EFFECTS OF EXERCISE TRAINING ON AGING-RELATED NAD⁺/SIRT1 PATHWAY IN MIDDLE-AGED AND AGED MICE

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ABSTRACT

Background and objective
The purpose of this study was to investigate the effects of regular exercise training on nicotinamide adenine dinucleotide/sirtuin 1 (NAD⁺/SIRT1) signaling protein levels in skeletal muscles of middle-aged and old-aged mice.

Material and methods
Experimental animals were 40 male C57BL/6 mice out of which 20 were 38-week-old (middle-aged) and the other 20 were 58-week-old (aged). They were divided into four groups: middle-aged control (MC), middle-aged exercise (ME), aged control (AC), and aged exercise (AE) groups (n = 10, each group). ME and AE groups performed exercise training five times weekly for 8 weeks using animal treadmill, after which gastrocnemius muscles were excised and analyzed.

Results
After 8 weeks of intervention, protein levels of AMP-activated protein kinase (AMPK), SIRT1, forkhead box protein 1 (FOXO1), and NAD⁺ levels were significantly lower in AC group than in MC group (p < 0.05). In addition, AMPK, SIRT1, FOXO1, NAD⁺, and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) levels were significantly higher in ME and AE groups that exercised for 8 weeks than in MC and AC groups that did not exercise (p < 0.05).

Conclusion

These results suggest that aging and exercise training have opposite effects on the NAD^+/SIRT1 pathway in gastrocnemius muscles and that exercise training can be effective in up-regulation of the aging-related NAD^+/SIRT1 pathway.

Keywords: *aging; AMPK; exercise training; FOXO1; NAD⁺; PGC-1α; SIRT1*

INTRODUCTION

It is not yet clear what mechanism causes the aging process, but mitochondrial dysfunction has been suggested as one of the main factors that accelerate aging.[1] It has recently been reported that nicotinamide adenine dinucleotide (NAD^+) levels in cells or tissues were reduced with increasing age and that this reduction due to aging led to cell dysfunction.[2] In addition, aging may reduce the level of sirtuin 1 (SIRT1), which inhibits the activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and forkhead box protein 1 (FOXO1), which causes mitophagy and reduces oxidative metabolism and antioxidant defense.[3] In addition, the reduction in NAD^+ and SIRT1 decreases mitochondrial unfolded protein response (UPRmt), which prevents cell aging,[4] and activates hypoxia-inducible factor 1-alpha (HIF-1α), thereby inhibiting mitochondrial gene expression and setting off aging.[3]

Although it is not clear whether NAD^+ reduction is due to increased consumption or decreased production of NAD^+, some mechanisms have proposed that increasing NAD^+ levels can alleviate the negative health effects of aging and exercise can activate NAD^+ and SIRT1.[5–7] Specifically, exercise induces rapid consumption of adenosine triphosphate (ATP), relatively increasing adenosine monophosphate (AMP) and activating AMP-activated protein kinase (AMPK).[5,6] Activation of AMPK increases NAD^+ ratio and induces activation of SIRT1. AMPK can phosphorylate PGC-1α directly.[7] While phosphorylated PGC-1α is deacetylated by

SIRT1 that is activated along with FOXO1,[8] this mechanism is reported to control mitochondrial biosynthesis and lipid metabolism gene expression,[9] strengthen antioxidant defense abilities to delay aging,[10] and prevent muscular dystrophy.[3] In addition, PGC-1α is involved in mitochondrial biosynthesis and it plays an important role in regulating energy metabolism.[11] Diaz et al.[12] suggested that the activation of PGC-1α has a positive effect on the prevention of aging-related diseases and the life extension in the mitochondrial disease model.

Previous studies have reported that administration of NAD^+ precursors such as nicotinamide mononucleotide (NMN) and caloric intake restrictions can induce an increase in NAD^+ levels, which may delay aging and inhibit the development of aging-related diseases. [1,3,13,14] As described above, although mechanisms have been proposed that exercise can increase NAD^+ levels, there have been few studies that investigate the changes in NAD^+ and SIRT1 regulation following exercise in skeletal muscles of different age groups. Meanwhile, it was suggested that acute high-intensity exercise can lead to excessive generation of reactive oxygen species (ROS) in the body.[15] The resulting ROS may accelerate aging and simultaneously deactivate AMPK in skeletal muscle through direct and/or indirect mechanisms.[6] Alternatively, moderate-intensity training is recommended for elderly subjects rather than high-intensity training since it reduces oxidative stress.[15] Additionally, it has been reported that the loss of skeletal muscle mass is mainly attributed to reduced type II muscle fibers.[16] Accordingly, this

needs to be verified for the gastrocnemius muscle. Therefore, this study aims to investigate the effects of moderate-intensity exercise training on the NAD$^+$ and SIRT1 pathway in gastrocnemius muscle for middle-aged mice when aging is being accelerated and in aged ones that aging proceeded.

METHODS

Animals and maintenance

Experimental animals were 40 male C57BL/6 mice out of which 20 were 38-week-old (middle-aged mice) and the other 20 were 58-week-old (aged mice), obtained from Samtako Bio Korea (Gyeonggi-do, Korea) and they were fed a standard laboratory diet (69.41% carbohydrate, 6.52% fat, and 24.34% protein; Research Diets, New Brunswick, NJ, USA) *ad libitum*. Four mice were housed per cage in the Dong-A University College of Medicine Animal Laboratory. The laboratory conditions were controlled for relative humidity (55 ± 5%), temperature (22 ± 2°), and light (12 h light/dark cycle; 07:00 light on, 19:00 light off). After 1 week of adaptation, the animals were randomly assigned 10 in each group, such as the middle-aged control (MC), middle-aged exercise (ME), aged control (AC), and aged exercise (AE) groups. The animal experiments were approved by the Dong-A University Medical School Institutional Animal Care and Use Committee (DIACUC-approval-13–21) and all the procedures were conducted in accordance with the committee guidelines.

Exercise intervention

The ME and AE groups were trained on an animal treadmill for 40 min/day, 5 days/week for 8 weeks using a modified exercise protocol described previously.[17,18] Exercise intensity consisted of 5 m/min (5 min), 10 m/min (30 min), and 5 m/min (5 min) at 0% slope from weeks 1 to 4 (low intensity). During weeks 5–8, exercise intensity was increased to 5 m/min (5 min), 14 m/min (30 min), and 5 m/min (5 min) at the same slope (moderate intensity).

Tissue sampling and analysis

To exclude the temporary exercise training effects, tissue sampling was conducted 48 h after the completion of the last exercise. Food was withdrawn from the mice cages 12 h prior to sacrifice. The gastrocnemius samples were excised after complete anesthesia using ethyl ether. The samples were immediately frozen in liquid nitrogen and stored at −80°C. As previously described,[19] to isolate protein from the gastrocnemius samples, 200 μl of radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 [NP-40], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail, and phosphatase inhibitor cocktail) was added. The tissue was homogenized and gastrocnemius tissue was lysed by incubation on ice for 30 min. The lysed gastrocnemius tissue was centrifuged at 13,000 rpm at 4°C for 30 min and the supernatant was transferred to a clean e-tube. The supernatant was quantified as 10 μg of a total protein sample using the BCA™ protein assay kit (PIERCE, USA). The protein was subjected to 12–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V. The membrane was blocked using 5% skimmed milk prior to overnight reaction at 4°C with primary antibody to AMPK (Cat. No. sc-398861), SIRT1 (Cat. No. sc-15404), PGC-1α (Cat. No. sc-13067), and FOXO1 (Cat. No. sc-374427) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by the secondary antibody reaction for 1 h at room temperature. Bound antibody was visualized by ECL solution (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the expression of protein was confirmed using ImageQuant™ LAS-4000 (GE Healthcare, Uppsala, Sweden). Each band level was normalized by comparing with β-actin (Cat. No. sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and total form. For NAD$^+$ analysis, gastrocnemius tissue protein was filtered through a 10 kD micro filter (Bio Vision, Milpitas, CA, USA) and it was analyzed with an NAD$^+$/NADH

quantitative kit (K958-400, Bio Vision, Milpitas, CA, USA).

Statistical analysis

Data were expressed as mean±standard error (SE) for all dependent variables. Analysis was performed using SPSS version 25.0 for Windows (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was used to verify the inter group differences in the results of body weight and tissue analyses. The Scheffe post-hoc test was used as a conservative locator of significant differences. All statistical significance levels (α) were set at 0.05.

RESULTS

Changes in body weight

Changes in body weight are shown in Figure 1. After 8 weeks of intervention, body weight was significantly different in all groups ($p<0.05$) except between AC and AE groups.

Changes in AMPK levels

Changes in AMPK levels of the gastrocnemius muscle are shown in Figure 2A. After 8 weeks of intervention, AMPK levels were significantly lower
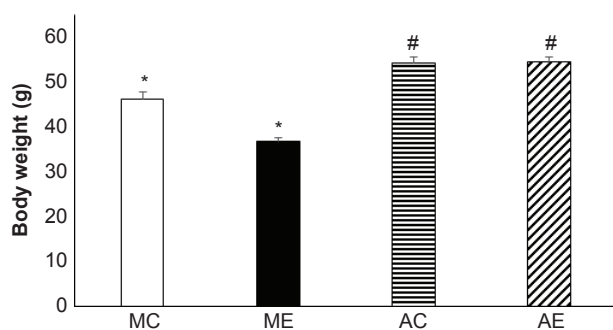

FIGURE 1 Changes in body weight after 8 weeks of intervention. Values are expressed as mean±SE, MC: middle-aged control, ME: middle-aged exercise group, AC: aged control group, AE: aged exercise group, *versus all groups ($p < 0.05$), and # versus MC and ME groups ($p < 0.05$).

in AC group than in MC group ($p = 0.045$). In addition, AMPK levels were significantly higher in ME and AE groups that exercised for 8 weeks than in MC and AC groups that did not exercise ($p = 0.040$; $p = 0.004$, respectively).

Changes in SIRT1 levels

Changes in SIRT1 levels of the gastrocnemius muscle are shown in Figure 2B. After 8 weeks of intervention, SIRT1 levels were significantly lower in AC group than in MC group ($p=0.048$). In addition, SIRT1 levels were significantly higher in ME and AE groups that exercised for 8 weeks than in MC and AC groups that did not exercise ($p=0.003$; $p=0.001$, respectively).

Changes in PGC-1α levels

Changes in PGC-1α levels of the gastrocnemius muscle are shown in Figure 2C. After 8 weeks of intervention, PGC-1α levels were significantly higher in ME and AE groups that exercised for 8 weeks than in MC and AC groups that did not exercise ($p=0.041$; $p=0.037$, respectively).

Changes in FOXO1 levels

Changes in FOXO1 levels of the gastrocnemius muscle are shown in Figure 2D. After 8 weeks of intervention, FOXO1 levels were significantly lower in AC group than in MC group ($p = 0.019$). In addition, FOXO1 levels were significantly higher in ME and AE groups that exercised for 8 weeks than in MC and AC groups that did not exercise ($p = 0.003$; $p = 0.001$, respectively).

Changes in NAD⁺ levels

Changes in NAD^+ levels of the gastrocnemius muscle are shown in Figure 2E. After 8 weeks of intervention, NAD^+ levels were significantly lower in AC group than in MC group ($p = 0.031$). In addition, NAD^+ levels were significantly higher in ME and AE groups that exercised for 8 weeks than in MC and AC groups that did not exercise ($p = 0.008$; $p = 0.044$, respectively).
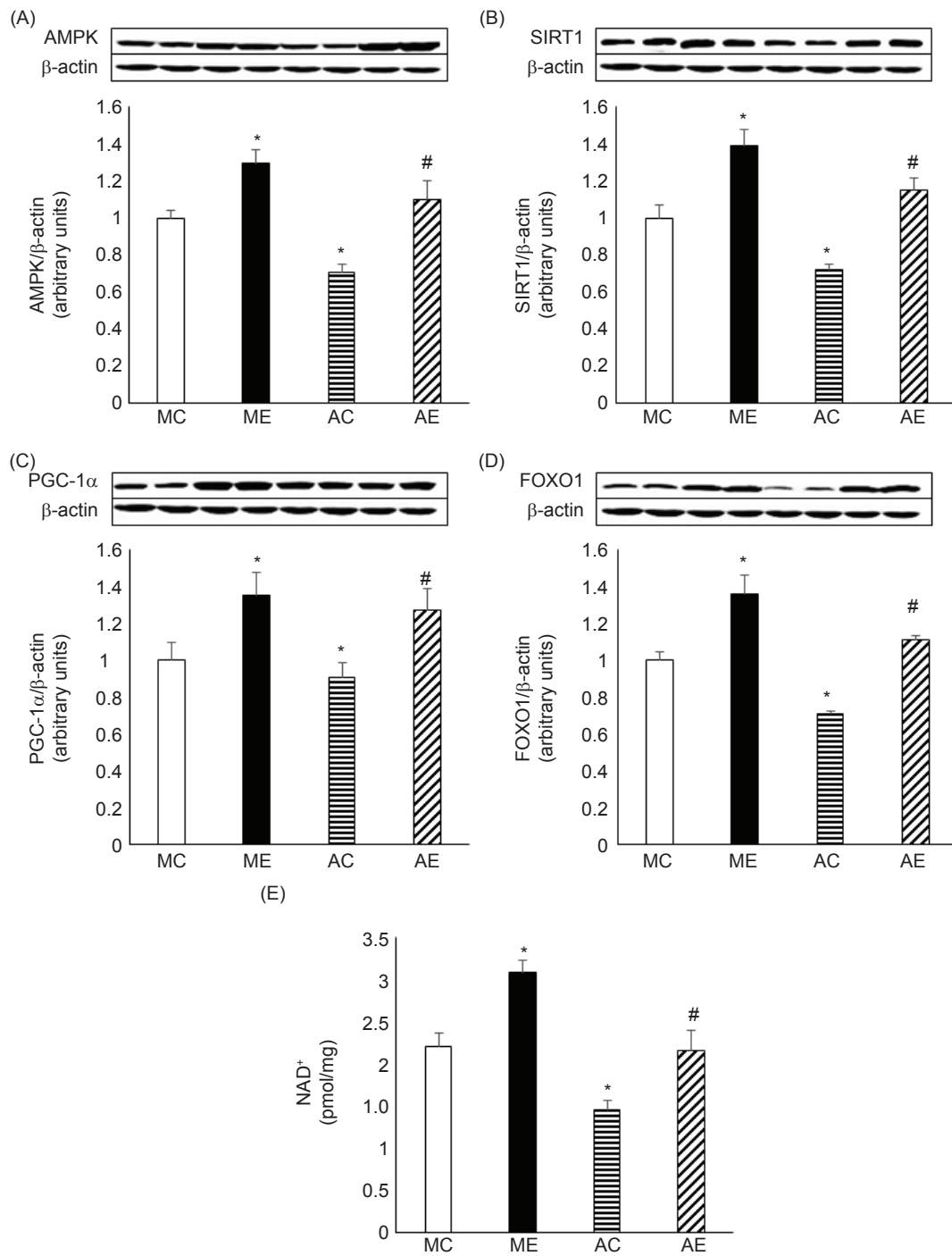
FIGURE 2 Changes in AMPK (A), SIRT1 (B), PGC-1α (C), FOXO1 (D), and NAD^{+} (E) levels after 8 weeks of intervention. Values are expressed as mean±SE, MC: middle-aged control, ME: middle-aged exercise group, AC: aged control group, AE: aged exercise group, *versus MC group (p<0.05), and #versus AC group (p < 0.05).

DISCUSSION

NAD^{+} is a universal and an essential coenzyme involved in the redox reaction of several cells, and is a substrate that regulates metabolic homeostasis and SIRT1 activity.[1,20] Changes in NAD^{+} levels have been reported to have a strong effect on energy metabolism and aging because they act as a substrate essential for the deacetylase activation of SIRT1 protein.[21,22] Imai and Yoshino[20] reported that NAD^{+} and SIRT1 activity may decrease with age and as NAD^{+} levels decrease, the incidence of aging-related diseases may also increase.[1–3] Therefore, several previous studies[13,14] have shown that administration of an NAD^{+} precursor or caloric restriction can increase NAD^{+} levels, while Katsyuba et al.[23] suggested that de novo NAD^{+} synthesis is effective in improving mitochondrial function and promoting health. In this study, the levels of AMPK, SIRT1, PGC-1α, FOXO1, and NAD^{+} protein in gastrocnemius muscles were analyzed to verify the effect of exercise on the aging-related NAD^{+}/SIRT1 pathway in middle-aged and aged mice. The results showed that the levels of AMPK, SIRT1, FOXO1, and NAD^{+} protein were significantly lower in the aged AC group than in the middle-aged MC group. These results suggest that AMPK, SIRT1, FOXO1, and NAD^{+} levels may decrease with age, which supports several previous studies[2,24,25] that reported a decrease in AMPK, SIRT1, FOXO1, and NAD^{+} levels due to aging. Specifically, AMPK not only plays a key role in maintaining energy homeostasis in the cell, but also induces various biological changes, such as anti-aging, through SIRT1 signaling,[26,27] and it has been reported that AMPK activation is reduced in aged skeletal muscles.[24] In a study by Kim et al.,[25] FOXO1—one of the FOXO families that regulate metabolism, cell cycle, cell death, and oxidative stress response— was significantly lower in old rats than in young rats. In addition, Fang et al.[2] suggested that NAD^{+} levels decrease with increasing age, leading to cell dysfunction as a consequence of aging.

On the other hand, according to the results of this study, ME and AE groups that exercised for 8 weeks showed significantly higher levels of AMPK, SIRT1, PGC-1α, FOXO1, and NAD^{+} than MC and AC groups that did not exercise. These results suggest that regular exercise training is effective for upregulation of the NAD^{+}/SIRT1 pathway regardless of age increase and activation of AMPK through exercise is likely to have played a major role. The increased ratio of AMP/ATP by muscle contraction is a major factor that induces skeletal muscle adaptation and AMPK monitors the concentration of AMP for cell energy homeostasis.[28] That is, as exercise leads to a decrease in ATP stored in skeletal muscle, energy demands for maintaining exercise increase rapidly. Along with this, an increase in AMP or NAD^{+} levels within the cell becomes an important factor in activating the metabolic system inside the mitochondria and cytoplasm.[5,29] Changes in these two factors have been proposed to be important substrates that activate AMPK and SIRT1, respectively.[30] Salminen and Kaarniranta[30] suggested that aging decreases AMPK activation but exercise is one of the factors that can activate AMPK and SIRT1. Han et al.[31] suggested that AMPK induces FOXO1 activation and deacetylation of PGC-1α, which results in maintaining cell homeostasis and preventing aging. In addition, according to previous studies,[23,32–35] while aging reduced NAD^{+} and PGC-1α levels, exercise intervention increased SIRT1 levels and regulated the proteins of PGC-1α and FOXO1, which had a positive impact on delayed mitochondrial metabolism and aging. Exercise also increased NAD^{+} protein levels, thereby regulating aging-related mechanisms.

Limitations in this study include the fact that the same exercise protocol was used in mice of different age groups which were middle-aged and aged. Therefore, future studies will require interventions with the same settings of exercise intensity and it is necessary to examine the changes in variables at various levels of tissue and/or muscle such as soleus muscle. In addition, in order to present clearer and more reliable results, future studies will require gene expression analysis through real-time PCR (qPCR) and measurement of phospho-AMPK levels.

CONCLUSIONS

In conclusion, aging can downregulate the NAD$^+$/SIRT1 pathway by lowering the levels of AMPK, SIRT1, FOXO1, and NAD$^+$ protein in the gastrocnemius muscle, whereas exercise training is effective in upregulating NAD$^+$/SIRT1 pathway regardless of increase in age.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

FUNDING

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