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Research article

Expression of Heat Shock Proteins (HSPs) in Aged Skeletal Muscles Depends on the Frequency and Duration of Exercise Training

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Abstract
The skeletal muscle in aged rats adapts rapidly following a period of exercise. This adaptation includes structural remodeling and biochemical changes such as an up-regulation of antioxidant enzymes, content of stress and heat shock proteins (HSPs). However, the associated—molecular mechanisms mediating different types of exercise training-induced adaptations are not yet completely understood. Therefore, the purpose of this study was to investigate the effects of duration and frequency exercise on the expression of HSPs, antioxidant enzymes, and mitogen-activated protein kinase (MAPKs) in the skeletal muscles of aged rats. Young (3-month-old) and aged (20-month-old) male Sprague-Dawley rats were randomly assigned to 6 groups and extensor digitorum longus (EDL; fast twitch muscle fiber) and soleus (SOL; slow twitch muscle fiber) skeletal muscles were collected immediately. The expression pattern of HSPs in skeletal muscles was decreased in old groups compared with young groups. Especially, HSPs showed lower expression in SOL than EDL muscle. Interestingly, HSPs in aged rats was increased significantly after S1 (single long-duration; 1×30 min, 5 days/week for 6 weeks) and M1 types (multiple short-duration; 3×10 min·day^{-1}, 5 days·week^{-1} for 6 weeks) than S2 (single long-duration; 1×30 min, 3 days/week for 6 weeks) and M2 (multiple short-duration; 3×10 min·day^{-1}, 3 days·week^{-1} for 6 weeks) types of exercise training. Also, superoxide dismutase (SODs) showed similar expression as HSP did. On the contrary, the p-ERK and p-JNK were down regulated. In addition, p-p38 level in the SOL muscle was activated markedly in all exercise groups. These results demonstrate that increasing of HSP expression through duration and frequency exercise can lead to protection and training-induced adaptation against aging-induced structural weakness in skeletal muscles.

Key words: Aging, multiple short-duration exercise training, single long-duration exercise training, heat shock protein, superoxide dismutase, mitogen-activated protein kinase.

Introduction

Aged skeletal muscles become smaller and weaker, which are also more susceptible to injury and take considerably longer to repair (Menshikova et al., 2006; Pasini et al., 2012). Previous studies have shown that the regular exercise leads to training-induced adaptation of aged muscles through contractile protein synthesis (Menshikova et al., 2006; Ogura et al., 2011). This adaptation includes structural remodeling and biochemical changes such as an up-regulation of antioxidant enzymes, content of stress and heat shock proteins (HSPs) (McArdle and Jackson, 2000; Vasilaki et al., 2003).

HSPs play an important role in cellular processes, such as cell survival, proliferation and prevention of apoptosis against oxidative stress and aging (Koh and Escobedo, 2004; Vasilaki et al., 2003; Weber et al., 2012). HSPs are classified into hsp 27, 60, 70 and 90 according to the molecular weight. The expression of HSPs in skeletal muscles increases with exercise training, and it causes inhibition of apoptosis and cytoskeletal protection, which are needed for maintaining homeostasis and preventing myotube degeneration (Gabai and Sherman, 2002). Furthermore, HSPs correlates with high expression of SODs, which acts as a protective mechanism against oxidative stress in skeletal muscles after exercise (Murlasits et al., 2006; Zembron-Lacny et al., 2008). Among signaling pathway, MAPKs are general mediators of the stress response during exercise training (Nader and Esser, 2001). ERK1/2, p38 and JNK of MAPKs families are activated by various environmental stresses and related to activation of HSPs (Cargnello and Roux, 2011). Study about the exercise-induced production of HSPs in the skeletal muscle of aged rats is important as it may provide a valuable insight into the molecular mechanisms which can increase muscle mass against age-related stressors by regular exercise (Morton et al., 2009). Previous studies have demonstrated that HSP may differ in aged animal skeletal muscles following a period of duration and frequency exercise as well as numerous muscle structural and biochemical changes including increased antioxidant enzymes activity (McArdle and Jackson, 2000; Nader and Esser, 2001; Noble et al., 2008). Nevertheless, the effects of duration and frequency of exercise on the expression of HSPs in the skeletal muscle of aged animals are unknown. Therefore, the purpose of this study was to investigate whether different types of exercise training affect the expression of HSPs, SODs, and MAPKs in skeletal muscles of aged rats.

Methods

Animals

Male Sprague-Dawley rats [young (4 months old) and aged (21 months old) rats] were kept in a room that had an inverted 12h light/dark cycle under standard conditions of temperature and humidity. The animals were divided into the following 8 groups: young control (YC, n = 6) and old control (OC, n = 6); single long-duration exercise-trained old groups (OS1, n = 7): 1×30 min, 5 days·week^{-1} for 6 weeks; second type of single long-duration exercise-trained old groups (OS2, n = 8): 1×30

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min, 3 days·week^{-1} for 6 weeks; multiple short-duration exercise-trained old groups (OM1, n = 8): 3×10 min·day^{-1}, 5 days·week^{-1} for 6 weeks; second type of multiple short-duration exercise-trained old groups (OM2, n = 8): 3×10 min, 3 days/week for 6 weeks. The rats in the exercise groups were subjected to a treadmill physical training program. A standard laboratory animal chow (56.8% carbohydrates, 22.5% proteins, 3.5% lipids and 17.2% other nutrients; Korean animals) and water were given ad libitum. The experiment was approved by the Ethical Committee of the Chonbuk National Universityv (CBU: 2012-0046) for care and experimentation of laboratory animals.

Physical training protocol

The training protocol has been described previously (Ogura et al., 2011). In the first week of the preliminary experiment, the rats were adapted to the treadmill (Omnipacer model LC-4, Omnitech, Columbus, OH), and Exercise consisted of 5~10 min at a speed of 5~10 m·min^{-1} with an incline of 0°. This program comprised of running on a treadmill for 30 (30×1) min, 5 days or 3 days per week for 6 weeks, and for 30 (10×3) min, 5 days or 3 days per week for 6 weeks at a maximal intensity or speed by aging rats (10 m·min^{-1}, 5° incline). Electric shocks were used sparingly to motivate the animals to run. Electrical shocks were applied to the metal grid behind the lane to stimulate the rats that failed to run spontaneously. The rats in the non-exercise group remained in their cages. Rats were sacrificed 48 h after their last exercise bout to minimize the influence of the last bout of exercise. Samples of blood and extensor digitorum longus (EDL; fast type) and soleus (SOL; slow type) skeletal muscles were collected. Separated serum and skeletal muscles were stored at –80°Cbefore determining cytokine levels and protein activity.

Western blot analysis

The total proteins were extracted from soleus and EDL muscle tissues using a lysis buffer containing 150 mmol·L^{-1} NaCl, 5 mmol·L^{-1} EDTA, 50 mmol·L^{-1} Tri-HCl (pH 8.0), 1%-NP 40, 1 mmol·L^{-1} aprotinin, 0.1 mmol leupeptin, and 1 mmol·L^{-1} pepstatin and quantified according to the Bradford dye-binding procedure (Bio-Rad, Hercules, CA, USA). A total protein of 20 µg was electrophoresed on 8% SDS-polyacrylamide gel under denaturing conditions, and then transferred to a Hybond-P membrane (Amersham, Arlington, IL, USA) with a Miniprotean II system (Bio-Rad, Hercules, CA, USA). After blocking with 5% skimmed milk in phosphate buffered saline (PBS), the membranes were incubated with the appropriate antibody [i.e. HSP27 (ADI-SPA-803, Enzo Life Sciences Inc, NY, USA); HSP60 (SC-13966, Santa Cruz Biotech, Santa Cruz, CA, USA); HSP70 (ADI-SPA-810, Enzo Life Sciences Inc, NY, USA); HSP90 (ADI-SPA-836, Enzo Life Sciences Inc, NY, USA); p-ERK (#9102, Cell signaling Beverly, MA, USA); p-p38 (SC-7975-R, Santa Cruz Biotech, Santa Cruz, CA, USA); p-JNK (#9251, Cell signaling Beverly, MA, USA); Mn-SOD (SC-30080, Santa Cruz Biotech, Santa Cruz, CA, USA); Cu/Zn SOD (ADI-SOD-100, Enzo Life Sciences Inc, NY, USA); ES-SOD (SC-32220, Santa Cruz Biotech, Santa Cruz, CA, USA); HO-1 (SC-136960, Santa Cruz Biotech, Santa Cruz, CA, USA) and tubulin (Sigma-Aldrich, St. Louis, MO, USA)] at a 1:1,000 dilution in either 1% skim milk or 3% BSA for 4 or 24 h at 4°C or room temperature. After washing with PBS containing 0.1% Tween-20, once for 15 min and twice for 5 min, the membranes were incubated with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase at a 1:3,000 dilution in PBS for 1 h at room temperature. After the final wash, the immune-reactive bands were visualized with chemiluminescent detection using LAS-4000 CCD Imaging system (Fujifilm Corp. Tokyo, Japan). The protein expression levels were analyzed with immageQuanT TL 1D gel analysis programme (GE Healthcare Bio-Science AB, Sweden).

Statistical analyses

The group comparisons were assessed by one-way analysis of variance (ANOVA) and Student-Newman-Keuls post hoc test. Null hypotheses of no difference were rejected if p-values were $p < 0.05$. The values are presented as mean ± SD.

Results

Morphological characteristics

Age-related skeletal muscle mass is characterized as the loss of muscle fibers including type II fiber of EDL muscle (Pasini et al., 2012). In this study, EDL muscle mass was decreased in OC group compared to YC group. 6 weeks of exercise training types significantly increased the weight of the EDL and SOL muscle fibers in aged rats (Figure 1B. C). In particular, OS1 and OM1 groups were increased significantly compared with OS2 and OM2 groups ($p < 0.05$). However, Body weight didn't show any difference in old groups despite exercise training (Figure 1A).

The expression of HSPs after different types of exercise training in aged rats

The HSP27, 60, 70 and 90 were decreased significantly in OC group compared to the YC group ($p < 0.05$). HSP27 was increased markedly by almost all exercise groups in EDL and SOL muscles. In particular, OS1 and OM1 groups showed significantly increased than OS2 and OM2 groups in expression of HSP27 ($p < 0.05$) (Figure 2A). Similarly, the HSP60, 70 and 90 levels were increased markedly in OS1 and OM1 groups of old groups ($p < 0.05$) (Figure 2B, C, D). Above all, the HSPs levels were mostly increased in OS1 group than OM1 group ($p < 0.05$).

The expression of antioxidants after different types of exercise training in aged rats

The Mn-SOD showed different pattern of expression with exercise training in the EDL and SOL muscles. Mn-SOD in SOL muscle was increased significantly in all exercise groups. In particular, OS1 group showed significantly increased expression than OS2 group. However, in EDL muscle, all exercise groups showed reduction pattern ($p <$

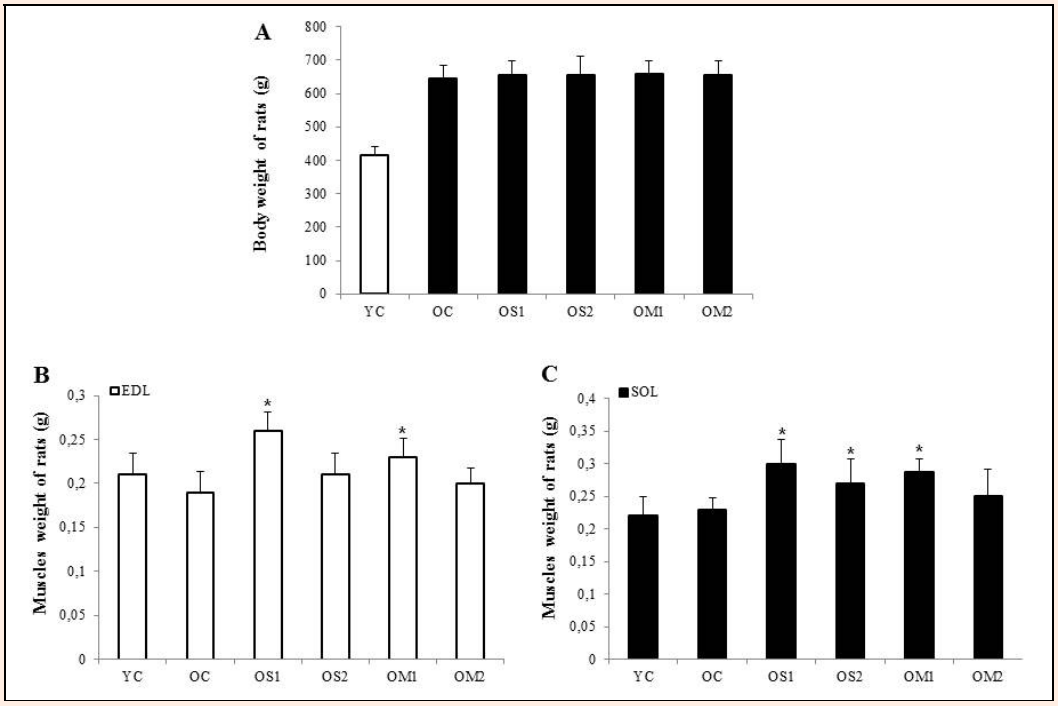

Figure 1. The body (A) and EDL (B), SOL (C) muscle weights were determined in young and old rats after 6 weeks of exercise training. The values are presented as mean ± standard deviation of animals per group ($p < 0.05$). The groups are young control (YC), old control (OC), single long-duration exercise-trained (OS1; 5 days·weeks^{-1}, 1×30 min/day, OS2; 3 days·weeks^{-1}, 1×30 min·day^{-1}), and multiple short-duration exercise-trained (OM1; 5 days·weeks^{-1}, 3×10 min·day^{-1}, OM2; 3 days/weeks, 3×10 min·day^{-1}). EDL muscle; extensor digitorum longus, SOL muscle; soleus. The symbols * indicates = significantly different from the OC group ($p < 0.05$).

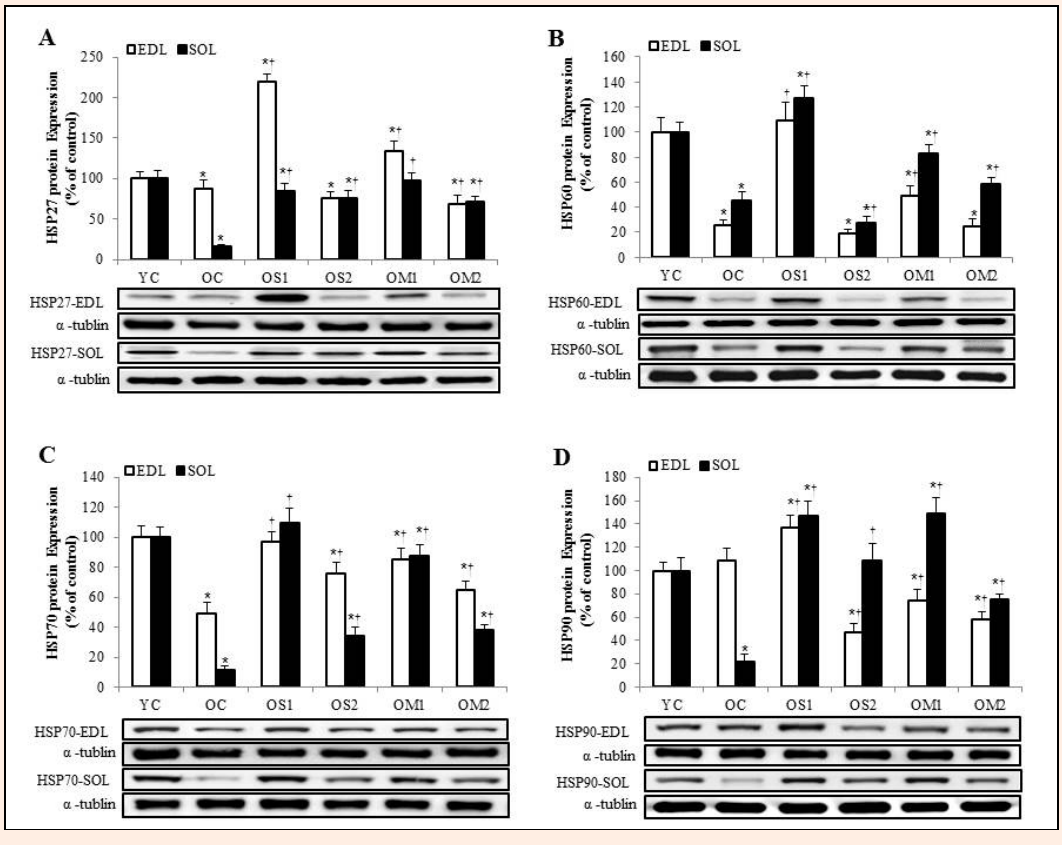

Figure 2. The expression levels of HSP27 (A), HSP60 (B) HSP70 (C), and HSP90 (D) proteins in the extensor digitorumlongus (EDL) and soleus (SOL) muscles. Data are presented as percentage of mean YC values. Representative blots are shown. The symbols * indicates = significantly different from the YC group ($p < 0.05$). The symbol † indicates = significantly different from the OC group ($p < 0.05$).

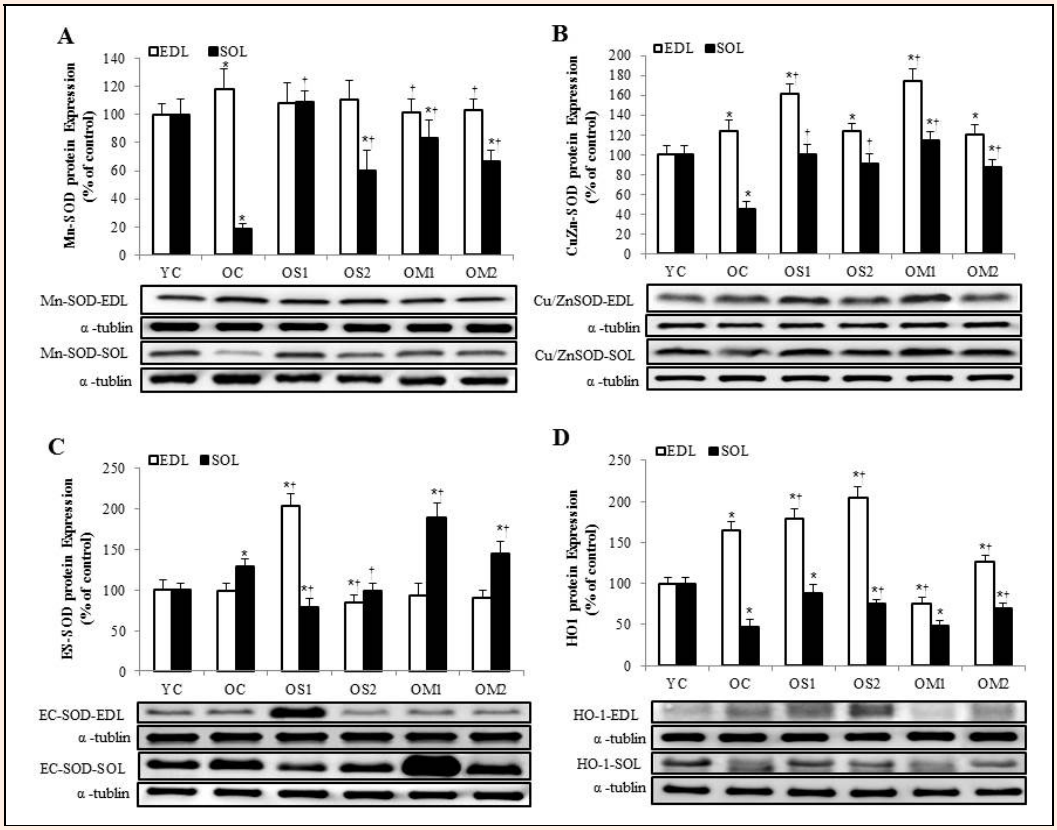

Figure 3. The expression levels of Mn-SOD (A), Cu/Zn-SOD (B) ES-SOD (C), and HO-1 (D) proteins in the extensor digitorum longus (EDL) and soleus (SOL) muscles. Data are presented as percentage of mean YC values. Representative blots are shown. The symbol * indicates = significantly different from the YC group ($p < 0.05$). The symbol † indicates = significantly different from the OC group ($p < 0.05$).

0.05) (Figure 3A). The Cu/Zn-SOD was increased significantly after exercise training in both EDL and SOL muscles ($p < 0.05$) (Figure 3B). Also, EC-SOD in EDL muscle was increased significantly in OS1 group. In contrast, EC-SOD in the SOL muscle was increased significantly in OM1 group ($p < 0.05$) (Figure 3C). In addition, heme oxygenase-1 (HO-1) in both EDL and SOL muscles were increased significantly in OS1 exercise training group ($p < 0.05$) (Figure 3D).

The expression of MAPKs after different types of exercise training in aged rats

The MAPKs showed different pattern of expression according to the muscle types, age, and exercise (Figure 4A, B, C). p-ERK in SOL muscle was inhibited significantly in OS1 and OM1 groups. In particular, OM groups were decreased markedly in both EDL and SOL muscles ($p < 0.05$) (Figure 4A). Also, p-p38 in EDL muscle was decreased significantly in the OS2 and OM groups. Conversely, p-p38 level in SOL muscle was increased markedly in all exercise groups ($p < 0.05$) (Figure 4B). Moreover, p-JNK level was decreased significantly in OS1 and OS2 groups of the EDL and SOL muscles ($p < 0.05$) (Figure 4C).

Discussion

In this study, HSPs, SODs and MAPKs showed different pattern of expression on the types of exercise training and muscle types. The HSPs are usually named according to their molecular weight (McArdle and Jackson, 2000) and prevent muscle damage caused by exercise training and aging (McArdle et al., 2002; Murlasits et al., 2006). Recent studies showed that HSPs have demonstrated different expression patterns according to the duration of exercise, intensity, and muscle types (Noble et al., 2008; Paulsen, 2007). Especially, they show high expression in slow-twitch oxidative skeletal muscle such as the soleus (Noble et al., 2006; 2008). Therefore, the expression of HSPs may differ according to the characteristics of the skeletal muscle- and exercise-specific, which is highly sensitive to aging-induced oxidative stress (Lollo et al., 2013; Noble et al., 2008; Paulsen, 2007). In addition, hemo oxygenase-1 (HO-1) is recognized as a major heat shock and stress response protein in cellular defense against oxidative stress (Elbirt and Bonkovsky, 1999). In this study, the expressions of HSPs and HO-1 were lower in aged rats compared to young rats. However, HSPs showed a remarkable increase in skeletal muscles by exercise training. In particular, they were elevated markedly by means of OS1 and OM1 training in SOL muscle. Above all, the HSP levels were mostly increased in the OS1 group rather than the OM1 group. These results suggest that the S1 and M1 types of exercise methods (5days/week) have a similar recovery capacity against aging- and exercise-induced oxidative stress in highly

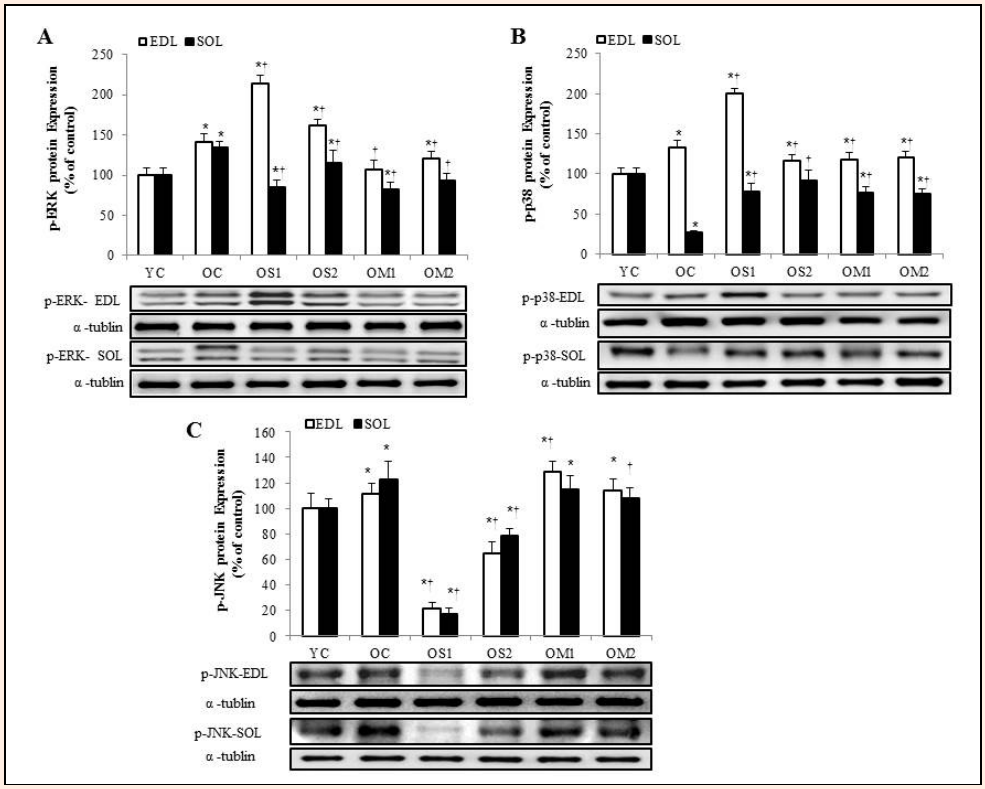

Figure 4. The expression levels of p-ERK (A), p-p-38 (B), p-JNK (C) proteins in the extensor digitorum longus (EDL) and soleus (SOL) muscles. Data are presented as percentage of mean YC values. Representative blots are shown. The symbol * indicates = significantly different from the YC group ($p < 0.05$).The symbol † indicates = significantly different from the OC group ($p < 0.05$).

oxidative muscles such as the soleus muscle of old rats. This study resulted in important and beneficial finding that HSP expression appears to be proportional to the duration and frequency of exercise training.

Skeletal muscles of aged rats are significantly susceptible to age-induced oxidative damage (McArdle and Jackson, 2000). This suggests that for improvement in the muscles, antioxidant capacity might be important following exercise (Nader and Esser, 2001). This study is consistent with the previous study which showed that the level of SODs is lower in old group compared with young group without exercise training. However, the level of SODs showed increased expression in aged rats by exercise training. In particular, the level of SODs elevated higher in OS1 and OM1 groups than OS2 and OM2 groups in both EDL and SOL muscle. It is clear that activation of SODs has associated with duration and frequency of exercise as well as the type of skeletal muscle. These findings suggest that antioxidant enzyme activity in aging may be differently activated following various types, intensity, and duration of exercise training. It is possible that the function of HSPs might have relevance to activation of SODs following exercise training in aging muscle. However, OS and OM groups in EDL muscle showed abbreviated expressions of Mn-SOD protein due to the exercise training. Because, it may be possible that the maladjustment state by the exercise caused high levels of oxidative stress in EDL muscle of aged rats.

MAPKs are known to be related to activation of HSPs. This pathway is activated by various environmental stresses and growth factors (Carlson et al., 2001). Also, it is involved in exercise-induced adaptations in skeletal muscle (van Ginneken et al., 2006). Kramer and Goodyear (2007) demonstrated that the role of MAPK activation by exercise is the transcriptional regulation of redox status in the skeletal muscle. Furthermore, exercise caused an increase in the phosphorylation of the ERK1/2, p38 and JNK proteins in rats (Cargnello and Roux, 2011). Widegren et al. (2000) reported that phosphorylation of ERK1/2 is rapidly decreased during recovery after exercise. In addition, p-38 is a stress-activated kinase that responds to cellular stress, such as metabolic stress and inflammation response (Carlson et al., 2001; Nader and Esser, 2001). Previous reports suggested that JNK level in the skeletal muscle of aged rats reduced following exercise training with activation of SOD and phosphorylation of p38 in cell survival (Kostenko and Moens, 2009; Launay et al., 2006; Lee et al., 2005). Consistent with previous report, expression of p-ERK was decreased significantly after exercise training. In particular, expression of p-ERK (its molecules was markedly reduced in the OS1 and OM1 groups compared with OC group in the SOL muscle. Also, the expression patterns of p-p38 appeared similar to HSPs expression. In addition, p-p38 was increased markedly in the SOL muscle compared to the EDL muscle after exercise training. Simultaneously, the p-JNK was decreased markedly in the OS1 group of the EDL and SOL muscles.

Taken together, HSPs are known to be activated via MAPK pathway such as phosphorylation of p38 and

ERK1/2 due to exercise stimulus (Widegren et al., 2000). Thus, these results suggest that the expression of HSPs and SODs was profoundly associated with regulation of MAPKs for adaptation of skeletal muscle after regular exercise training. Perhaps these results indicate that the expression of HSPs and MAPKs in skeletal muscles elicited functional adaptations by exercise training which is evident effect of high duration and frequency of exercise in aged rat.

Conclusion

This study resulted in induction of HSPs and SODs expression by high duration and frequency of exercise training such as single long-duration exercise training (S1) and multiple short-duration (M1) types with concomitant MAPKs pathway depending on the type of muscles. Collectively, findings from the present study suggest that the frequency and duration of exercise training could affect the functional adaptation and protection against aging-induced weakness of skeletal muscles through changing expression of related molecules. Also, benefits elicited by physical exercise appear proportional to the training frequency and duration. This valuable information may have important clinical efficacy. Further studies will be required to elucidate how the recovery period after exercise affects the expression of HSPs and MAPKs in skeletal muscles.

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Key points

- The expression of heat shock proteins (HSPs) in aged rats was increased significantly after single long-duration (S1) and multiple short-duration (M1) types than S2 and M2 types of exercise training in soleus (SOL) skeletal muscles.
- Superoxide dismutase (SODs) showed similar expression as HSPs did. On the contrary, the p-ERK and p-JNK were down regulated. In addition, p-p38 level in the SOL muscle was activated markedly in all exercise groups.
- Induction of HSPs and SODs by high duration and frequency of exercise training such as S1 and M1 types with concomitant MAPKs pathway depending on the type of muscles.
- The frequency and duration of exercise training could affect the functional adaptation and protection against aging-induced structural weakness of skeletal muscles through changing expression of related molecules.

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