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ORIGINAL ARTICLE

Masticatory Functional Load Increases the mRNA Expression Levels of ACTN2 and ACTN3 and the Protein Expression of α-Actinin-2 in Rat Masseter Muscle

Çiğneme Fonksiyonel Yükü Fare Masseter Kasında ACTN2 ve ACTN3'ün mRNA Ekspresyon Düzeylerini ve α-Aktin-2'nin Protein Ekspresyonunu Artırır

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ABSTRACT

Objectives: α-actinins play structural and regulatory roles in cytoskeletal organization. They form a lattice structure that secures actin in thin filaments, which generate and transmit muscle contractile forces. The morphological and biochemical characteristics of rat masseter muscles are known to change reactions to masticatory functional loads, but their effect on α-actinins remains unknown. This study aimed to determine the response of α-actinins to masticatory functional loads.

Materials and Methods: Twenty-four male Wistar rats aged 3 weeks were divided randomly into 3 groups of liquid diet (LD), soft diet, and hard diet (HD). The rats were then sacrificed at the end of 8 weeks. The middle part of superficial masseter muscles was examined to investigate the masticatory effect of functional load on the mRNA expression levels of ACTN2 and ACTN3 and the protein expression levels of α-actinin-2 and α-actinin-3.

Results: The mRNA expression levels of ACTN2 and ACTN3 and the protein expression levels of α-actinin-2 of the HD group were significantly higher than those of the LD group, which served as the control group.

Conclusion: Masticatory functional load organizes the mRNA expression levels of ACTN2 and ACTN3 and the protein expression levels of α-actinin-2 in rat masseter muscles through stimuli during muscle physiological adaptation.

Key words: α-actinins, masseter muscles, masticatory function

ÖZ

Amaç: α-aktininler, hücre iskeleti organizasyonunda yapısal ve düzenleyici roller oynarlar. Aktin, kas kasılma kuvvetlerini üreten ve ileten ince filamentler halinde sabitleyen bir kafes yapısı oluştururlar. Sıçan masseter kaslarının morfolojik ve biyokimyasal özelliklerinin çiğneme fonksiyonel yüküne karşı verilen tepkiyi değiştirdiği bilinmektedir, ancak bunların α-aktininler üzerindeki etkisi bilinmemektedir. Bu çalışma, α-aktininlerin çiğneme fonksiyonel yüküne karşı verdiği tepkiyi belirlemeyi amaçlamıştır.

Gereç ve Yöntemler: Üç haftalık 24 erkek Wistar sıçan rastgele sıvı diyet (LD), yumuşak diyet ve sert diyet (HD) uygulanan gruplar olmak üzere 3'e ayrıldı. Sıçanlar daha sonra 8 haftanın sonunda sakrifiye edildi. Yüzeyel masseter kaslarının orta kısmı, fonksiyonel yükün ACTN2 ve ACTN3'ün mRNA ekspresyon seviyeleri ve α-aktin-2 ve α-aktin-3'ün protein ekspresyon seviyeleri üzerinde çiğnemenin etkisini araştırmak için incelendi.

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Bulgular: HD grubunun ACTN2 ve ACTN3'ün mRNA ekspresyon seviyeleri ve α-aktin-2'nin protein ekspresyon seviyeleri, kontrol grubu olan LD grubununkilerden anlamlı şekilde daha yüksekti.

Sonuç: Çiğneme fonksiyonel yükü, kas fizyolojik adaptasyonu sırasında uyarılar yoluyla, fare masseter kaslarında ACTN2 ve ACTN3'ün mRNA ekspresyon seviyelerini ve α-aktin-2'nin protein ekspresyon seviyelerini düzenler.

Anahtar kelimeler: α-aktininler, masseter kasları, çiğneme fonksiyonu

INTRODUCTION

Animal studies have revealed a straight causal relation between the transformation of masticatory muscle function induced by substituting diet consistency and muscular changes in a complete masticatory system. Kiliaridis and Shyu[1] reported that the strength of masticatory muscles after tetanic stimulation is lower with a soft diet (SD) food than with a hard diet (HD) food. The population of satellite cells in the masseter muscle with a reduced masticatory function is small.[2] A decrease in the physical consistency of diets can increase the fiber diameter, muscle mass, and cross-sectional area of type 2B fibers.[3-7] The masseter muscles of animals fed with SD have smaller proportion and cross-sectional area of fibers that co-expressing myosin heavy chain (MyHC)-I and MyHC-cardiac alpha than those in the control animals.[8]

α-actinins, which belong to the actinin-binding protein group, are classified into muscle and non-muscle isoforms. α-actinin-cross-linked actin filaments are located on the Z disk of sarcomeres to help stabilize and maintain the architecture of the contraction of skeletal muscles.[9] α-actinins participate in a wide range of signal transduction complexes by interacting with other proteins to accelerate physiological changes.[10] It is considered an important structural component associated with the contractile muscle of force generation and transmission as in the maintenance of regular myofibrillar arrays. α-actinins in the skeletal muscle have 2 isoforms, namely, α-actinin-2 and α-actinin-3.[11-13] α-actinin-2 is found in entire muscle fibers, including cardiac muscles and the brain, whereas α-actinin-3 is limited to most fast-contracting fibers, e.g., type 2. Both actinins initiate energy production of force-generating glycolytic at a high speed.

Through interactions with calcineurin signals, α-actinin-3, which is encoded by *ACTN3*, can contribute to muscle function to influence the proportion of fiber types during growth.[14] α-actinin-3 deficiency (XX) may influence the decrease in the performance of muscle strength, power, and endurance of elite athletes and general population.[15] Ogura et al.[16] claimed that the total protein level of α-actinin-2 increases in the plantaris and in the white and red gastrocnemius muscles, but no significant disparity is observed in the α-actinin-3 expression after an exercise training. Khaledi et al.[17] found that the mRNA and protein expression levels of actinins change in response to progressive resistance training. This finding shows that the mRNA expression levels of α-actinin-2 and α-actinin-3 are upregulated after 8 weeks of exercise in female Sprague-Dawley rats. The protein expression of α-actinin-2 significantly enhanced in the training group although no difference is detected in the protein expression of α-actinin-3.[17]

However, few studies have described the link of α-actinins to masseter muscle activities. Zebrick et al.[18] demonstrated the mRNA expression level of the masseter muscle differs from that in ACTN3 single nucleotide polymorphism genotypes. In the sagittal and vertical classifications of malocclusion, the frequency of ACTN3 genotypes significantly differs. In skeletal class 2 malocclusion, the clearest association is the enhancement of 577XX genotype. This genotype also produces fast type 2 fibers with small diameters within masseter muscles.[18] These results indicate that some aspects of muscle function may be affected by ACTN3 genotypes, such as α-actinin-3, to enhance the forceful and fast skeletal muscle contraction. The deficiency of α-actinin-3 implies the need of α-actinin-3 for rapid muscle contractions and optimal force.

However, the response of α-actinins to masticatory muscles is still unknown. The contraction velocity and maximum force generation of a closing jaw's muscle responsible for chewing are influenced by a decrease in the masticatory functional load during development. Our study aimed to examine the effect of masticatory functional load on the mRNA and protein expression levels of ACTN2 and ACTN3 in the masseter muscle of rats.

MATERIALS AND METHODS

Twenty-four 3-week-old male Wistar rats (body weight=approximately 60 g) were randomly classified into 3 groups, which contained eight rats in each group. In group 1 (control group), a liquid diet (LD) was given to the rats fed with a blended mixture of pellets and water with a ratio of 1:4. In group 2, a SD was prepared for the rats fed with a slurry mixture of pellets tempered in water with a 1:1 ratio. In group 3, the rats were fed with regular rat pellets set as a HD group. All the groups were fed ad libitum and given water. The rats were separately placed in suspended metal cages without other materials or objects that could be a masticatory stimulus. Every week, the body weight and physical condition were measured and recorded to monitor the rats' condition. After 8 weeks of examination, all the rats were anesthetized with pentobarbital sodium at a fetal overdose of 50 mg/kg and sacrificed through exsanguination. Then, the middle part of the rats' superficial masseter muscles was dissected, frozen in liquid nitrogen, and stored at -85°C. This experimental protocol was approved by the Ethics Committee for the Health Research of Universitas Brawijaya, Malang, Indonesia (269/EC/KEPK/07/2017).

Analysis of the mRNA expression levels of ACTN2 and ACTN3

RNA was isolated using a total RNA purification kit (Jena Bioscience, Jena, Thuringia, Germany). cDNA was synthesized with an iScript™ cDNA synthesis kit (Bio-Rad, Hercules, California, USA) under the following conditions:

priming at 25˚C for 5 min, reverse transcription at 46˚C for 20 min, and enzyme inactivation at 95˚C for 1 min. Quantitative real-time reverse transcription PCR (qRT-PCR) was conducted using SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, California, USA) with the following primer sequences: ACTN2 F: 5'-CTATTGGGGCTGAAGAAATCGTC-3' and ACTN2 R: 5'-CTGAGATGTCCTGAATGGCG-3'. ACTN3 F: 5'-AGAAACAGCAGCGGAAAACC-3' and ACTN3 R: 5'-CAGGGCTTTGTTGACATTG-3'.[17] β-actin was chosen for quantitative data normalization, with the primers sequences as follow: β-actin F: 5'-ACCATGTACCCAGGCATTGC-3' and β-actin R: 5'-CACACAGAGTACTTGCGCTC-3'.[17] ACTN3 was amplified under the following conditions: enzyme activation at 95˚C for 3 min, denaturation at 95˚C for 5 sec, and annealing at 54.9˚C for 1 min (45 cycles). ACTN2 and β-actin were amplified under the following conditions: enzyme activation at 95˚C for 3 min, denaturation at 95˚C for 5 sec, annealing for 1 min at 57.3˚C (45 cycles). For all qPCR experiments comparative quantitation measured by a CFX96™ real-time PCR detection system (Bio-Rad, Hercules, California, USA).

Analysis of the protein expression levels of α-Actinin-2 and α-Actinin-3

Protein expression was analyzed via Western blot with α-actinin-2 (N1N3) and α-actinin-3 antibody (GeneTex, Irvine, California, USA). Protein bands in the gel were transferred to a PVDF membrane overnight and blocked with skim milk for 1 h. The membrane was incubated with 1:1000 anti-actinin antibody diluted in 1% PBS-skim milk overnight. Afterward, a secondary antibody was added, and BCIP/NBT was used as a substrate. Bands were analyzed using Quantity One (Bio-Rad, Hercules, California, USA).

Statistical analysis

Data were presented as mean ± standard error of the mean (SEM) and analyzed via One-Way ANOVA with Tukey's post hoc test. Differences were considered statically significant when $p<0.05$.

RESULTS

No significant differences were found in the rat body weight among the three experimental groups after 8 weeks (Table 1). The mean rat body weights in the LD, SD, and HD groups were 179.88±0.48, 179.30±0.75, and 179.64±0.72, respectively.

Table 1. Data are presented as mean body weight (grams) ± SD in liquid, soft, and hard diet groups for 8 weeks of the experiment (n=8 per group)

Week	2nd	4th	6th	8th
Diet	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Liquid	79.06±0.26	136.91±0.18	158.31±0.49	179.88±0.48
Soft	78.64±0.47	136.5±0.46	157.89±0.45	179.30±0.75
Hard	78.96±0.19	136.95±0.21	158.51±0.52	179.64±0.72

SD: Standard deviation

mRNA expression of ACTN2 and ACTN3

The mRNA expression levels of ACTN2 and ACTN3 in the 3 experimental groups were assessed through qPCR. In the HD group, the mRNA expression levels of ACTN2 and ACTN3 from the masseter muscle significantly increased by mean factors of 2.29 (SEM: 0.41) and 2.19 (SEM: 0.2), respectively, in contrast to the LD group. In the SD group, the mRNA expression levels of ACTN2 and ACTN3 were upregulated, but they were not significantly different from that in the LD and HD groups ($p<0.005$) (Figure 1).

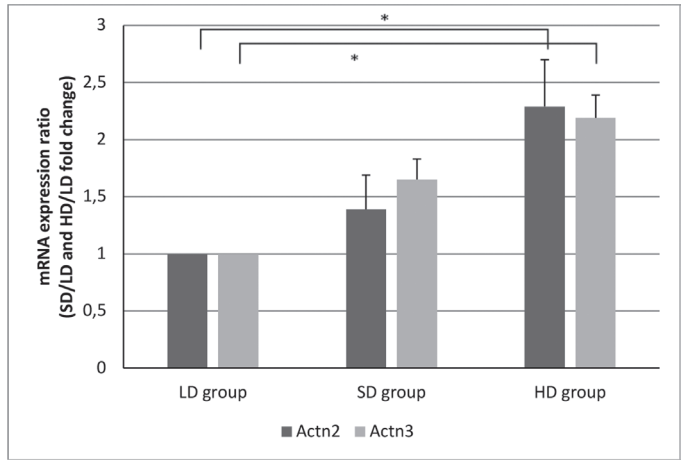

Figure 1. mRNA expression levels of ACTN2 and ACTN3 in masseter muscles shown as fold change compared with those in the liquid diet group as the control group. Statistical analysis was performed via One-Way ANOVA with Tukey's post-hoc test. *Significant with $p<0.05$. LD: Liquid diet SD: Soft diet HD: Hard diet

Protein expression of α-actinin-2 and α-actinin-3

The effects of masticatory muscle function on the protein expression levels of α-actinin-2 and α-actinin-3 in the 3 experimental groups were assessed through Western blot. The protein expression level of α-actinin-2 in the HD group (18.45, SEM: 0.78) was significantly higher than that in the LD group (14.40, SEM: 0.44; Figure 2A). The protein levels of α-actinin-3 in the HD group also increased compared with that in the SD and LD groups by mean factors of 16.75 (SEM: 0.72), 14.16 (SEM: 0.91) and 14.66 (SEM: 0.97), respectively. However, no significant difference in the protein expression levels of α-actinin-3 was observed in the 3 groups (Figure 2B).

DISCUSSION

This study investigated the functional influence of masticatory muscles on the mRNA expression levels of ACTN2 and ACTN3 and the protein expression levels of α-actinin-2 and α-actinin-3 with the consistency of diet variation. In the 3 experimental groups, no transformation was observed in the masticatory pattern in response to the consistency of diet variations. No significant body weight differences were observed in the LD, SD, and HD groups during the 8-week experiment. The mRNA expression levels of ACTN2 and ACTN3 were upregulated as the consistency of diet increased in the SD and HD groups compared with that in the LD group. The protein expression of α-actinin-2

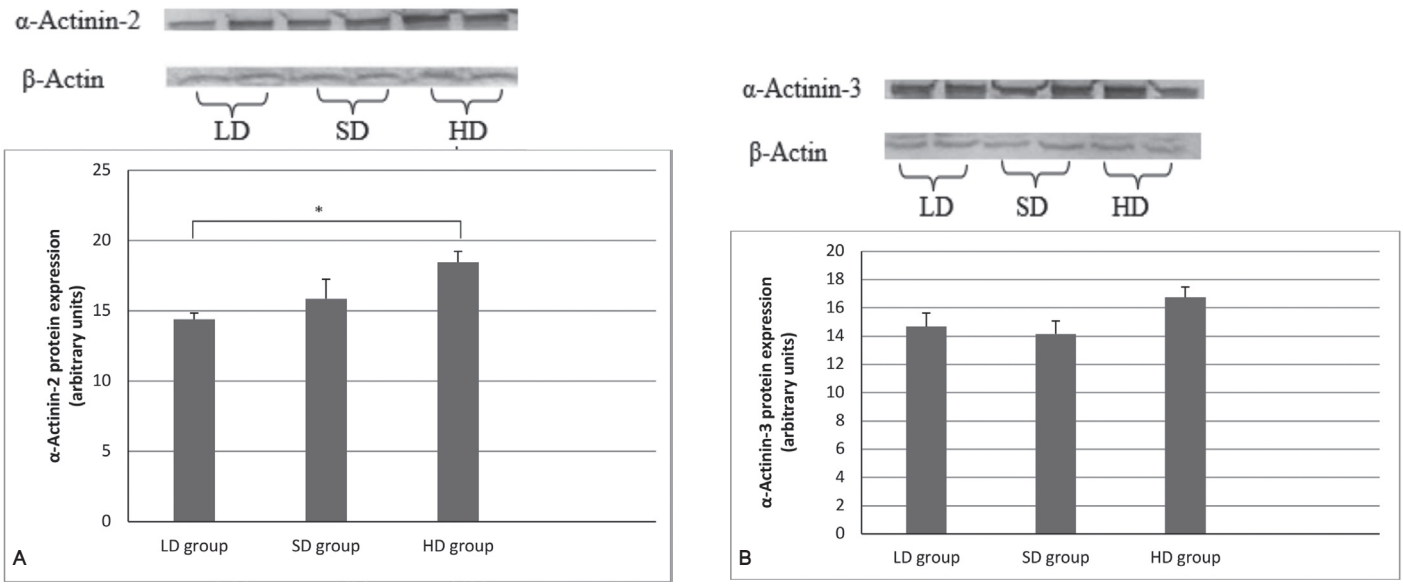

Figure 2. Western blot analysis. A) α-actinin-2 and B) α-actinin-3 protein expression in the LD, SD, and HD groups. β-actin was used as the sample loading control. The bar graphs on the right represent means ± SEM. *Significant with $p<0.05$. LD: Liquid diet SD: Soft diet HD: Hard diet, SEM: Standard error of the mean

increased as the consistency of diet increased. However, the expression levels of ACTN2, ACTN3, and α-actinin-2 in the LD group significantly differed from that in the HD group. However, the difference was not significant when the LD and HD groups were compared with the SD group. The expression levels of α-actinin-3 did not significantly differ among the three groups.

A previous study showed that about 16%-18% of the global population lacks α-actinin-3 possibly because of homozygosis for the common null of ACTN3 577X polymorphism.[19] This phenomenon is also associated with untrained adolescents, elite athletes, and young adults with low sprint performance, low muscle strength, and weak muscle power.[13] In the human skeletal muscle, the total protein level of α-actinins decreases after irregular exercise and recovers systematically in 7-8 days once exercise is complete.[20] This finding suggests that α-actinin-3 may be considered an important structural component to optimize forceful and rapid muscle contractions.[13]

The results of this study were similar to those of several animal studies on the effect of physiological stimuli on cellular α-actinins. Khaledi et al.[17] examined the effects of progressive resistance training on the gene and protein levels of ACTN3 and ACTN3. They found that the mRNA levels of ACTN2, ACTN3, and α-actinin-2 increase, but no transformation occurs in the protein expression of α-actinin-3. Ogura et al.[21] observed the effects of endurance exercise training on rats by using α-actinin-2 and α-actinin-3 levels. After exercise for 8 weeks on a treadmill, the α-actinin-2 expression in the plantaris muscles is slightly higher than the α-actinin-3 expression. They also demonstrated that α-actinin isoforms respond to other physiological stimuli. Therefore, the α-actinin-3 expression is slightly higher than the α-actinin-2 expression after hind limb unloading.[22]

Diet food consistency in laboratories changes the strength level of biting demands, masticatory activity, and behavior. It changes the composition and diameter of fiber types in animal masticatory muscles.[1,3-5] In the present study, physiological stimulation through masticatory functional load revealed that cellular α-actinins were involved in the masseter muscle. Zebrick et al.[18] used the masseter muscle obtained via orthognathic surgery to examine the expression and genetic variation in ACTN2 and ACTN3 and determined their associations with musculoskeletal malocclusion phenotypes. Masseter muscle samples from 60 subjects who underwent orthognathic surgery included the following vertical and sagittal classifications: class 2 and class 3 open bite, class 2 and class 3 deep bite, and class 2 and class 3 normal bite malocclusions. Their results demonstrated that the ACTN3 polymorphism R577X is related to class 2 and deep bite skeletal malocclusions. In masseter muscles, α-actinin-3 is lost with the small diameter of type 2 fiber. Real-time PCR demonstrated that the mRNA expression of ACTN3 is almost undetected with the 577XX genotype, and the expression level of ACTN2 remains unchanged. Therefore, ACTN2 may not compensate the loss of α-actinin-3 in masseter muscles.[18]

The adoption of consistency of diet variations is based on histological, morphological, and biochemical alterations in muscle fiber types. In the SD group, type 2A had a smaller percentage and type 2B had a larger percentage in the anterior deep masseter than those in the normal diet group.[1] Fundamentally, α-actinin-2 is found in the entire fibers of the skeletal muscle, whereas the α-actinin-3 expression is limited to type 2 fast-contracting skeletal muscle fibers.[11,13] Ogura et al.[22] indicated that the α-actinin-3 expression enhances in terms of the relative content of type 2 MyHC and fast myosin levels after hind limb unloading. Exercise training changes MyHC from 2B to 2A. Their study also suggested that changes in MyHC composition may affect the enhancement of the aerobic capacity of skeletal muscles after training.[22] The line

of fiber-type-specific gene expression-activated α-actinin-3 defines the type and size of fibers by binding to the calsarcin family of signaling proteins on the Z disk, which binds to the signaling protein calcineurin.[23,24]

This masticatory functional load showed that reactions were similar to those in skeletal muscle models. This finding indicated that the mRNA expression of ACTN2 and ACTN3 and the protein expression of α-actinin-2 were altered during masticatory muscle function. The mRNA expression of ACTN2 and ACTN3 and the protein expression of α-actinin-2 were significantly changed as the masticatory functional load increased between the HD and LD groups. Non-significant differences were shown in the SD group compared with the LD and HD groups. This difference likely indicated that the masticatory functional load in the SD group was insufficient to induce mRNA and protein expression. However, further investigation on the differences in the expression levels of ACTN2 and ACTN3 among LD, SD, and HD groups should be performed, which increased in time or remained stable is needed.

CONCLUSION

In summary, the mRNA expression levels of ACTN2 and ACTN3 and the protein expression level of α-actinin-2 are set in the rat masseter muscle as the masticatory functional load increases. Even though cellular α-actinins of the masseter muscle likely adapt to functional changes, the underlying mechanism should be further elaborated.

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