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RESEARCH ARTICLE

Exogenous nicotinamide supplementation and moderate physical exercise can attenuate the aging process in skeletal muscle of rats

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Abstract Nicotinamide (NAM) could enhance the availability of NAD^+ and be beneficial to cell function. However, NAM can inhibit the activities of SIRT1 and PARP. The effect of NAM supplementation on the aging process is not well known. In the present study exogenous NAM (1–0.5% in drinking water) was supplemented for 5 weeks and in the last 4 weeks moderate treadmill running was given to 5 mo and 28 mo old rats. The content of SIRT1 was not effected by NAM treatment alone. However, the activity of SIRT1, judged from the acetylated p53/p53 ratio, increased in both NAM treated age groups, suggesting beneficial effects of exogenous NAM. This was confirmed by the finding of increased PGC-1α and pCREB/CREB ratio in the gastrocnemius muscle of old but not young NAM treated animals. Our data suggest NAM administration can attenuate the aging process in skeletal muscle of rats, but NAM administration together with exercise training might be too great challenge to cope with in the old animals, since it leads to decreased levels of SIRT1.

Keywords Aging · Exercise · Nicotinamide · SIRT1

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Introduction

SIRT1 and other sirtuins with deacetylase activity (SIRT2, SIRT3, SIRT5), deacetylate lysine residues of target proteins at the expense of NAD^+, generating deacetylated lysine, *2′-O-acetyl-ADP-ribose* and NAM. NAD^+ is crucial to normal cellular metabolism and enzymes like sirtuins, DNA repair by poly(ADP-ribose) polymerase -1(PARP-1) or lactate dehydrogenase (LDH) can significantly deplete NAD^+ levels, which jeopardizes cellular function. Moreover, among these enzymes there is a competition for NAD^+. Administration of exogenous nicotinamide (NAM) could enhance the availability of NAD^+, however, to produce NAD^+ from NAM requires energy (Belenky et al. 2007; Jia et al. 2008; Lee et al. 2013; Liu et al.

2014). NAM can be converted to nicotinamide mononucleotide by nicotinamide phosphoribosyl transferase (NAMPT). In order to preserve cellular NAD^+ levels in critical conditions NAM can inhibit the activities of SIRT1 and PARP, to save energy for survival (Kerr and Ford 1991). On the other hand, NAM could be also protective in anoxic conditions (Chong et al. 2005) or in hypoxia/reoxygenation situations (Shen et al. 2004). Therefore, in severe metabolic conditions NAM administration could have inverse effects, while when energy is available exogenous NAM can optimize cellular NAD^+ levels. On cell lines, it has been reported that NAM supplementation caused cellular senescence via SIRT1 inhibition (Zheng et al. 2014). However, it remains to be shown that administration of exogenous NAM to animals leads to inhibition of sirtuins.

During aging there is a decrease in the biosynthesis of NAD^+ (Prolla and Denu 2014). Hence, exogenous NAM could have beneficial effects, but this has yet to be reported. It has been noted that sirtuins, especially SIRT1, can directly influence brain function (Barhwal et al. 2015; Fujitsuka et al. 2016; Koltai et al. 2011; Sarga et al. 2013; Torma et al. 2014; Tulino et al. 2016). SIRT1 appears to be involved in neuronal stem cell differentiation (Ma et al. 2014), synaptic plasticity (Michan et al. 2010) and metabolism (Li et al. 2008).

We and others have shown that exercise increases the activity and content of SIRT1 (Hart et al. 2014; Kang et al. 2013; Koltai et al. 2011; Marton et al. 2015; Radak et al. 2011; Suwa et al. 2008), while aging decreases NAD levels and NAMPT concentration (Koltai et al. 2010). Moreover, it has also been reported that exercise training can attenuate the age associated decline in NAD and NAMT levels in skeletal muscle of rats (Koltai et al. 2012). Ferrara and co-workers (Ferrara et al. 2008), reported that aging decreases and exercise increases SIRT1 activity in the heart of but not in the adipose tissue and exercise training reversed the age-associated oxidative stress as well (Ferrara et al. 2008). Interesting investigation was done by Conti et al. (Conti et al. 2012) in which endothelial cells, were exposed to oxidative stress, and conditioned with sera from athletes regularly participating in different sports. Data revealed that different types of exercise training induced different molecular effects in terms of survival, and it turned out that poststress activity of Sirt1 was significantly increased in T-endothelial cells (Conti et al. 2012).

To our knowledge information is not available on the possible, if any, effect of exogenous NAM administration on exercise trained aged skeletal muscle. We were influenced by the hypothesis that regular NAM administration and moderate levels of physical exercise could attenuate the progress of aging. Alternatively, we could not discard the possibility that exogenous NAM could inhibit SIRT1 activity, and hence, alter the wide array of SIRT1 signaling pathways.

Methods

Animals and training protocol

Twenty four young (5 month old) and twenty four 28 month old female Wistar rats were used in the study and grouped into young control (YC), young exercised (YE), young control with NAM administration (YCN), young exercised with NAM administration (YEN) old control (OC), old exercised (OE), old control with NAM administration (OCN), and old exercised with NAM administration (OEN).

NAM was administered through the drinking water of the animals at a concentration of 0.5–1.0%, for 5 weeks. The administration of NAM started with 1% in the drinking water (Toth 1983), but after a significant loss in body weight of old rats (30–40%), we decreased the NAM levels to 0.5% which attenuated the loss in body weight. Food and water were available ad libitum and the consumed amount was monitored daily.

Exercised rats were introduced to treadmill running five days per week for four weeks with the running speed set at 10 m/min for 10 min the first week, 20 min for the second week, 30 min for the third and fourth weeks. This running load is very moderate, but old rats could not tolerate higher intensities. The same exercise protocol was applied to old and young animals.

At the end of the study, the rats were anaesthetized with intraperitoneal injections of ketamine (50 mg/kg) and perfused with 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.

Gastrocnemius 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.

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.

Ten to 50 micrograms of protein were 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 (1:1000 #ab110304 Abcam SIRT1; 1:500 #ab131442 Abcam p53; 1:2000 #06-758 Upstate acp53; 1:500 #ab37299 Abcam (PBEF) NAMPT, 1:1000 #9197 Cell Signaling CREB (48H2); 1:1000 #9198 Cell Signaling pCREB Ser133 (87G3), 1:500 #sc-13067 PGC-1α (H-300); 1: 15000 #T6199 Sigma α-tubulin). 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 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 α-tubulin, which served as an internal control.

Statistical analyses

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

Results

The body mass of OE rats was significantly less than that of OC group ($p < 0.05$), although significantly lower body mass was measured in both NAM treated group around the 19th and 24th days of treatment. By the end of the 5th week the body mass of NAM treated animals were comparable to OC group (Fig. 1).

The data on the content of SIRT1 in young and old animal muscle treated with NAM and exercise training is very complex. In young animals training increased the protein content of SIRT1 (Fig. 2), while the combined effects of NAM and training resulted in decreased levels of SIRT1 compared to the NAM treated non-exercising group. In aged muscle, similarly to young the lowest level of SIRT1 protein was detected in the NAM treated exercise group. SIRT1 activity was assessed with the ratio of acetylated and total amount of p53, because p53 is exclusively deacetylated by SIRT1. Our data revealed that aging decreases the activity of SIRT1 ($p < 001$, Fig. 3). In young animals, the applied treatment changed the Acp53/p53 ratio only in YEN group, where decreased activity was observed. On the other hand, in old animals, to our surprise, NAM treatment and NAM treatment plus exercise decreased the Acp53/p53 ratio.

NAMPT is an enzyme which catalyzes nicotinamide mononucleotide (NMN) formation from NAM and therefore, an important enzyme in NAD synthesis. NAMPT levels decreased with aging and exercise training but NAM administration did not change the concentration of this enzyme (Fig. 4).

Transcription coactivator peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) is a master regulator of mitochondrial biogenesis, and the data revealed that the protein content of this co-activator increased in the gastrocnemius of NAM and NAM trained groups of old animals (Fig. 5). Besides the link between SIRT1 and PGC-1α, SIRT1 can also regulate the activity of transcription factor cAMP-responsive element (CRE)-binding protein (CREB). The ratio of pCREB/CREB increased significantly in trained old muscle, while in young groups NAM treatment caused a significant decrease (Fig. 6).

Discussion

NAM administration resulted in lower food intake and weight gain, which was probably due to methyl-group deficiency (Kang-Lee et al. 1983). NAM administration (100 mg/kg/day for 8 weeks) resulted in increased lipid levels and decreased glycogen content in skeletal muscle of rats, which was associated with increased levels of lipids in the muscle, and probably due to reduced capacity of free fatty acid oxidation (Qi et al. 2016). NAM (vitamin B3) is the only vitamin

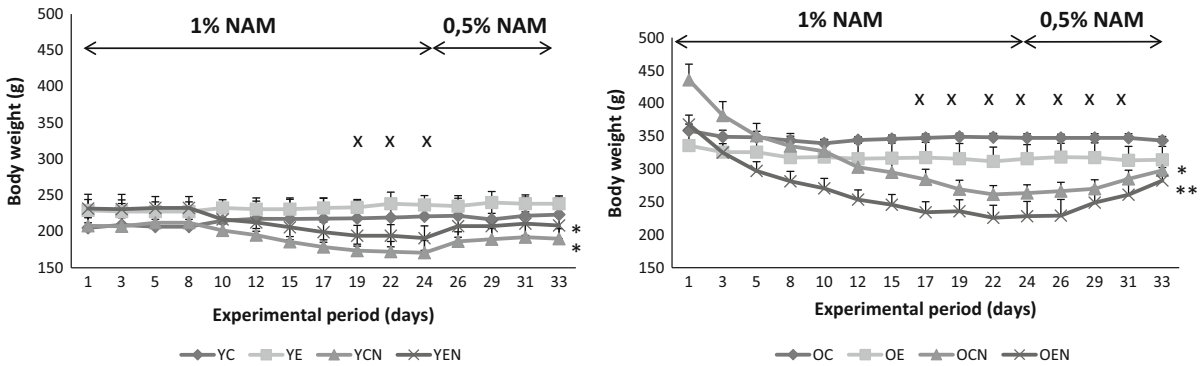

Fig. 1 Changes in body mass. *Note:* The body mass of the NAM treated animals changed significantly during the experimental period. Results are expressed mean ± SD. *p < 0.05, **p < 0.01, N = 6 in each group

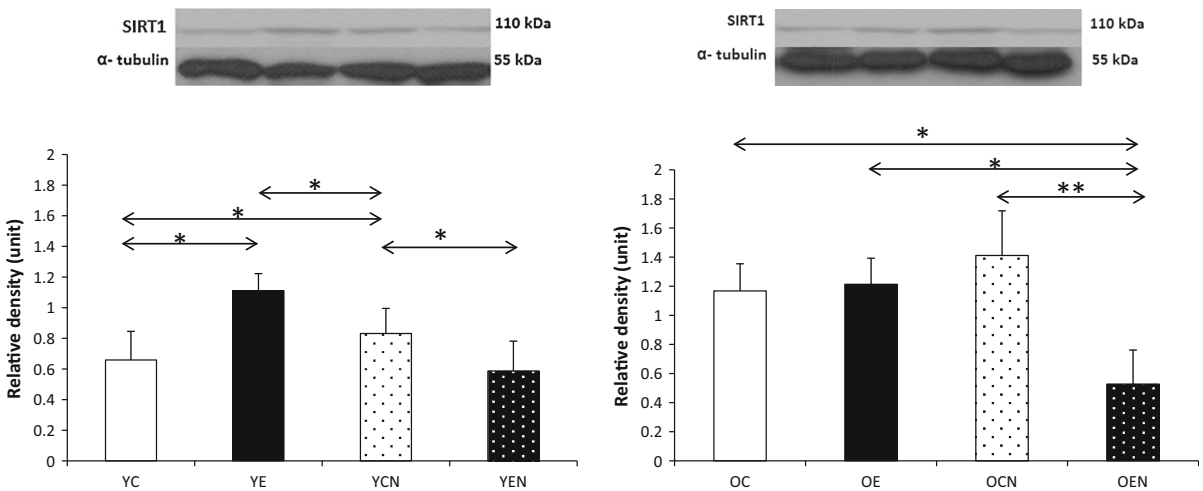

Fig. 2 Levels of SIRT1 contents. *Note:* Histone deacetylase SIRT1 content change with age, exercise training and NAM administration. Results are expressed mean ± SD. *p < 0.05, **p < 0.01, N = 6 in each group

which can be synthesized. It is made from L-tryptophan through kynurenine. Besides being important to NAD synthesis, NAM can inhibit the activity of SIRT1, the enzyme which appears to be important in a wide range of physiological processes including mitochondrial biogenesis (Koltai et al. 2012), DNA repair (Sarga et al. 2013), metabolism (Morales-Alamo and Calbet 2016) and apoptosis (Joseph et al. 2013; Radak et al. 2013), among others.

Exercise has been shown to alter SIRT1 content in skeletal muscle and SIRT1 is believed to be important for adaptation (Koltai et al. 2010). Our suggestion is that NAM administration can influence the behavior of SIRT1 in exercise-induced adaptation and young and old rats react differently to exercise and a NAM challenge.

NAM treatment increased SIRT1 content in skeletal muscle in both, of old and young animals, while the response to exercise training and NAM treatment was similar in both groups. Because p53 is deacetylated by SIRT1 the Acp53/p53 ratio was used to assess the activity of SIRT1 (Chung et al. 2015; Feng et al. 2015; Kim et al. 2016; Li et al. 2015; Song et al. 2015; Weidele et al. 2017) and the results showed that exercise and NAM treatment as well as the combined effects of these increased the activity of SIRT1 in old muscle. In terms of exercise training this is similar to our earlier results (Koltai et al. 2010, 2012) however, the intensity of training was much less than that used in those studies. Interestingly, NAM, which can inhibit SIRT1 in certain concentrations, did not alter SIRT1 activity, suggesting that in skeletal muscle of aged

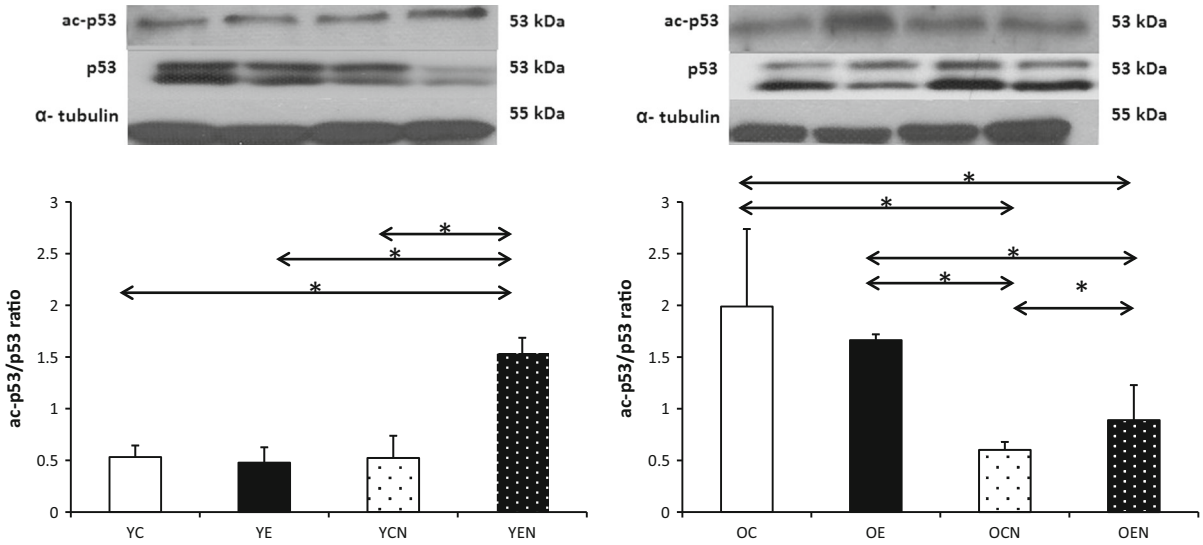

Fig. 3 Total and acetylated p53 levels. *Note:* P53 acetylation levels were measured to assess the activity of SIRT1. Bars show ac-p53/p53 ratio. Results are expressed mean ± SD. *p < 0.05, **p < 0.01, N = 6 in each group

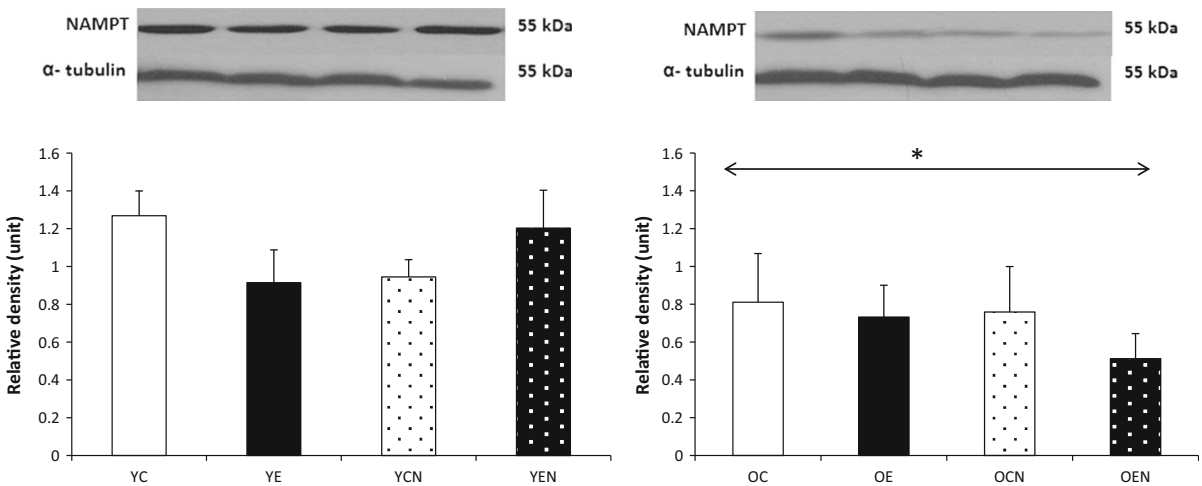

Fig. 4 NAMPT levels in young and old skeletal muscle. *Note:* NAMPT is important to NAD biosynthesis and the levels were measured by immunoblots. Results are expressed mean ± SD. *p < 0.05, **p < 0.01, N = 6 in each group

rats, the supplemented NAM possibly increased the concentration of NAD, which served as energy for SIRT1 activity. The difference between the response of young and old skeletal muscle to exercise and NAM treatment, could be due to the fact that this exercise program was too mild in the young groups to cause adaptation. The lack of the response to NAM in young animals could be due to the fact that the base level of NAD is much higher in young groups than old ones (Koltai et al. 2010).

The content of NAMPT, which plays a crucial role in NAD synthesis supports the showed different response to exercise and NAM treatment in young and old rats. In aged group NAMT levels decreased significantly in double treated group compared to control levels. The mechanism behind the age associated different response is not known, it could be due to different tolerance to exercise intensity, despite of the fact that NAMT levels were not modified by exercise training. The combined effects of NAM

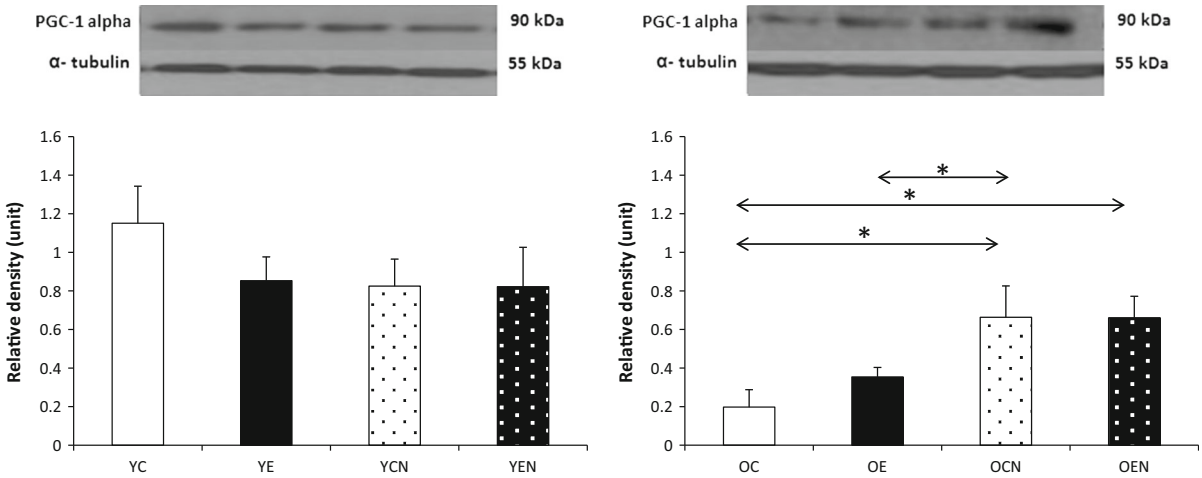

Fig. 5 PGC-1 alpha concentration. *Note:* PGC-1 alpha is a master regulator of mitochondrial biogenesis. Moderate level of exercise training increased the levels of PGC-1 alpha in aged skeletal muscle. Results are expressed mean ± SD. *p < 0.05, **p < 0.01, N = 6 in each group

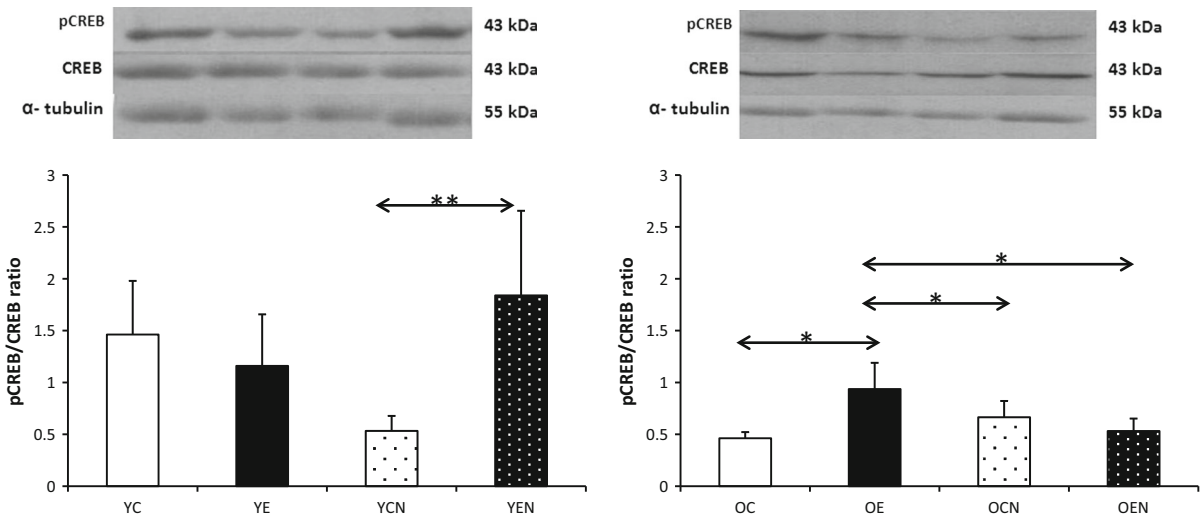

Fig. 6 pCREB-CREB ratio levels in young and old skeletal muscle. *Note:* CREB transcription factor is responsible for the regulation of number of genes. Results are expressed mean ± SD. *p < 0.05, **p < 0.01, N = 6 in each group

treatment and exercise could provide different environment on young and old muscle, which can associated with different NAMT levels.

The applied exercise load, which failed to cause adaptation in young animals, increased PGC-1α content in old rats, and NAM alone increased the content of PGC-1α. These results suggest that, in aged skeletal muscle NAM treatment associated increases in NAD concentration could initiate beneficial responses in mitochondrial content. It must be noted, that the rats were sacrificed at 33 months, which is thought to correspond to about 85 years of age in humans. At this age even moderate levels of exercise could be effective, and NAM treatment appears to be beneficial.

CREB transcription factor is activated by the phosphorylation of serine residues within their *N*-terminus, which enhances their transcriptional activation. Protein kinase A as well as AMPK could be responsible of this activation (Thomson et al. 2008). CREB activation occurred only in old trained groups, suggesting that AMPK mediated activation could take

place, resulting in adapting response to exercise training which directly can activate mitochondrial biogenesis (Hart et al. 2013). There are only limited data on exercise related activation of CREB in aged skeletal muscle, but the findings are similar to ours (Kang et al. 2013).

Our data revealed that the response of young and old skeletal muscle to moderate running training and NAM supplementation is different, when measured by the activation of SIRT1, PGC-1a, and CREB.

NAM administration at this concentration did not cause inhibition of SIRT1, but appears to be beneficial and attenuates the aging process in skeletal muscle.

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