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Exercise training increases anabolic and attenuates catabolic and apoptotic processes in aged skeletal muscle of male rats

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ABSTRACT

Aging results in significant loss of mass and function of the skeletal muscle, which negatively impacts the quality of life. In this study we investigated whether aerobic exercise training has the potential to alter anabolic and catabolic pathways in the skeletal muscle. Five and twenty eight month old rats were used in the study. Aging resulted in decreased levels of follistatin/mTOR/Akt/Erk activation and increased myostatin/Murf1/2, proteasome subunits, and protein ubiquitination levels. In addition, TNF-α, reactive oxygen species (ROS), p53, and Bax levels were increased while Bcl-2 levels were decreased in the skeletal muscle of aged rats. Six weeks of exercise training at 60% of VO2max reversed the age-associated activation of catabolic and apoptotic pathways and increased anabolic signaling. The results suggest that the age-associated loss of muscle mass and cachexia could be due to the orchestrated down-regulation of anabolic and up-regulation of catabolic and pro-apoptotic processes. These metabolic changes can be attenuated by exercise training.

1. Introduction

The skeletal muscle is crucial for movement and also plays an important role in sugar and fat metabolism, and immune response. Age-associated loss in function and mass of the skeletal muscle is well documented (Bijlsma et al., 2012; Reid and Fielding, 2012). However, the causative mechanism(s) controlling this complex process is not well understood. Enhanced generation of inflammation (Degens, 2010), aging-related increases in the level of reactive oxygen species (ROS) (Hiona and Leeuwenburgh, 2008), altered metabolism (Lawler and Hindle, 2011), and increased rates of protein degradation (Witt et al., 2008) are also on the list of potential causative factors of sarcopenia. Indeed, it has been reported that administration of exogenous tumor necrosis factor alpha (TNF-α) leads to a significant decrease in the mass of the skeletal muscle (Llovera et al., 1993). This cytokine can interfere with the contractile properties of the skeletal muscle causing decreased force generating capacity (Reid et al., 2002). Inflammation can readily increase the concentration of ROS, which above certain levels jeopardizes cellular function (Ji, 2007; Langen et al., 2003; Radak et al., 2005). Recently it has been reported that myostatin, which is a negative regulator of muscle growth and is induced in aged skeletal muscle (Bowser et al., 2013; Brioche et al., 2013), can also add to higher levels of ROS (Sriram et al., 2011). Increased levels of myostatin can readily reduce protein synthesis (Hitachi et al., 2014) and it appears that the rate of protein degradation is enhanced in aged skeletal muscle (Goto et al., 2007). It has also been shown that the ubiquitin–dependent proteasome system can be activated with aging (Radak et al., 2002), and recent information indicates that muscle RING finger 1/2 (Murf1/2), which is a ubiquitin ligase, could have an important role in aging skeletal muscle (Sacheck et al., 2007). Thus, it is obvious that the mechanism(s) affecting muscular atrophy is very complex and extremely complicated.

Physical exercise has been shown to retard age-associated loss of muscle mass (Dickinson et al., 2013) and supplementation of growth hormone (Brioche et al., 2013; Nass, 2013).

Therefore the aim of the present study was to obtain a picture of the signaling anabolic, catabolic and apoptotic pathways of aged skeletal muscle. The role of aerobic exercise training on these pathways was investigated.

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2. Methods

2.1. Animals and training protocol

Twelve young (three month old) and twelve eight month old male Wistar rats were used in the study and grouped into young control (YC), young exercised (YE), old control (OC), and old exercised (OE).

The investigation was carried out according to the requirements of The Guiding Principles for Care and Use of Animals, EU, and approved by the local ethics committee. Exercised rats were introduced to treadmill running for three days; then for the next two weeks the running speed was set at 10 m/min, with a 5% incline for 30 min/day. The running speed and duration of the exercise were gradually increased to 60% of VO2max of the animals. As a result, by the final week of the six week training program, young animals ran at 22 m/min, on a 10% incline, for 60 min, whereas old animals ran at 13 m/min, and a 10% incline for 60 min.

At the end of the study, the rats were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and perfused by 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). This procedure was carried out two days after the last exercise session to avoid the metabolic effects of the final run.

Quadriceps muscle was carefully excised and homogenized in buffer containing 137 mM NaCl, 20 mM Tris–HCl, pH 8.0, 2% NP 40, 10% glycerol and protease inhibitors. The protein content was measured by the Bradford method using BSA as a standard, and the samples were stored at −80 C.

2.2. Estimation of oxidant levels and redox active iron

Intracellular oxidant and redox-active iron levels (Kalyanaraman et al., in press) were estimated using modifications of the dichlorodihydrofluoresceindiacetate (H_2DCFDA) staining method (Radak et al., 2004). In brief, the H_2DCFDA (Invitrogen-Molecular Probes #D399) was dissolved to a concentration of 12.5 mM in ethanol and kept at −80 °C in the dark. The solution was freshly diluted with potassium phosphate buffer to 125 μM before use. For fluorescence reactions, 96-well black microplates were loaded with potassium phosphate buffer (pH 7.4) to a final concentration of 152 μM/well. Then 8 μl diluted tissue homogenate and 40 μl 125 μM dye were added to achieve a final dye concentration of 25 μM. The change in fluorescence intensity was monitored every 5 min for 30 min with excitation and emission wavelengths set at 485 nm and 538 nm (Fluoroskan Ascent FL). The fluorescence intensity unit was normalized with the protein content and expressed in relative unit production per minute.

2.3. Western blots

Ten to fifty micrograms of protein was electrophoresed on 8–12% v/v polyacrylamide SDS-PAGE gels. Proteins were electrotransferred onto PVDF membranes. The membranes were subsequently blocked and after blocking, PVDF membranes were incubated at room temperature with antibodies (1:500 #sc-6884 Santa Cruz GDF-8/11 (C-20); 1:500 #sc-30194 Santa Cruz Follistatin (H-114); 1:500 #sc-32920 Santa Cruz MuRF1 (H-145); 1:500 #sc-49457 Santa Cruz MuRF2 (N-15); 1:1000 #9272s cell signaling Akt; 1:1000 #9271s cell signaling Phospho-Akt (Ser473); 1:500 #sc-8319 Santa Cruz mTOR (H-266); 1:1000 #5536 cell signaling Phospho-mTOR (Ser2448); 1:1000 #9102 cell signaling p44/42 MAPK (Erk1/2); 1:1000 #9106 cell signaling Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204); 1:500 #sc-1350 Santa Cruz TNFα(N-19); 1:500 #sc-526 Santa Cruz Bax (P-19); 1:500 #sc-492 Santa Cruz Bcl-2 (N-19); 1:500 #sc-1311 Santa Cruz p53 (C-19); 1:1000 #2459 cell signaling PSMA6; 1:1000 #3936 cell signaling Ubiquitin (P4D1); 1:500 #sc-15404 Santa Cruz SIRT1 (H-300); 1:500 #sc-69359 Santa Cruz COX4 (D-20); 1:500 #sc-7159 Santa Cruz cytochrome c (H-104); 1:2000 #sc-81178 Santa Cruz β-Actin (ACTBD11B7)). After incubation with primary antibodies, membranes were washed in TBS-Tween-20 and incubated with HRP-conjugated secondary antibodies. After incubation with the secondary antibody, membranes were repeatedly washed. Membranes were incubated with a chemiluminescent substrate (Thermo Scientific, SuperSignal West Pico Chemiluminescent Substrate #34080) and protein bands were visualized on X-ray films. The bands were quantified by ImageJ software, and normalized to β-actin, which served as an internal control.

2.4. Statistical analyses

Statistical significance was assessed by Kruskal–Wallis ANOVA followed by the Mann–Whitney U test in the case of those variables where post-hoc analysis was adequate. The significance level was set at $p < 0.05$.

3. Results

3.1. The effects of aging

Aging resulted in a significant decrease in the protein content of cytochrome C (Fig. 1A) and COX4 (Fig. 1B), indicating decreased mitochondrial content. The ROS levels were appraised using the H_2DCFDA staining method, and an age-associated increase was detected (Fig. 2). Myostatin, which is a negative regulator of muscle growth significantly increased with aging ($p < 0.01$) (Fig. 3A). An age-associated decrease in the follistatin levels, which is the antagonist of myostatin, was observed in the OC group compared to YC (Fig. 3B). The ratio of pmTOR/mTOR and pAkt/Akt did not change significantly as a result of aging (Fig. 3C, D). However the ratio of pERK/ERK increased in the aged control group compared to young controls (Fig. 3E).

The assessment of protein degradation was made by measuring Murf1, Murf2, proteasome subunit alpha (PSMA6), and protein ubiquitination. Generally, all of these markers increased with aging (Fig. 4A–D). Degradation of proteins is associated with apoptosis and an increase in p53 levels was detected as a result of aging (Fig. 5A). Bax is a pro-apoptotic protein and an age-associated increase in this protein was found in the skeletal muscle ($p < 0.01$) (Fig. 5B). TNF-α is an adipokine which can relate to apoptosis and it has been found unaltered with aging (Fig. 5C). Bax induces apoptosis by binding the Bcl-2 family, which was found to be significantly lower in aged muscle than in young muscle (Fig. 5D). SIRT1 is anti-apoptotic protein, which levels were not altered by aging (Fig. 5E).

3.2. The effects of exercise training

Six week running training at the intensity of 60% of VO2max resulted in an adaptive response in mitochondrial enzymes with significant elevation of cytochrome C levels in both young and aged groups. The training program eliminated the age-associated loss of cytochrome C (Fig. 1A) and COX4 (Fig. 1B). Exercise training did not significantly change the levels of ROS. Aerobic exercise training did not change the myostatin levels (Fig. 3A), however eliminated the age-associated increase. In accordance with this change, the follistatin levels increased by training in aged animals (Fig. 3B).

Exercise increased the pmTOR/mTOR levels in aged groups, while no statistical alteration was present in young groups, and this was true for pAkt/Akt ratio (Fig. 3C, D). However, exercise prevented the age related increase in the ratio pERK/ERK (Fig. 3E). Exercise training decreased the protein levels of Murf1 aged groups compared to aged control rats (Fig. 4A), while exercise decreased the levels of Murf2 in both age groups (Fig. 4B).

Interestingly a statistical increase in PSMA6 and ubiquitination levels was found between young control and young exercise rats (Fig. 4C, D), while in aged groups exercise does not significantly altered the levels of PSMA6 and protein ubiquitination. Exercise training did not

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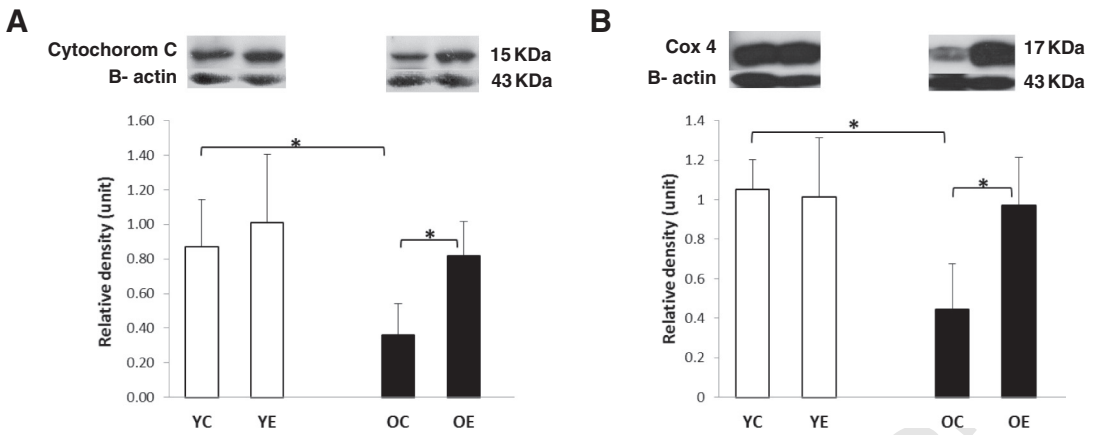

Fig. 1. The levels of cytochrome C and COX 4. Mitochondrial content was evaluated by cytochrome c and COX 4. Groups: YC, young control; YE, young exercised; YEI, young exercised IGF-1 treated, OC, old control; OE, old exercised, OEI, old exercised IGF-1 treated. Values are means ± SE for six animals per group. *$p < 0.05$, **$p < 0.01$.

result in significant alteration of p53, Bax, TNF-α and SIRT1 levels (Fig. 5. A, B, D, E); the only statistical difference in these apoptotic markers was that exercise decreased the Bcl2 levels in the young group compared to young control rats (Fig. 5C).

4. Discussion

Age-associated loss in function and size of the skeletal muscle leads to a decreased quality of life. The findings of the present study suggest that the loss of muscle mass is due to the decreased activity of anabolic pathways and increased activity of catabolic pathways in the skeletal muscle.

The follistatin mediated anabolic pathway was found to be down-regulated in aged skeletal muscle. The IGF pathway is known to promote myogenesis (Rosen et al., 1993), and follistatin mediated inhibition of myostatin causes enhanced expression of IGF-1 (Gilson et al., 2009) and activation of anabolic pathways, probably through an IGF-receptor (IGF-IR). Data from the present study demonstrate that aging results in the down-regulation of follistatin mediated pathways. This finding is in accordance with the observation, that administration of follistatin results in increased muscle protein synthesis (Suryawan et al., 2006). Aerobic exercise has been shown to elevate the serum levels of follistatin (Gorgens et al., 2013), while exercise can activate Akt and Erk pathways (Boonsong et al., 2007; Fuentes et al., 2011; Pasiakos et al., 2010; Williamson et al., 2006), leading to enhanced production of follistatin (Chen and Ruiz-Echevarria, 2013). In the present study we have observed that exercise could counteract with the effects of aging on follistatin levels, and this could be an important means by which regular exercise could attenuate sarcopenia.

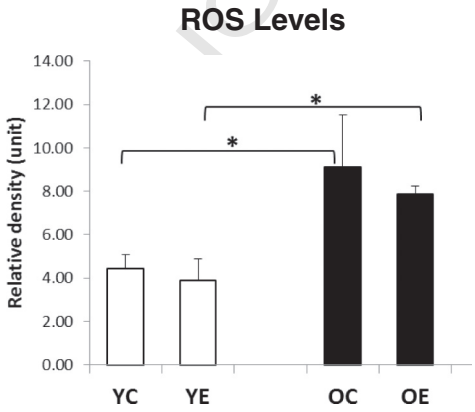

Fig. 2. The evaluation of ROS content. The measurement of ROS levels was done by fluorescent detection of H2DCFDA. Groups: YC, young control; YE, young exercised; YEI, young exercised IGF-1 treated, OC, old control; OE, old exercised, OEI, old exercised IGF-1 treated. Values are means ± SE for six animals per group. *$p < 0.05$, **$p < 0.01$.

The significant decrease in mass of the skeletal muscle could be also due to the enhanced level of catabolic processes. Myostatin is a powerful negative regulator of muscle growth. Myostatin signaling results in the activation of Smad2 and Smad3 and consequently the regulation of MyoDas well as the ubiquitin-associated degradation (Attisano et al., 2001). This pathway is activated in aged skeletal muscle, suggesting the involvement of myostatin in age-associated muscle loss. Indeed, blockage of myostatin also curbs the activity of catabolic pathways (Thomas and Mitch, 2013). On the other hand, cancer-associated cachexia has been shown to increase myostatin and Murf2 levels in the skeletal muscle (Bonetto et al., 2009). These data suggest a functional link between myostatin and Murf(s) mediated catabolism. Murf1 and Murf2 are ubiquitin ligases but results from work using Murf1 transgenic mice suggest that Murf1 can interfere with the ROS production of mitochondria in the cardiac muscle (Mattox et al., 2014). Similar interaction could be present in the skeletal muscle. Murf1/Murf2 has been implicated in the remodeling of type-II fibers in the skeletal muscle (Moriscot et al., 2010) as these fibers lose more total area and function than type-I fibers during the aging process (Deschenes, 2004; Pak and Aiken, 2004). The increased level of Murf1/Murf2, hence, can be a compensatory mechanism to try to remodel these fibers, which includes degradation of damaged fibers. Aging resulted in increased levels of ROS, which are initiators/consequences of muscle wasting (Eley et al., 2008) and closely related to the activation of apoptosis (Favier et al., 2008). It has been reported that age-associated increases in p53 in the skeletal muscle leads to the mitochondrial release of cytochrome c and apoptosis (Tamilselvan et al., 2007). In the present study aging resulted in increased levels of pro-apoptotic proteins p53 and Bax and down-regulation of anti-apoptotic Bcl-2 protein.

Exercise associated decrease in the levels of p53 and Bax in proteins could counteract the age-mediated pro-apoptotic pathways. SIRT1 is considered to be an anti-apoptotic protein (Radak et al., 2013). However, an age-associated alteration of this protein was not observed, although exercise training increased the content of this protein in the older group. We have previously reported, using the same animals, that exercise increased the activity of SIRT1 (Koltai et al., 2010). However, it is not clear if that finding affects the anti-apoptotic role of SIRT1. In addition, it has to be mentioned that the role of sirtuins in aging is very complex, and sirtuins belong to the vitagen family together with heat shock proteins and thioredoxin (Calabrese et al., 2010, 2011, 2012; Cornelius et al., 2013). The U-shape dose response curve, which is often called hormesis, is very representative to oxidants, oxidative damage and vitagens, and without question vitagens could play an

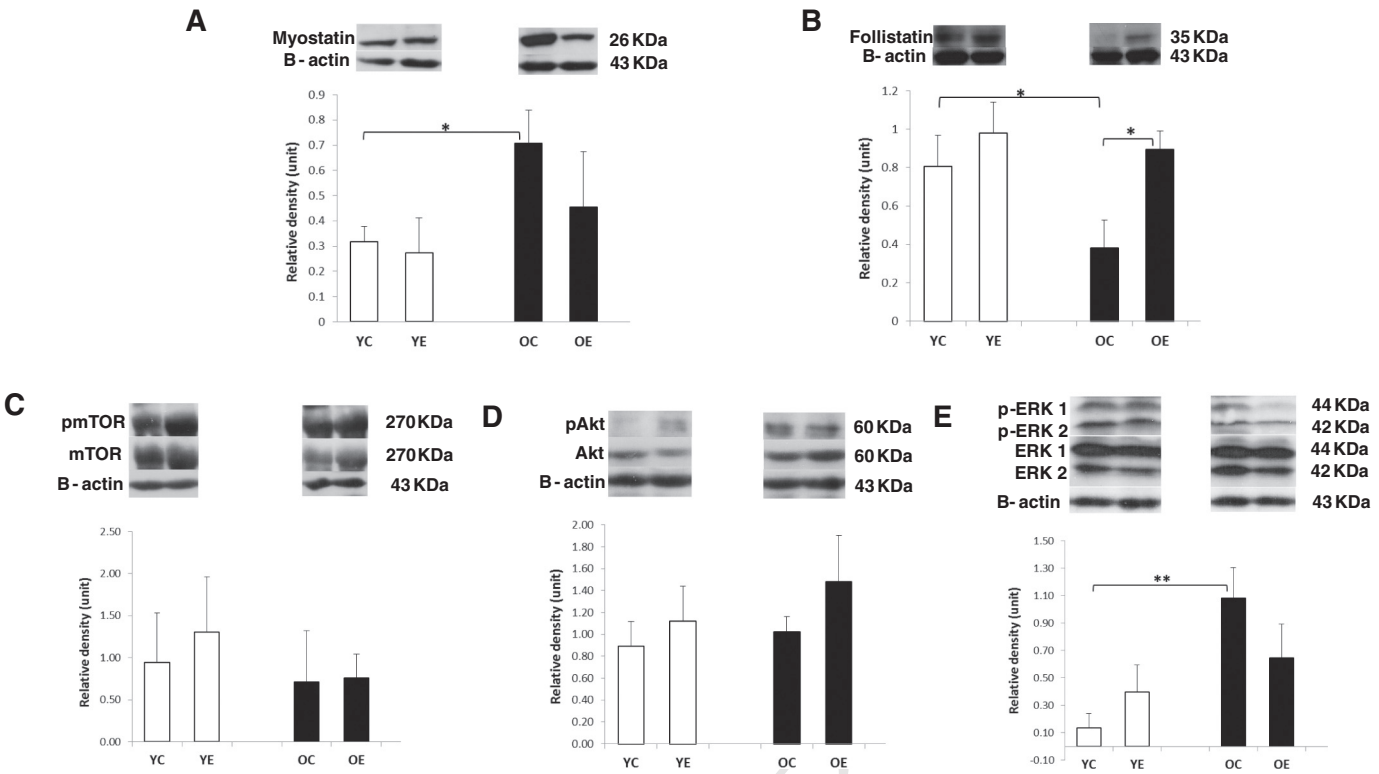

Fig. 3. Anabolic factors of the skeletal muscle. Myostatin (A) and follistatin (B) levels were evaluated by Western blot. The activities of mTOR (C), Akt (D) and ERK (E), were measured by the ratio of phosphorylated and total levels of mTOR, Akt and ERK. Groups: YC, young control; YE, young exercised; YEI, young exercised IGF-1 treated, OC, old control; OE, old exercised, OEI, old exercised IGF-1 treated. Values are means ± SE for six animals per group. *$p < 0.05$, **$p < 0.01$.

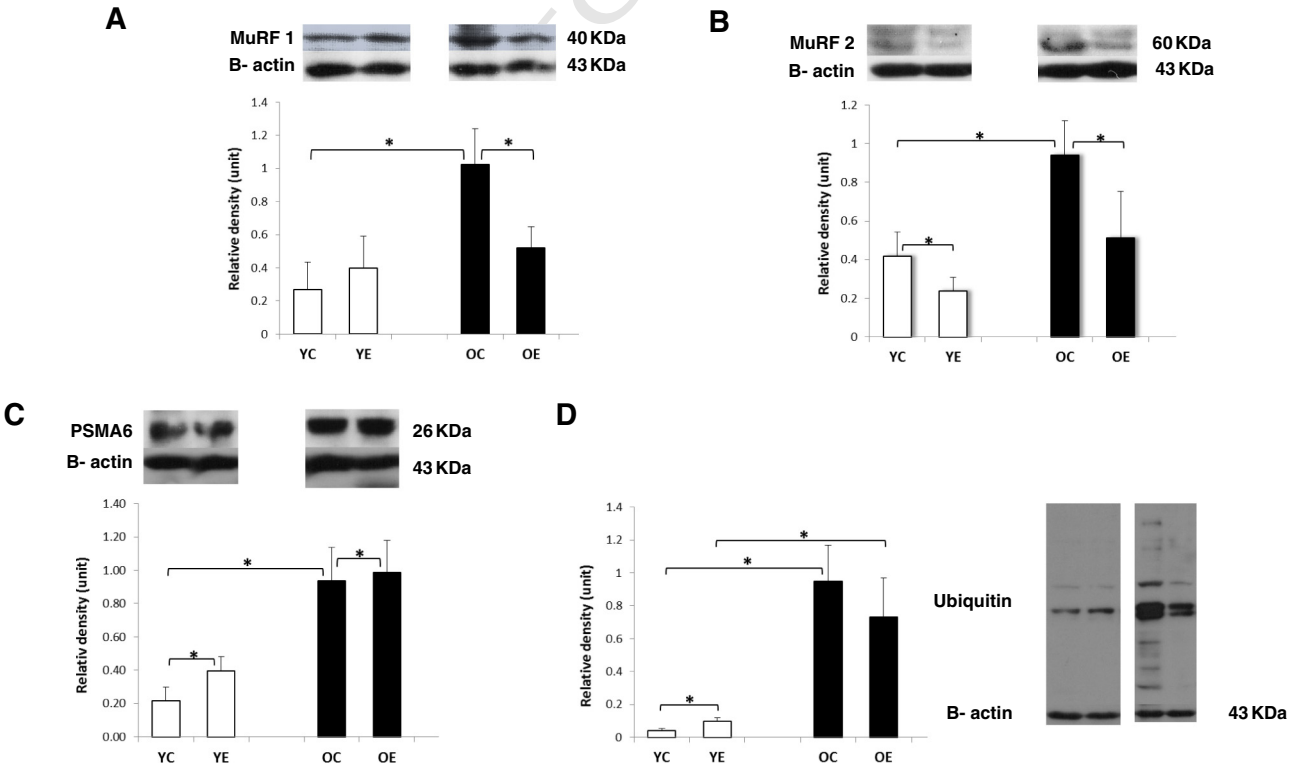

Fig. 4. Catabolic factors of the skeletal muscle. MuRF1 (A), MuRF2 (B), PSMA6 (C) and protein ubiquitination (D) levels were evaluated as markers of protein degradation. Groups: YC, young control; YE, young exercised; YEI, young exercised IGF-1 treated, OC, old control; OE, old exercised, OEI, old exercised IGF-1 treated. Values are means ± SE for six animals per group. *$p < 0.05$, **$p < 0.01$.

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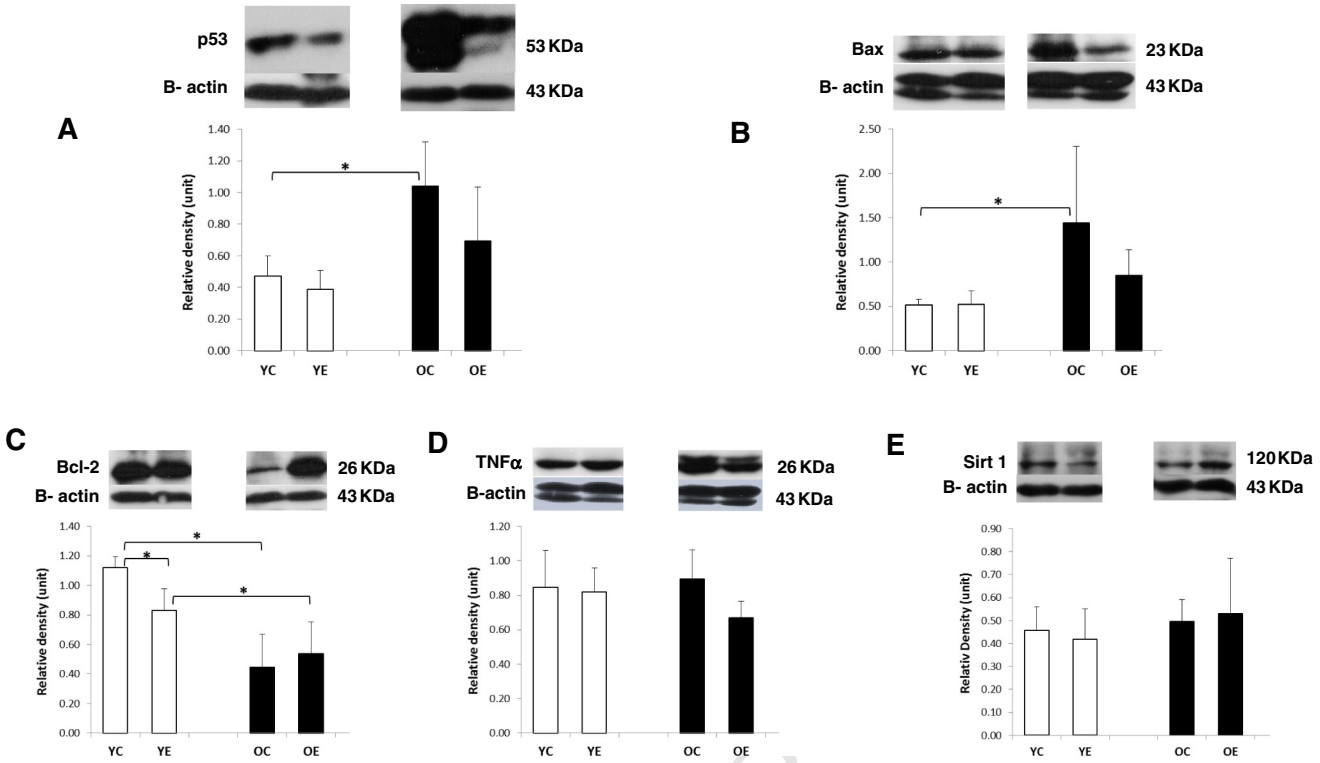

Fig. 5. Proaptotic and anti-apoptotic markers in the skeletal muscle. Pro-apoptotic factors p53 (A), BAX (B), and TNF-a (C) and anti-apoptotic factors Bcl-2 (D) and SIRT1 (E) were measured by immunoblot. Groups: YC, young control; YE, young exercised; YEI, young exercised IGF-1 treated, OC, old control; OE, old exercised, OEI, old exercised IGF-1 treated. Values are means ± SE for six animals per group. *p < 0.05, **p < 0.01.

important role in aging process (Calabrese et al., 2007; Radak et al., 2011). Nevertheless, the role of SIRT1 in age-associated loss of muscle mass needs further verification.

In conclusion, we report that aging results in significant decreases in anabolic processes of the skeletal muscle by activation of the follistatin pathway. This finding, together with the data that show enhanced activation of myostatin, Murf1/2, PMSA6, protein ubiquitinating pathway, and apoptosis in the skeletal muscle of aged animals, suggests that the age-associated loss in muscle mass is a result of altered protein synthesis and degradation. Exercise training can reverse the decline in anabolic processes and increases in catabolic and apoptotic processes, and serves as an important tool to fight sarcopenia and cachexia.

Conflict of interest

There is no conflict of interest regarding the manuscript.

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