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Progressive Resistance Training Modulates the Expression of ACTN2 and ACTN3 Genes and Proteins in the Skeletal Muscles

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Abstract Purpose: Mammalian skeletal muscle has the two isoforms of actin binding protein, α-Actinin-2 and α-Actinin-3, which are located in the skeletal muscle Z-line where they cross-link the actin thin filaments. There is a common stop codon polymorphism R577X in the *ACTN3* gene. Several association studies have demonstrated that the *ACTN3* R577X genotype influences athletic performance. The response of α-Actinins to resistance exercise training is little understood. Methods: Female Sprague-Dawley rats were assigned to control (C; n = 10) and resistance training (T; n = 12) groups. Training consisted of climbing a ladder carrying a load suspended from the tail. After training, fast (Flexor halluces longus, FHL) and slow (Soleus) hind limb muscles from each group was examine to study the effect of resistance training on muscle mass. Gene expression and protein levels of both *Actn3*, *Actn2* were examined. Results: The resistance trained group had a significantly greater absolute muscle mass in FHL (P=0.011). We also found that *Actn3* and *Actn2* gene expression levels increased significantly in FHL and Soleus muscles by mean factors of 2.16, and 2.91, respectively. α-Actinin-2 protein expression increased significantly in training group (P=0.025) while, α-actinin-3 protein expression remained similar in training & control groups (P=0.130). The most important finding of this study showed that both α-actinin-3 and α-actinin-2 mRNA levels were up-regulated after 8wk of resistance training (P≤0.05). Conclusion: Our results provide a new insight into the impact of progressive resistance training and evaluating the role of α-actinins responsiveness.

Keywords: *vertical ladder, α–actinins, sarcomere, Z-line, resistance training*

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1. Introduction

The contractile apparatus of skeletal muscle is composed of repeating units (sarcomeres) that contain ordered arrays of actin-containing thin filaments and myosin-containing thick filaments. The Z-lines are electron-dense bands, perpendicular to the myofibrils that anchor the thin filaments. α–Actinins are a major component of the Z-line in skeletal muscle and are structurally related to dystrophin. α-actinins have several functional domains: a N-terminal actin-binding domain, a central rod domain, and a C-terminal region that contains two potential calcium-binding EF hand motifs [15]. During evolution, gene duplication and alternative splicing events have resulted in the generation of considerable functional diversity within the α-actinins family. This diversity is most marked in mammals, where four separate α-actinins encoding genes produce at least six distinct protein products, each with an unique tissue-expression profile [19]; two of which are primarily expressed in skeletal muscle: α-Actinin-2 and α-actinin-3 [15,16]. In human muscle, α-actinin-2 is expressed in all muscle fibers, while α-actinin-3 has more specialized expression and is restricted to the fast, glycolytic muscle fibers responsible for rapid force generation [19]. The sarcomeric α-actinins are major components of the Z-disc in skeletal muscle, although they account for less than 20% of Z-disc weight [10]. The stability of the Z-discs and the skeletal muscle cytoskeleton is the result of a complex network of interactions, and *in vitro* studies have suggested that the C-terminus of sarcomeric α-actinins plays a crucial role in the maintenance of Z-Line integrity and myofibrillar organization [26]. Based on this, α-actinins are considered an important structural component of muscle contractile force generation and transmission, as well as maintenance of the regular myofibrillar arrays [22].

Human α-actinins isoforms, α-actinin-2 and α-actinin-3, are encoded by *ACTN2* and *ACTN3* genes, respectively [19]. Interestingly, 16% of the global human population is completely α-actinin-3-deficient due to homozygosity for a common null polymorphism in *ACTN3* (R577X) [26]. Genotype frequencies have been investigated among elite athletes in different sports from various nations [1,8,18,23,24,25]. These studies demonstrated that the

frequency of the 577X null allele is significantly lower in elite sprint and power athletes than in controls, suggesting that α-actinin-3 is required for optimal muscle performance at high velocity. In the general population the effects of α-actinin-3 deficiency in response to various exercise training regimes has also been investigated [5,7]. Non-athletic *ACTN3 577RR* individuals were found to have greater muscular strength than *ACTN3*-deficient *577XX* humans [29].

An *Actn3* knock-out (KO) mouse model mimics the human findings and have been found to be weaker than WT mice [17]. KO mice were also found a compensatory upregulation of α-actinin-2 (two-fold), improved oxidative capacity and, enhanced resistance against skeletal muscle fatigue [16,17,27]. It has been recently demonstrated that this is likely due to increase calcineurin activity associated with α-actinin-3 deficiency and upregulation of α-actinin-2. These results showed the skeletal muscle fibers transformation from fast glycolytic to slow oxidative metabolism [27].

Exercise training is well known to change significantly skeletal muscle properties [22]. Hypertrophy occurs as an adaptive response to load-bearing exercise, and as a result of an enhanced rate of protein synthesis [12]. This increase in protein synthesis enables new contractile filaments to be added to the pre-existing muscle fiber, which in turn enables the muscle to generate greater force [12]. Despite the important role of α-actinins in sarcomere for producing force [16,21], little is known about the influence of resistance training on the expression of α-actinins in skeletal muscle tissue. To our knowledge most studies rely on the genetic influence of the R577X polymorphism on physical performance or response to training. However, few studies have focused on *ACTNs* transcript or protein levels in response to exercise training. Yu et al. [31] demonstrated that total protein level of α-actinins in human skeletal muscle was decreased following eccentric exercise but gradually recovers 7–8 days after exercise completion [31]. In rat fast and slow skeletal muscle Ogura et al [22] examined the effects of sprint-type exercise training regimen on α-actinin-2 and α-actinin-3 protein expression level. Relative to untrained rats, exercise training increased the expression level of α-actinin-2, but no change was found in α-actinin-3. Their results suggested that changes in α-actinin-2 production may be related to increase aerobic capacity for skeletal muscle after training.

Since most studies suggest that α-actinin-2 and α-actinin-3 are important determinants in skeletal muscle function leading to athletic performance, the properties of α-actinin-3 and α-actinin-2 in response to resistance exercise training and hypertrophy are of interest to this field. Considering that increases in muscle load stimulates the expression of different proteins in contractile machinery, it is still unclear whether α-actinins have a similar response to hypertrophy. Therefore, this study aimed to investigate the impact of hypertrophy induced by resistance training on protein and gene expression levels of α-actinin-3 and α-actinin-2 in rat skeletal muscles.

2. Materials and Methods

2.1. Ethics Statement

Animal care and protocols were in accordance with and approved by the Institutional Animals Ethics Committee of University of Tehran(Code:AL_236457) and were conducted according to the guiding principles for animal care and ethics course [2].

2.2. Animals

Female Sprague-Dawley rats(n=22, initial body mass:169.25±9 gr age:3month)were obtained from a licensed laboratory animal vendor in Razi Vaccine and Serum Research center (Ministry of Jihad Keshavarzi, Karaj, Iran). on arrival at our laboratory, all animals were provided with the standard rodent food and water *ad libitum* and housed in an environmental-controlled room [23 ± 1°C, 55 ± 5% relative humidity; 12: 12 h light-dark photoperiods (lights on 09:00–21:00 hours)]. Following one week of acclimation, animals were assigned to either a control (C; n = 10, 171 ± 4 g) or training group (T; n = 12, 169 ± 12 g).

2.3. Resistance Training (RT)

Rats underwent progressive resistance exercise which involved climbing a ladder, 110cm long, and 2cm grid, at a standard incline of 85 degrees. The animals were positioned to ensure that they performed each sequential step, with one repetition along the ladder involved 26 steps by the subject (or 13 steps per limb). Initially the rats were motivated to climb the ladder by touching their tail to initiate movement. At the top of the ladder the rats reached a housing chamber (20×20×20 cm) where they were allowed to rest for 120 seconds. Three days following familiarization with the ladder climbing, RT rats began a high intensity progressive resistance exercise regimen whereby weights were attached to their tails. The load apparatus was secured to the tail by wrapping the proximal portion of the tail with a self-adhesive foam strip. The first training session consisted of 4 to 8 ladder climbs while carrying progressively heavier loads with the, initial climb consisting of a load that was 75% of the animal's body weight [14]. Upon successful completion of this load, additional 30-g weight was added to the load apparatus. This procedure was successively continued until the load was reached to the level that the subject could not climb the entire length of the ladder. Failure to complete a climb was defined as the inability to make progress up the ladder following three touching the tail as a shock. The highest load successfully carried the entire length of the ladder was considered as the rat's maximal carrying capacity for that training [14]. Subsequent training sessions consisted of 4 to 9 ladder climbs. During the first 4 ladder climbs, rats carried, 50% 75%, 90% and 100% of their previous maximal carrying capacity (MCC) (Hornberger TA Jr, 2004), respectively. This training regimen was repeated once every 3 days for 8 weeks, a total of 20 training sessions. The control animals were handled on the same days and times as the trained groups in order to minimize any stress attributable to handling [14].

2.4. Tissue Collection

Following resistance training, the animals were anaesthetized with the pentobarbital sodium (50 mg kg) until a surgical plane of anesthesia was reached. The Soleus and Flexor hallucis longus(FHL) muscles were removed and weighed carefully; then placed in RNAlater

(Applied Biosystems, USA) or immediately covered in cryo-preservation medium (Tissue-Tek, ProSciTech) and snap froze in partially thawed isopentane. Muscles were stored in a freezer at - 80ºC until analysis. The animals were scarified by removal of the heart.

2.5. Antibodies

All antibodies to α-actinins were a gift from Prof. Kathryn North (Melbourne. Australia). The α-Actinin-2 was analyzed using the rabbit antibody 4B3 at 1:200 000 for Western blot (WB), and the α-Actinin-3 was analyzed using the rabbit antibody 5B3 at 1:12 000 for WB. For secondary antibodies, we used Alexa Fluor 555 goat anti-mouse IgM (1:250 dilution; Molecular Probes) and Alexa Fluor 488 goat anti-mouse IgG (1:200 dilution; Molecular Probes).

2.6. Sample Preparation for Detection of mRNA Expression

Muscle samples for the extraction of total RNA was available from 22 female rats from control and training groups. Total RNA was extracted from tissue samples using the RNaeasy Mini Kit (Qiagen, GmbH, and Hilden, Germany). Spectrophotometric analyses (Gensesys 10 UV) of RNA concentration and purity (UV246 260/280 and UV 260/230 ratios) were performed and extracts were stored at -80°C until used for RT-PCR analysis.

2.7. Quantitative RT- PCR

First strand cDNA synthesis was performed using QuantiTect® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). Equal amounts of RNA samples (1µg/reaction) were reverse transcribed in triplicates according to manufacturer's instructions. For qPCR experiments, QuantiTect® SYBR Green PCR Kit (Qiagen, GmbH, and Hilden, Germany) was used with specific PCR primers as described in Table1. β-Actin was selected for the normalization of quantitative data. Reactions were run in 10µl volumes on a Rotor-Gene™ 6000 real-time analyzer (Corbett Research, Qiagen, GmbH, and Hilden, Germany) for 45 cycles.

2.8. Data Analysis

For all qPCR experiments comparative quantitation among control and training samples was performed by REST 2009 (Relative Expression Software Tool, Qiagen, GmbH, and Hilden, Germany) based on Pair Wise Fixed Reallocation Randomization Test® [Pfaffl et al. 2002]. Charts were generated by GraphPad Prism 5 (GraphPad Software Inc., La Jolla, USA).

Table 1. Primers used for *Actn2* and *Actn3*

Target	Accession	Forward	Reverse
ACTN2	NM_001170325	5'- CTATTGGGGCTGAAGAAATCGTC -3'	5'- CTGAGATGTCCTGAATGGCG-3'
ACTN3	NM_133424	5'- AGAAACAGCAGCGGAAAACC -3'	5'- CAGGGCTTTGTTGACATTG -3'
βACTIN	NM_031144	5'- ACCATGTACCCAGGCATTGC -3'	5'-CACACAGAGTACTTGCGCTC -3'

2.9. Immunoblotting

Equal sample loading was evaluated using intensity of myosin and actin bands on pre-cast mini-gels (Invitrogen) stained with Coomassie Blue Brilliant (Sigma-Aldrich, USA) and total myosin is shown as a loading control [16,26]. Samples adjusted for loading were separated by SDS–PAGE on pre-cast minigels, transferred to polyvinylidene fluoride membranes (Millipore, USA), which was then blocked with 5% skim milk/1× PBS/ 0.1% Tween-20, then probed with indicated antibodies and developed with ECL chemiluminescent reagents (Amersham Biosciences, USA). Primary antibodies used included, α-actinin-2 (1:500 000) and α-actinin-3 (1:12 000).

2.10. Statistical Analysis

The data are presented as mean ± SD and normality tested with Kolmogorov–Smirnov test. The differences between control and training groups analyzed using T-Test. $P < 0.05$ was considered statistically significant**.** All statistical analyses were performed using the statistical software SPSS 11.0.

3. Results

3.1. Effect of Resistance Training on *Actn2* and *Actn3* mRNA Expression

In order to detect changes in mRNA expression levels as a function of resistance training, mRNA samples were isolated for both training and control groups. Comparison of muscle samples from the training and control groups using qPCR showed significant upregulation of *Actn2* and *Actn3* mRNA expression in the training group by mean factors of 2.16 (SEM=0.16, 95% CI, 1.65 to 2.68), and 2.91 (SEM=0.43, 95% CI, 1.92 to 3.91) respectively (Figure 1 A and Figure 1B).

3.2. Effect of Resistance Training on α-actinin-2 and α-actinin-3 Protein Expression

We assessed levels of α-actinin-2 and α-actinin-3 protein expression in resistance trained group compared to the control group using the Western blot .In the slow Soleus muscle, similar to transcript changes, we found a significant greater level of α-actinin-2 in the trained group as compared to untrained controls (Figure 2.B). In contrast, in the fast FHL muscle we found no difference in the level α-actinin-3 protein expression in trained vs. control groups (Figure 2.A), despite the previously observed increase in mRNA expression levels.

3.3. Effect of Resistance Training on Body and Muscle Mass

No significant differences in the final body weights among the groups were observed (control: 202±9, training: 207±9) after 8 weeks of resistance training. As indicated in previous studies, this model of training Flexor hallucis longus (FHL) (mainly fast) is highly responsive to training stimulus [14]. In agreement with previous studies, we found significant increase in FHL muscle mass with

concomitant changes in body weight in trained group (P<0.001). Muscle mass, an one of the hypertrophic index [14], increased after progressive resistance training [14]. Despite a significant increase in FHL muscle mass (P<0.001), the slow soleus muscle [4] mass was no difference in the trained group compared to the controls (P=0.341) (Figure 4.A). Maximal weight carried by training group demonstrated performance improvements in trained group (P<0.001) (Figure 4.B).

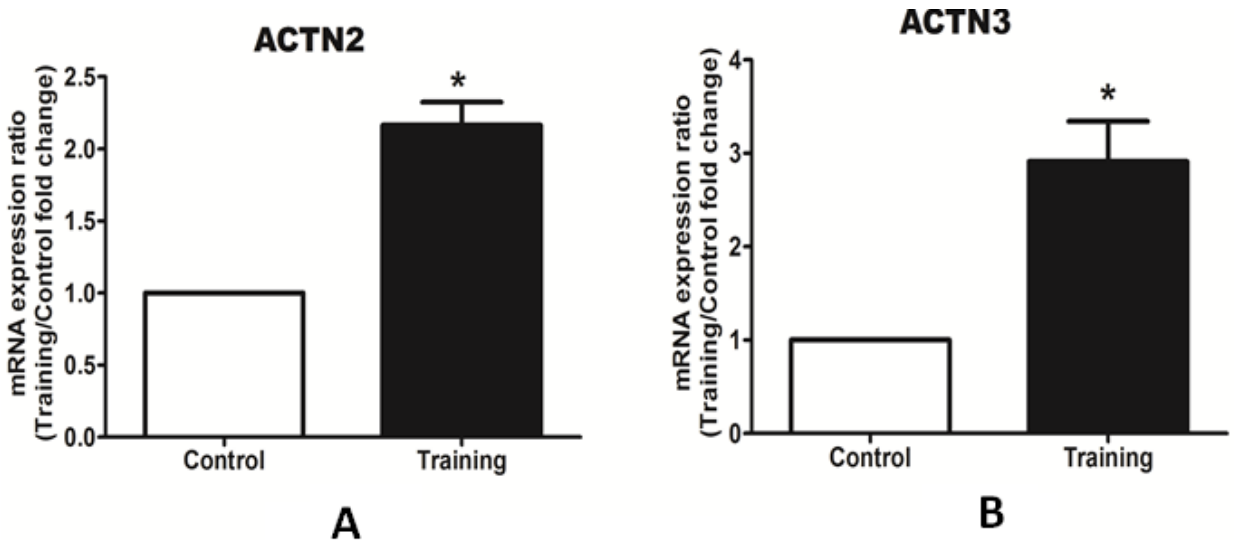

Figure 1. (A) Expression of *Actn2* in Soleus muscle and (B) *Actn3* mRNA in FHL muscle of training group shown as fold change compared to the control group. Statistical analysis is tested using pair wise fixed reallocation randomization test. * Significant with $P < 0.05$

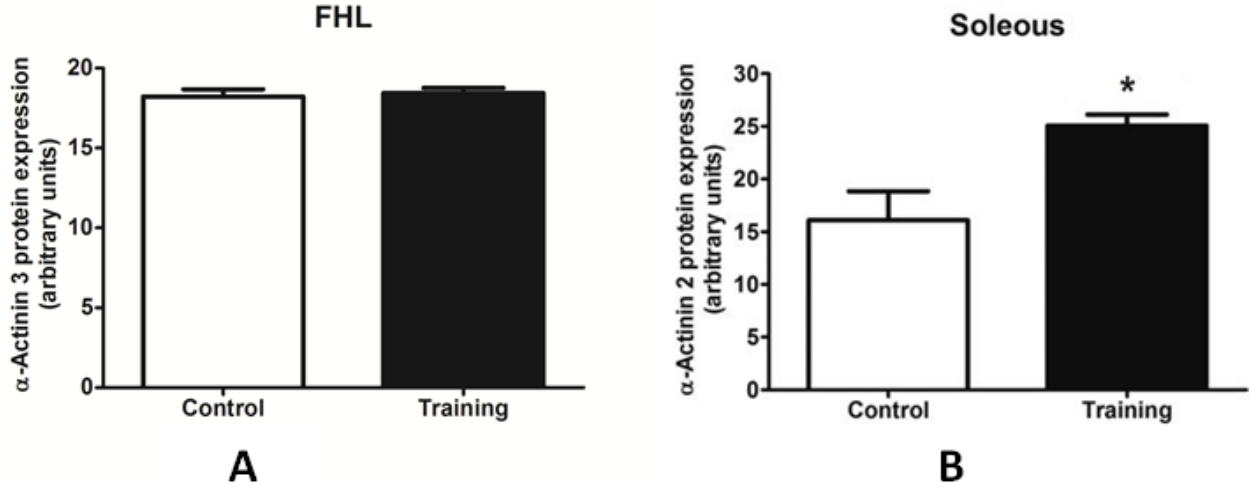

Figure 2. Measurement of protein expression levels for α-actinin-2 (A) and α-actinin-3 (B). Statistical analysis by T-Test* Significant with $P < 0.05$

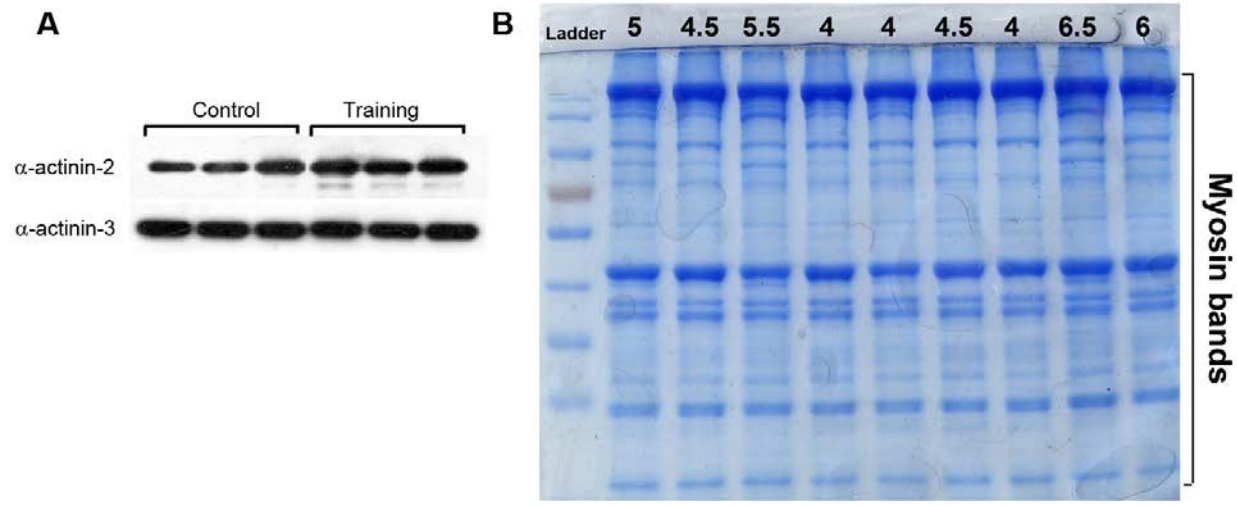

Figure 3. Up-regulation of α-actinin-2 proteins is observable in training group after 8 weeks of progressive resistance training. Western blot images showed no changes in α-actinin-3 among groups after 8 weeks of training (A). Total myosin is shown as a loading control for equal sample loading (B)

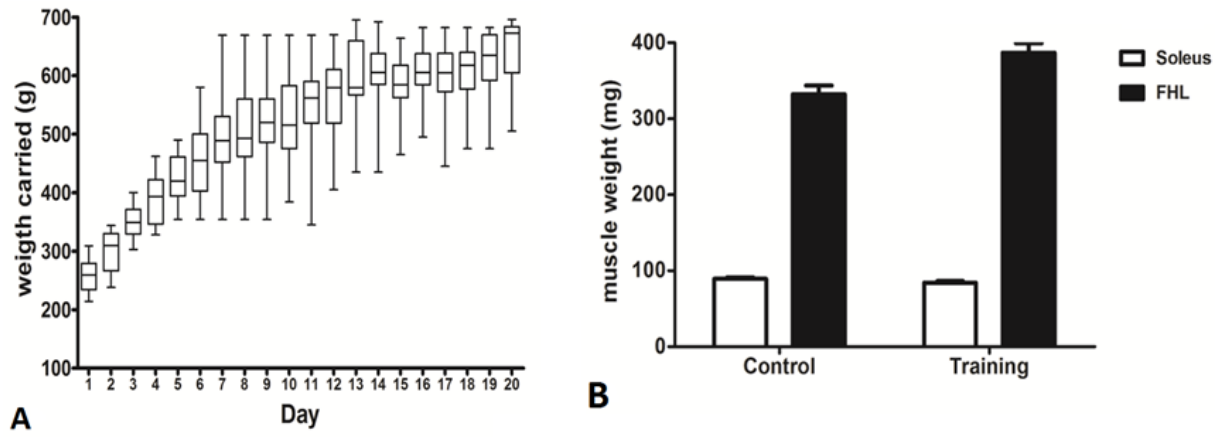

Figure 4. Changes in body and muscle mass. Muscles from rats in the both control and training groups were isolated and weighted (mean ± SD, P < 0.01) (A). Maximal carrying load per training session over the course of 20 training sessions (8 weeks). Values are expressed as mean ± SD (B)

4. Discussion

This study investigated the effects of progressive resistance training on *Actn2* and *Actn3* mRNA and α-actinin-2 and α-actinin-3 protein expressions in rat skeletal muscles. Unfortunately ethical committee does not approve human muscle biopsy for basic research and we used rats for this study. We examined Flexor hallucis longus (FHL) and Soleus muscles, which are the fast and slow twitch muscles. Our results showed that both *Actn2* and *Actn3* mRNA are upregulated in both the fast and the slow-twitch predominant muscles after 8 weeks of progressive resistance training and induced skeletal muscle hypertrophy. Our exercise training regimen induced hypertrophy in rat FHL muscle, indicated by increases in muscle mass and maximal weight carried during 8 weeks (Figure 4). A Previous study indicated that a single nucleotide polymorphism (SNP) of the *ACTN3* gene may be associated with muscle power performance [1,15,16,30]. This may imply that the α-actinin-3 isoform is critical in any activity calling for extraordinary speed or power. But still much remains to be done to understand really the potential contractile role of α-actinins. Therefore, it is important to determine the effect of physiological stimuli on cellular α–actinins. Also, we employed hypertrophic protocols from previous studies [3,12,13,14], which affect contractile and non-contractile apparatus in skeletal muscle in order to shed light on the role of α–actinins in this context.

Previous studies which emphasized the effects of different exercise training protocols on α-Actinins levels did not demonstrate an increase in both *ACTN3* and *ACTN2* [20,22]. Norman et al [20] investigated a bout of isokinetic exercise on *ACTN2* and *ACTN3* mRNA expression levels in different *ACTN3* genotypes. They showed that mRNA levels of *ACTN3* increased after exercise regimes in RR individuals but *ACTN2* mRNA did not increase significantly in the exercised group. In addition, they found that the expression of *ACTN2* affected by the content of *ACTN3*, which implies that α-actinin-2 may compensate for the lack of α-actinin-3 and hence counteract the phenotypic consequences of the deficiency. They also concluded that α–actinins do not play a significant role in determining muscle fiber-type composition [20]. Our findings are in agreement with this study in that we show how the α-actinins respond to resistance exercise stimuli. This may refer that the stimuli which is necessary for α-actinin-3 response to exercise depends on the type of resistance training performed. Other studies about the effect of resistance training emphasized strength gain with different genotypes and did not investigate any mRNA and protein measurements [5]. Clarkson et al [5] indicated 12 weeks of resistance training resulted in differential strength gain in individuals with different *ACTN3* genotypes [5]. Another study utilised a 10-week resistance training program in older men and women and demonstrated different response of quadriceps muscle strength in response with different *ACTN3* genotypes [6]. They found no association between *ACTN3* R577X genotype and muscle phenotype in men. Women homozygous for the mutant allele (577X) demonstrated greater absolute and relative 1-RM gains compared with the homozygous wild type (RR) after resistance training when adjusted for body mass and age. There was a trend for a dose-response with genotype such that gains were greatest for XX and least for RR (24). Also Delmonico et al [6] indicated the both women and men with RR gained more muscle power after 10 weeks of unilateral knee extensor strength training than in the XX group [6].

Another rat study investigating the effect of 9-week sprint treadmill training protocol on sarcomeric α-Actinins protein expression levels found sprint training increased the expression level of α-Actinin-2, but did not influence the expression level of α-actinin-3 [22]. Despite performing sprint training (typically associated with anaerobic performance), muscle shifted from IIb to IIa myosin and also increased aerobic enzyme activity to indicate the muscle was improving the aerobic capacity. Consistent with aerobic adaptation shifts, there were increases in the expression of α-actinin-2 with no change in α-actinin-3. Our progressive resistance training protocol resulted in increased mass of the Fast FHL muscle but not the slow Soleus muscle. This is consistent with previous studies using similar training protocols [9,14]. In the trained (heavier) FHL, this muscle showed an increase in transcript of *Actn3* but no change in *Actn3* protein. While the Soleus showed no change in mass, there was an increase in *Actn2* transcript and protein level. This suggests that in response to this weighted resistance training, similar to Ogura et al's [22] sprint training, there is an increase in *Actn2* level but no evidence for altered protein levels of *Actn3*. These differences in α-actinins

responses during adaptation suggest that these proteins may have different mechano-sensing properties and post-transcriptional control and should be analyzed in humans with reference to *ACTN3* genotypes. While some previous studies demonstrated that *ACTN3* genotypes may impact adaptation to training [22] the levels of *ACTNs* before and after power or endurance training have not been measured. Whether the impact of training on *ACTN3* gene expression may be limited to gene expression but not protein levels needs to be addressed in *ACTN3* RR and RX genotypes in combination with the response of *ACTN2*. Whether *ACTN3* XX individuals are able to upregulate their *ACTN2* levels is also relevant for understanding the Z-line response with training. Multiple factors, such as training intensity and frequency, muscle fibre type composition, and sex, may account for some of the contrasting finding reported. Recently, Seto et al. [27] showed that α-actinin-2, which is differentially expressed in α-actinin-3 deficient muscle, has higher binding affinity for calsarcin-2, a key inhibitor of calcineurin activation. α-Actinin-2 competes with calcineurin for binding to calsarcin-2, resulting in enhanced calcineurin signaling and reprogramming of the metabolic phenotype of fast muscle fibers [27]. One of the signaling pathways in skeletal muscle hypertrophy is Ca^{2+}/ calmodulin (CaM)-dependent phosphatase calcineurin (Cn). In addition to the role of Cn signaling in the determination of muscle fibre type characteristics, this phosphatase is known to play an important role in muscle hypertrophy [28]. If α-actinin-2 is related to calcineurin signaling [27], we speculate that the increase in α-actinin-2 levels may play a role in modulating the hypertrophic signaling pathway. Garton et al. [11] indicated *Actn3* KO mice had significantly less reduction in hind limb muscle mass and lean mass following immobilization [11]. They examined muscle fibre size, and demonstrated that the differential effect was most pronounced in type 2B fibres, where α-actinin-3 is normally expressed. Deficiency of α-actinin-3 in 2B fibres reduced the rate of atrophy [11]. They hypothesized that the presence or absence of α-actinin-3 would have a local effect in response to muscle atrophy, irrespective of the muscles' innervation status [11]. Considering the properties of Soleus muscle (slow twitch), our results indicate that α-actinin-3 deficiency may alter α-actinin-2 protein expression at Z-line to compensate the absence of α-actinin-3. [11] suggested in times of stress, α-actinin-2 at the Z-line resists proteolysis, resulting in the decreased atrophy response seen in α-actinin-3 deficient muscle. An alternative explanation is resistance properties of α-actinin-2 probably help Soleus muscle in response to progressive resistance training.

5. Conclusion

In conclusion, in agreement with previously published findings [11,16,25,26,27,29]=on mice and humans, we have shown that actinins mRNA and protein levels change in response to exercise in female Sprague-Dawley rats. However it is not yet clear whether the observed changed in actinins expression are primary or secondary response. Considering that actinins have been shown to interact with proteins that are associated with muscle remodeling and myofibrillar organization [27], we believe additional studies are warranted to elucidate fully the impact of resistance training on α-actinins response. Our knowledge about the effect of different training's modules on actinins proteins in the field of exercise science will complete after further investigation about the mechanisms that stimulate these proteins.

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Conflict Of interest

The authors declare no conflicts of interests.

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