Masticatory Functional Load Increases the mRNA Expression Levels of ACTN2 and ACTN3 and the Protein Expression of α-Aktin-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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Objektifler: 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 bioyokimyasal özelliklerinin çiğneme fonksiyonel yüküne karşı verilen tepkiyi değiştirmediği bilinmektedir, ancak bunların α-aktininler üzerindeki etkisi bilinmemektedir. Bu çalışma, α-aktininlerin çiğneme fonksiyonel yüküne karşı verdiği etkisini araştırmayı amaçlamaktadır.

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

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 bioyokimyasal özelliklerinin çiğneme fonksiyonel yüküne karşı verilen tepkiyi değiştirmediği bilinmektedir, ancak bunların α-aktininler üzerindeki etkisi bilinmemektedir. Bu çalışma, α-aktininlerin çiğneme fonksiyonel yüküne karşı verdiği etkisini belirlemeyi amaçlamıştır.

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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.\(^3\) 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.\(^8\)

\(\alpha\)-actinins, which belong to the actinin-binding protein group, are classified into muscle and non-muscle isoforms. \(\alpha\)-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.\(^9\) \(\alpha\)-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. \(\alpha\)-actinins in the skeletal muscle have 2 isoforms, namely, \(\alpha\)-actinin-2 and \(\alpha\)-actinin-3.\(^11\) \(\alpha\)-actinin-2 is found in entire muscle fibers, including cardiac muscles and the brain, whereas \(\alpha\)-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, \(\alpha\)-actinin-3, which is encoded by ACTN3, can contribute to muscle function to influence the proportion of fiber types during growth.\(^16\) \(\alpha\)-actinin-3 deficiency (XX) may influence the decrease in the performance of muscle strength, power, and endurance of elite athletes and general population.\(^16\) Ogura et al.\(^16\) claimed that the total protein level of \(\alpha\)-actinin-2 increases in the plantaris muscle, whereas \(\alpha\)-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.

However, few studies have described the link of \(\alpha\)-actinins to masticatory muscle activities. Zebbrick et al.\(^19\) 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 \(\alpha\)-actinin-3, to enhance the forceful and fast skeletal muscle contraction. The deficiency of \(\alpha\)-actinin-3 implies the need of \(\alpha\)-actinin-3 for rapid muscle contractions and optimal force.

However, the response of \(\alpha\)-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\(^14\) 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’-CTATTGGGGCTGAAAGAATGTC-3’ and ACTN2 R: 5’-CTGAGATTCCTGAAAGTCC-3’. ACTN3 F: 5’-AGAAACGCAAAAGGAAAACC-3’ and ACTN3 R: 5’-CAGGGCTTTGTTGACATTG-3’. 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’. 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, and annealing at 57.3°C for 1 min (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 (NIN3) 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 statistically 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 | Diet | 2w  | 4w  | 6w  | 8w  |
|------|------|-----|-----|-----|-----|
|      | 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 |

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).

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
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. This
phenomenon is also associated with untrained adolescents,
elite athletes, and young adults with low sprint performance,
low muscle strength, and weak muscle power. 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. This finding suggests
that α-actinin-3 may be considered an important structural
component to optimize forceful and rapid muscle contractions.
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. 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. 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.
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. In the present study, physiological stimulation
through masticatory functional load revealed that cellular
α-actinins were involved in the masseter muscle. Zebrick et al.
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.
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.
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. Ogura et al.
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. The line
of fiber-type-specific gene expression-activated \(\alpha\)-actinin-3 defines the type and size of fibers by binding to the calsinacin 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 \(\alpha\)-actinin-2 were altered during masticatory muscle function. The mRNA expression of ACTN2 and ACTN3 and the protein expression of \(\alpha\)-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 \(\alpha\)-actinin-2 are set in the rat masseter muscle as the masticatory functional load increases. Even though cellular \(\alpha\)-actins of the masseter muscle likely adapt to functional changes, the underlying mechanism should be further elaborated.

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