levels in muscle. Citrate synthase (CS) and COX activities were measured as recommended by the manufacture's protocol (Sigma–Aldrich).

2.9. Statistical analysis

Experimental data were expressed as mean ± SE and group comparisons were made by one-way or two-way ANOVA with Tukey's HSD *post hoc* test.

3. Results

To test whether aging and/or exercise intensity affect skeletal muscle inflammation, we examined pro-inflammatory markers, TNF-α, IL-1β and MCP-1 in both young (Y) and old (O) mouse TA muscles. The old non-exercise (O-NE) mice showed a 61% increase in TNF-α compared to the young control mice (Y-NE) ($P < 0.05$). Furthermore, HE increased the TNF-α significantly in both the young (52%, $P < 0.05$) and old (101%, $P < 0.05$) groups, whereas LE did not affect it (Fig. 1A). The basal level of IL-1β in the O-NE group was 2.3-fold higher than in the Y-NE group ($P < 0.05$). There was a 69% increase in IL-1β with HE in the old group ($P < 0.05$), whereas both LE and HE did not affect the IL-1β levels in the young group (Fig. 1B). The MCP-1 mRNA increased 2.7-fold in the O-NE vs. the Y-NE groups. Similarly to TNF-α and IL-1β, LE did not change the MCP-1 mRNA in both the young and old groups. However, there was a 70–80% increase in MCP-1 mRNAs by HE in both the young and old groups ($P < 0.05$) (Fig. 1C).

The mitochondrial ROS production rate remained unchanged with aging, however LE increased the ROS generation significantly in both young (34%, $P < 0.05$) and old (37%, $P < 0.05$) groups compared to their corresponding NE controls. Notably, HE enhanced the mitochondrial ROS production rate dramatically in the old group vs. the O-NE (1.3-fold, $P < 0.01$), significantly, although there was a 31% increase in ROS with HE in the young group ($P < 0.05$) (Fig. 1D). Redox status, expressed as the GSSG/GSH ratio, was determined as a marker for oxidative stress. There was a 48% increase in GSSG/GSH in the O-NE vs. the Y-NE group. Both LE and HE in young mice did not affect the ratio; however, in the old groups, the ratio in the HE was higher than in the NE and LE groups by 45% ($P < 0.05$) and 32% ($P < 0.05$), respectively. Furthermore, GSSG/GSH levels in the old group were significantly higher than in the corresponding young control ($P < 0.05$) (Fig. 1E). The oxidative

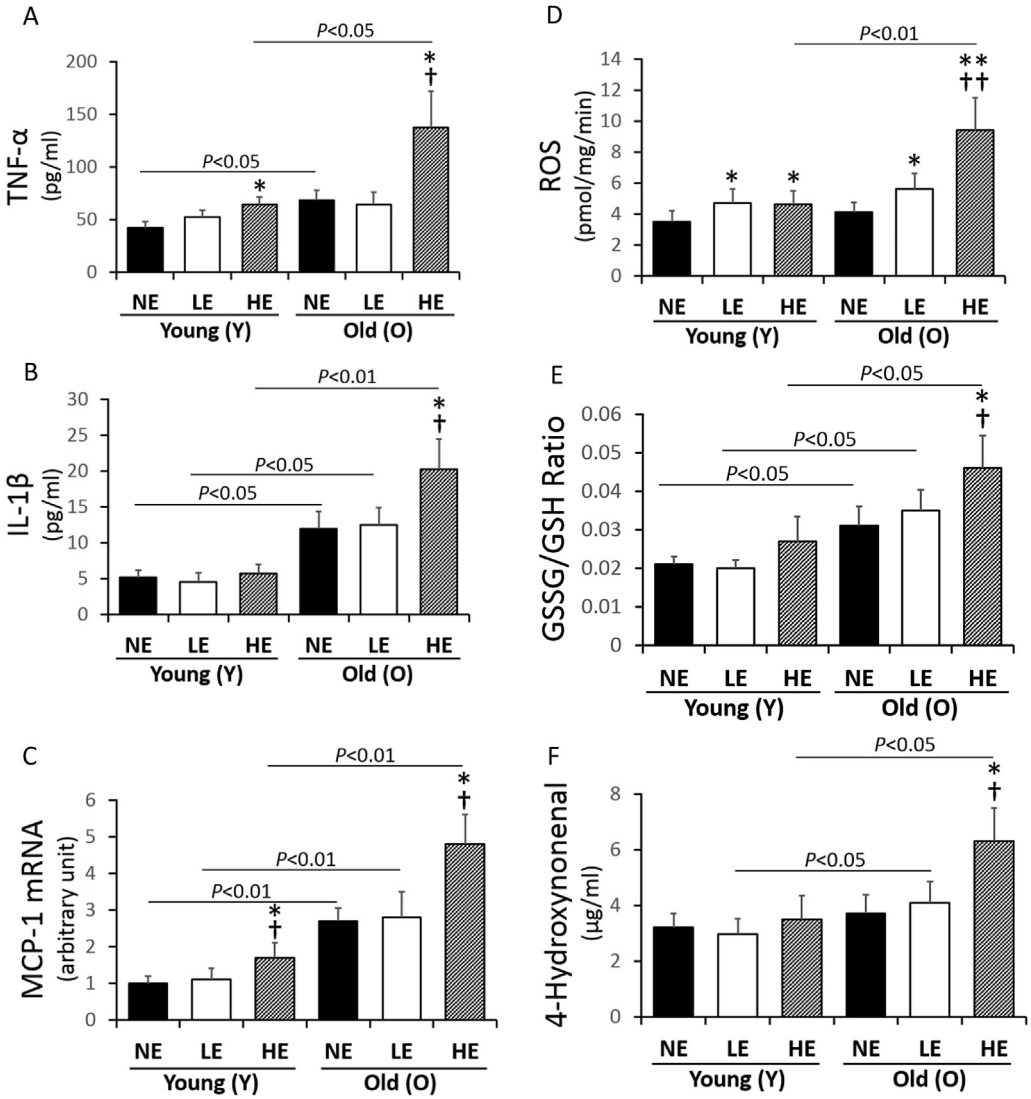


Fig. 1. Changes of proinflammatory cytokines and oxidative stress following low- or high-intensity exercise in young and old mouse soleus muscles. Proinflammatory cytokines, (A) TNF-α protein, (B) IL-1β protein and (C) MCP-1 mRNA levels were evaluated. (D) Levels of mitochondrial ROS production rate, (E) oxidized (GSSG) and reduced (GSH) glutathione ratio and (F) mitochondrial 4-hydroxynonenal were measured as oxidative stress markers. Values are the means ± SEM; NE, non-exercise control group ($N = 10$); LE, low-intensity exercise group ($N = 10$); HE, high-intensity exercise group ($N = 10$); *$P < 0.05$ vs. NE; **$P < 0.01$ vs. NE; †$P < 0.05$ HE vs. LE; ††$P < 0.05$ HE vs. LE; Two-way ANOVA with Tukey's HSD *post hoc* test.

damage marker, the 4-hydroxynonenal (4-HNE) level in mitochondria, was not changed in the O-NE group vs. the Y-NE. While both exercise types did not change the levels of 4-HNE in the young group, there was a 69% increase in 4-HNE for the O-HE, compared to the O-NE group ($P < 0.05$). The aged muscles showed a higher level of 4-HNE for both the LE (38%, $P < 0.05$) and HE (80%, $P < 0.05$) groups than in the corresponding young controls (Fig. 1F).

To determine mitochondrial biogenesis and function, we first observed the ratio of mtDNA to nDNA. There was a 46% reduction of the mtDNA:nDNA ratio in O-NE vs. Y-NE ($P < 0.05$). The young and the old LE groups increased the mtDNA:nDNA ratio by 47% and 86%, respectively ($P < 0.05$). We observed that the mtDNA:nDNA ratio was further increased by 35% in Y-HE vs. Y- LE ($P < 0.05$). The old group showed 86–94% increases in the mtDNA:nDNA ratio for both the O-LE and O-HE groups ($P < 0.05$) (Fig. 2A). We further assessed mitochondrial respiratory chain enzymatic activities to evaluate mitochondrial function. The CS activity was reduced by 30% in the O-NE vs. the Y-NE groups ($P < 0.05$). The young group showed ~2.5-fold increase in CS activities for both the LE ($P < 0.01$) and the HE group ($P < 0.01$) vs. the Y-NE. Also, CS activity in the old group was up-regulated by 53% in the LE compared to the NE, but there was no change in the HE group. CS activities in the old group were significantly lower than in the corresponding young control (Fig. 2B). We observed that COX activity was not significantly different between the Y-NE and the O-NE group, however ~2.2-fold increases in COX activities were observed in the Y-LE and the Y-HE groups ($P < 0.05$). In the old group, there was a 73% higher level of COX activity with LE vs. NE, however HE showed a 31% decrease in COX activity compared to LE ($P < 0.05$). The O-LE and O-HE groups showed a 35% and 59% decrease, respectively, in COX activity compared to the corresponding young control (Fig. 2C). Since the above results indicated that the HE regimen in the old group does not ameliorate mitochondrial dysfunction but evokes the inflammatory response, we next sought to understand how HE in aged muscle alters mitochondria biogenesis markers, PGC-1α and Tfam. Increased levels of total PGC-1α were observed in both LE and HE

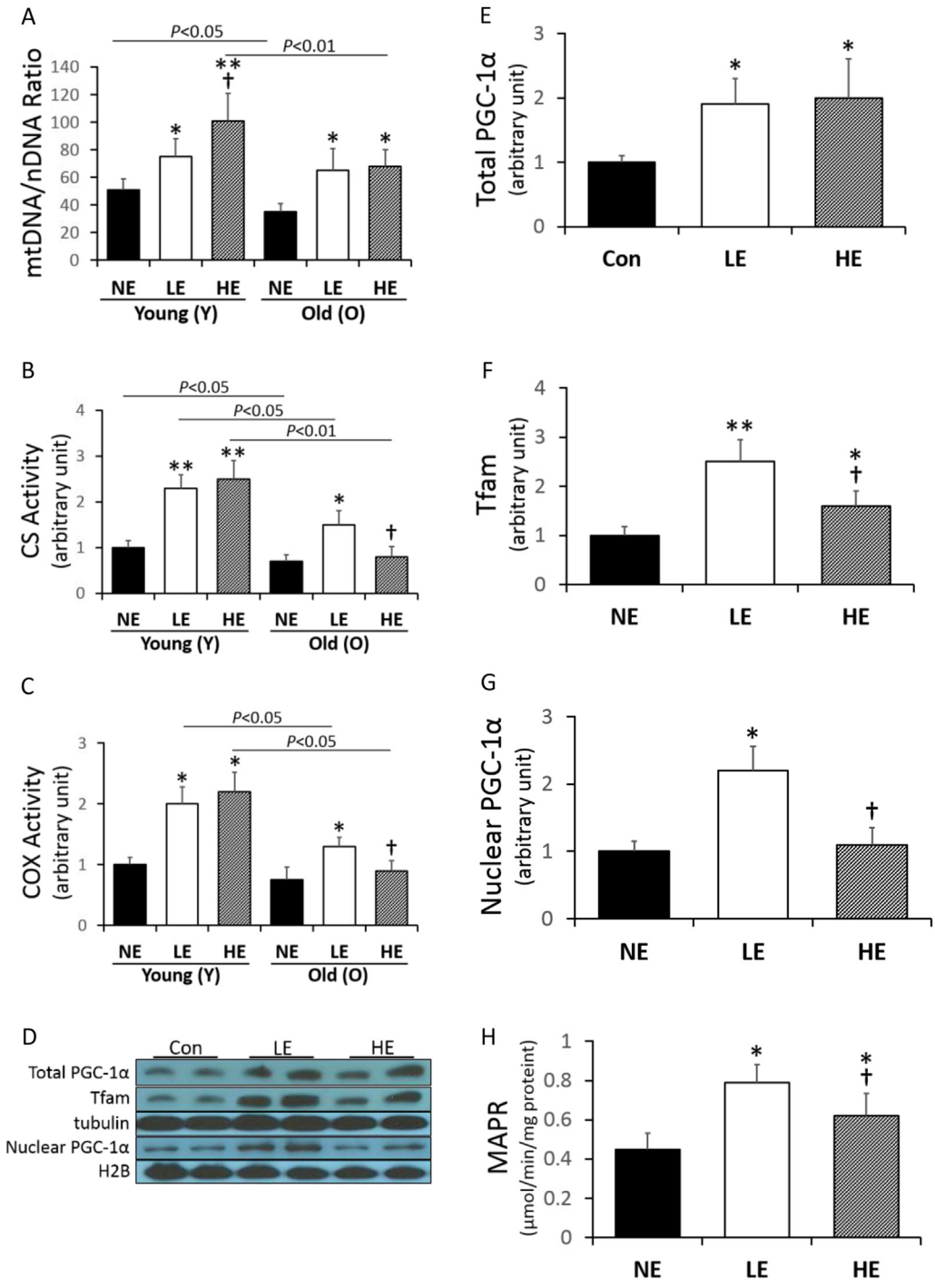
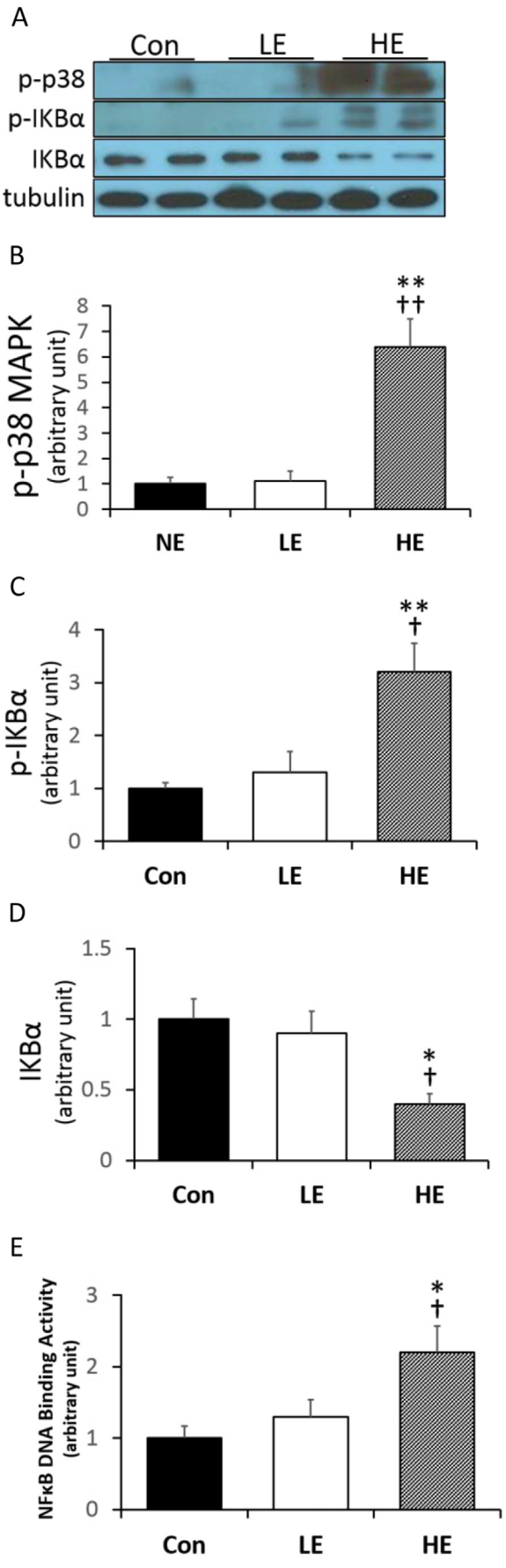


Fig. 2. Effect of low- and high-intensity exercise on mitochondrial biogenesis and function in mouse soleus muscles. (A) Mitochondria DNA (mtDNA) to nuclear DNA (nDNA) ratio, (B) citrate synthesis (CS) and (C) cytochrome *c* oxidase (COX) activity in young and old groups. (D) Representative Western blot images of total PGC-1α and Tfam in whole lysates, and nuclear PGC-1α in nuclear fractions in old groups. (E–G) The quantifications of total PGC-1α, Tfam, nuclear PGC-1α and (H) mitochondrial ATP production rate (MAPR) in old groups. Values are the means ± SEM; NE, non-exercise control group ($N = 10$); LE, low-intensity exercise group ($N = 10$); HE, high-intensity exercise group ($N = 10$); $^{*}P < 0.05$ vs. NE; $^{**}P < 0.01$ vs. NE; $^{\dagger}P < 0.05$ HE vs. LE; Two-way ANOVA (A–C) or One-way ANOVA (E–H) with Tukey's HSD *post hoc* test.

vs. NE in aged muscle by ~2-fold ($P < 0.05$) (Fig. 2D, E). The nuclear encoded mitochondrial protein (NEMP), Tfam was increased by 2.5- and 1.6-fold in LE ($P < 0.01$) and HE ($P < 0.05$) vs. NE, respectively, however it was reduced by 36% in HE compared to LE group ($P < 0.05$) (Fig. 2F). Moreover, we observed that the PGC-1α level in nuclear extraction was 2.2-fold higher in the LE vs. the NE group but was reduced by 50% with the HE vs. LE group in old mice ($P < 0.05$) (Fig. 2D, G). In parallel with the lower level of nuclear PGC-1α in the HE group compared to the LE in old mice, we also observed a 76% increase in MAPR with the LE vs. the NE group ($P < 0.05$), but it was reduced by 22% with the HE vs. LE ($P < 0.05$) (Fig. 2H).

Because p38 MAPK is involved in sensing the cellular redox state, phosphorylated (p)-p38 MAPK was evaluated in aged muscle. LE did not affect the p-p38 MAPK protein expression but it was



increased by 6.4-fold in the HE vs. the NE ($P < 0.01$) (Fig. 3A, B). Activation of the NFκB pathway was determined by measuring IκBα protein degradation and NFκB DNA binding activity. LE did not affect the level of p-IκBα protein content but it was significantly increased by 3.2-fold in the HE vs. the NE group ($P < 0.01$) (Fig. 3A, C). The IκBα protein content was not significantly changed by LE, whereas HE decreased the IκB content by 60% vs. NE ($P < 0.05$) (Fig. 3A, D). NFκB DNA binding activity was not changed by LE vs. NE. However, HE up-regulated the activities by 2.2-fold compared to NE ($P < 0.05$) (Fig. 3E).

To test whether exercise intensity affected mitochondrial fusion and fission, the protein levels of Mfn2 and Drp1 were quantitated. Mfn2 expression was not altered by LE but was decreased by 89% by HE vs. NE ($P < 0.01$) (Fig. 4A, B). There was no change in the fission protein, Drp1, with LE, but there was a 70% decrease in Drp1 in the HE vs. the NE ($P < 0.01$) (Fig. 4A, C). An indicator of mitophagic response, the p62 protein content was reduced by 35% in the LE vs. the NE ($P < 0.05$) but it was not changed in HE groups. Beclin-1 and the LC3-II to LC3-I ratio were not changed in the LE and HE vs. NE groups (Fig. 4).

4. Discussion

Strenuous exercise is known to be more susceptible to disturbance of cellular homeostasis caused by increased ROS generation and inflammation [25–27], however, it still has not been fully investigated whether such high intensity exercise leads to impairment of mitochondrial integrity in the senescent muscle. This study reveals that both LE and HE lead to an increase in mitochondrial copy number in the young and old groups. However, HE suppresses the up-regulation of mitochondrial function in the old group, although it is increased by LE. Thus, we suggest that excessive ROS and inflammatory cytokines generated by strenuous exercise 1) inhibit signals affecting mitochondrial function and/or 2) trigger mitochondrial degradation pathways. First, in regard to regulation of mitochondrial function, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) has emerged as a potent stimulator of mitochondrial biogenesis by co-activating nuclear encoded mitochondrial proteins (NEMPs) [29]. Previous studies have shown that aging attenuates PGC-1α pathways, resulting in a deficit of mitochondrial content and endurance training compensates for the decreases of PGC-1α pathways during aging [30]. It has been further reported that exercise-induced activation of the muscle PGC-1α pathway is blunted by the allopurinol treatment that reduces muscle ROS levels, suggesting the PGC-1α mediated mitochondrial pathway would be ROS sensitive [31]. However, although these are consistent for young mice in this study, the observations in old mice that there was an increased ROS along with an increased total PGC-1α expression, but suppressed mitochondrial function following strenuous exercise, rendered our interpretation more complex. Thus, we further observed the nuclear PGC-1α level as one of the determinants for its activation. To our surprise, unlike the total PGC-1α content, the level of nuclear PGC-1α protein remained unchanged in the HE compared to the NE group, though the levels were increased in the old group by LE. Thus, we postulated that excessive ROS generation seen in the old group may evoke inhibitory signals to suppress mitochondrial

Fig. 3. Redox-sensitive signaling pathways, p38 MAPK and NFκB are activated by high-intensity exercise in old mouse soleus muscles. (A) Representative Western blot images of phosphorylated (p)-p38, p-IκBα and IκBα. (B–D) The quantifications of p-p38 MAPK, p-IκBα and IκBα. (E) NFκB DNA binding activity. Values are the means ± SEM; NE, non-exercise control group ($N = 10$); LE, low-intensity exercise group ($N = 10$); HE, high-intensity exercise group ($N = 10$); $^{*}P < 0.05$ vs. NE; $^{**}P < 0.01$ vs. NE; $^{\dagger}P < 0.05$ HE vs. LE; $^{\dagger\dagger}P < 0.01$ HE vs. LE; One-way ANOVA with Tukey's HSD *post hoc* test.

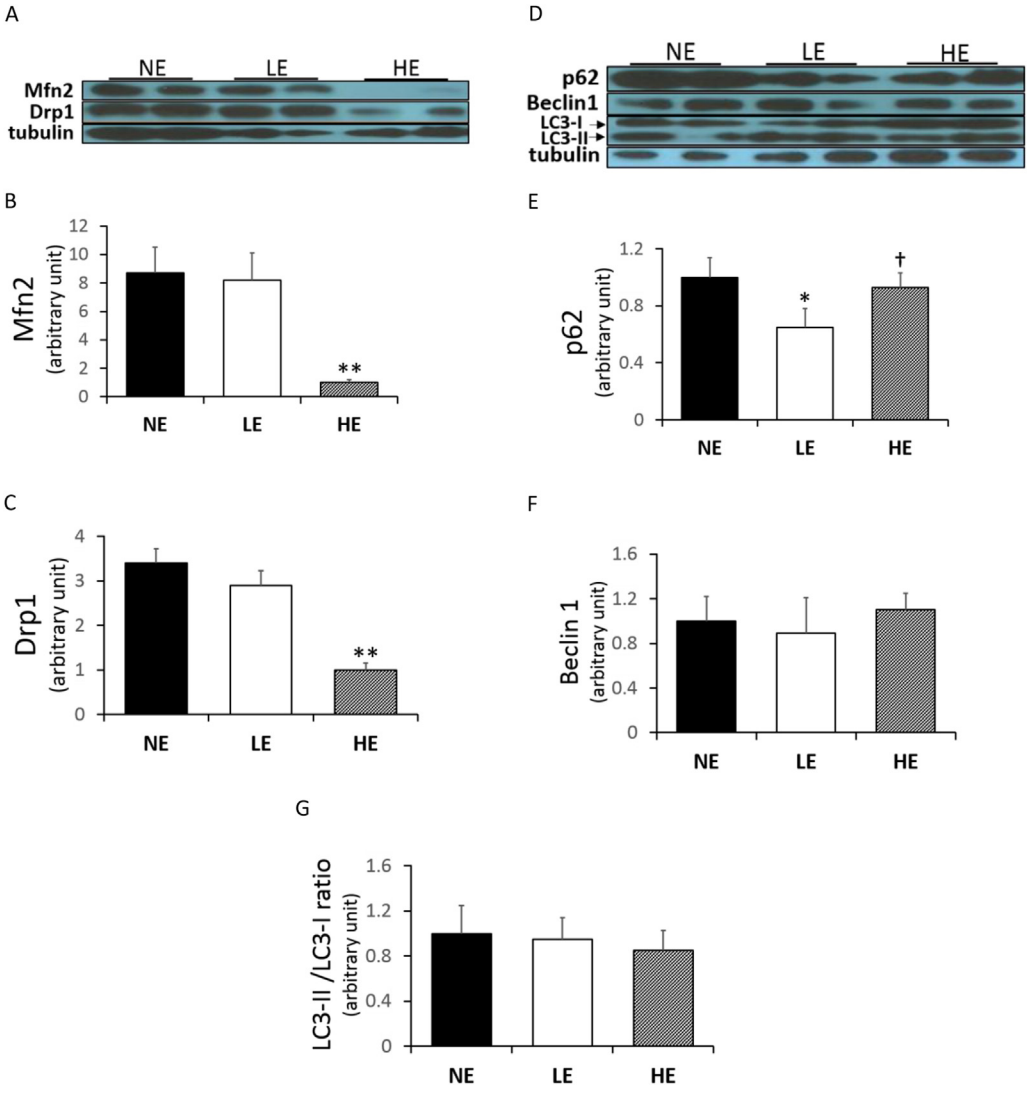


Fig. 4. Effect of low- and high-intensity exercise on mitochondrial dynamics and autophagy in old mouse soleus muscle. (A) Representative Western blot images of mitochondrial fusion and fission proteins. (B–C) The quantifications of Mfn1 and Drp1 proteins. (D) Representative Western blot images of mitochondrial autophagy proteins. (E–F) The quantifications of p62 and Beclin1 proteins. (G) LC3-II to LC3-I protein ratio. Values are the means ± SEM; NE, non-exercise control group ($N = 10$); LE, low-intensity exercise group ($N = 10$); HE, high-intensity exercise group ($N = 10$); $^{*}P < 0.05$ vs. NE; $^{**}P < 0.01$ vs. NE; $^{\dagger}P < 0.05$ HE vs. LE; One-way ANOVA with Tukey's HSD *post hoc* test.

function by reducing PGC-1α translocation into cell nuclear or activity. Since 1) the NFκB is known as an ROS sensitive factor and an excessive response would disrupt cellular homeostasis [32], and 2) previous evidence showed that PGC-1α interacts with a NFκB p65 subunit or reduced phosphorylation of p65, which may in turn inhibit the NFκB promoter or PGC-1α activities [33,34], this raises the possibility that HE-induced activated NFκB interacts with PGC-1α, thereby reducing PGC-1α nuclear translocation and/or transcriptional activity. However, it is still vague whether the activated NFκB directly interacts with or regulates PGC-1α nuclear translocation, although our observations implicate suppressed mitochondrial pathways with HE can be partly caused by reduced PGC-1α nuclear translocation.

Second, there would be increased mitochondrial degradation pathways by the strenuous exercise, facilitating changes in mitochondrial copy number and morphology, and, thereby, dysfunction. Disruption of mitochondria in terms of morphology can be explained by 1) suppression of mitochondrial fusion proteins that are essential for maintaining mitochondrial shape and integrity, or 2) alteration of *de novo* protein synthesis of mitochondrial fission or mitophagy pathways that contribute to mitochondrial fragmentation or degradation [35]. In the current study, rather than increasing fission protein, Drp1, we observed that HE in the old group decreased Drp1 as well as Mfn2, suggesting there are increased formations of enlarged mitochondria and reduced mitochondria turnover, which lead to mitochondrial dysfunction [36]. Indeed, a previous study demonstrated increased ROS levels from nutrient excess or palmitate exposure are deleterious to mitochondrial architecture and dynamics in muscle tissue and cells [37]. However, the study was performed with a different ROS source than our model, and there is still limited evidence showing a causative link between various biological sources of ROS and mitochondrial dynamics mechanisms *in vivo*. Nonetheless, our study provides initial insight into the role of exercise-induced ROS as a potential regulator of the mitochondrial dynamics network in senescent muscle. Since an aberrant ROS level as a mitochondrial waste product eventually leads to cytotoxicity and alteration of mitophagy pathways [38], we further tested whether the mitophagy markers such as p62, Beclin1 and LC3 are activated in response to exercise in the senescent muscle. However, our observation

showed that the mitophagy-associated proteins are not affected and this seems to be explained by the possibility that the elevated flux and thereby highly sustained levels of mitophagy factors in senescent muscles *per se* would have limited fluctuation following strenuous exercise. Taken together, it remains to be further explored 1) how excessive exercise-induced ROS or inflammation following HE mediates the fusion and/or fission pathways; 2) whether mitophagy flux can be changed in response to different exercise types or intensities; and 3) whether these results are paralleled by other biological sources of ROS in terms of mitochondrial degradation pathways in skeletal muscle aging.

In summary, these findings provide the new insight that strenuous exercise in senescent muscles suppresses up-regulating of mitochondrial function, with implications for the beneficial effect of low-intensity exercise for treating aging and age-related mitochondrial diseases.

Conflict of interest

None.

Acknowledgments

This work was supported by Hankuk University of Foreign Studies Research Fund of 2015.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.038.

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