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Effects of Increased Activity on Neuromuscular Junction in Aged Rat Diaphragms

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Abstract

SUZUKI, T., HISAMITSU, R. and MIYATA, H., Effects of Increased Activity on .Neuromuscular Junction in Aged Rat Diaphragms. Adv. Exerc. Sports Physiol., Vol.14, No.2 pp.31-36, 2008. To examine the age-related functional and morphological changes in neuromuscular junction, male adult rats were divided into young (3-month; n = 14), adult (1-year; n = 14) and old (2-year; n = 14) groups. Seven rats in each group were subjected to unilateral phrenicotomy to induce compensatory increased activity on intact hemidiaphragm. Neuromuscular transmission failure (NF) after 2 min repetitive contraction was evaluated by a comparison of forces generated by the phrenic nerve and muscle stimulations. The endplate area was analyzed by immunohistochemical staining procedure and confocal imaging. The relative NF to muscle fatigue was significantly lower in the old group than the young and adult groups, indicating a functional improvement of neuromuscular transmission with aging. The compensatory activated diaphragm indicated significantly larger endplate area and lower transmission failure than those in age-matched control groups in the young and adult groups, but not in the old group. These results indicated that functional and morphological plasticity of the neuromuscular junction decreased in old diaphragm muscle.

Keywords: aging, diaphragm, transmission failure, endplate area

Introduction

It has been well documented that human muscular strength decreases with aging, especially from the sixth decade of life. The decrease in strength is explained by the reduction in muscle mass (9). The decline in muscle mass is mediated by a reduction in the size and number of muscle fibers, especially type II muscle fibers (19), although the extent of the muscle mass loss varies among muscles. Furthermore, the intensity of daily physical activities is known to influence the degree of muscle mass wasting (8).

The diaphragm muscle is a special group of skeletal muscle that is rarely inactive since it is a major inspiratory muscle with a duty cycle, defined as the ratio of active to total times, of 40% (23), whereas the duty cycle of the extensor digitorum muscle is 2% (15). It has been reported that despite this remarkable activation history, age-related changes in the diaphragm muscles still occur. For example, the maximum isometric force production decreased by 13% in old rats as compared to young ones (5). Furthermore, Gosselin et al. (13) demonstrated that the age-related decline in diaphragm muscle specific force is caused by intrinsic factors other than changes in myosin heavy chain composition. It is thought that the impaired normal cycling of denervation-reinnervation and consequent increased proportion of hybrid fibers may be the underlying mechanism in the decreased strength and speed of muscle contraction (25). Therefore, the neuromuscular junction (NMJ) is considered one of the most important sites in the aging process. To date, however, little information has been published describing the age-related changes in NMJ function and its adaptation to increased activity.

For functional properties of NMJ, Miyata et al. (22) showed neuromuscular transmission improvement of the young diaphragm muscle to which activity level increased by 50%, induced by contralateral phrenicotomy. This improvement was accomplished without any changes in functional properties of phrenic motoneuron (24). It should, therefore, be of interest to examine to what extent aged NMJ of the diaphragm muscle can adapt to increased activity. The purpose of the present study was to examine whether aged NMJ can adapt to a compensatory increased activation (CAC) of the diaphragm muscle.

Material and Method

Animal treatment

All experimental and animal care procedures were approved by the Committee on Animal Care and Use in Yamaguchi University and followed the American Physiological Society Animal Care Guidelines.

Experiments were performed on 14 male Wistar rats in young (2-month; 281±34g), adult (11-month; 588±41g) and old (23-month; 620±99g) groups. Each group was then divided into control (CTL) and compensatory activation (CAC) groups. Surgical procedures were performed on the

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CAC group while animals were anesthetized with pentobarbital sodium (50mg/kg) by IP. Under an operating microscope, the right phrenic nerve was exposed. By transecting one centimeter of the right phrenic nerve, the left side of the diaphragm activity level was augmented. The same procedure was performed on sham controls, only omitting the nerve section. The wound of muscle and skin were sutured separately after confirming absence of hemorrhage at the surgical site. After one month (3-month, 1-year and 2-year age), there were no significant differences in body weight between CTL (366±28g, 599±53 and 615±93g, respectively) and age-matched CAC groups (355±31g, 601±42g, 620±103g, respectively). The animals were re-anesthetized with pentobarbital sodium, and the diaphragm muscle was excised. Muscle segments 5 mm wide were sectioned in a parallel direction with the muscle fiber from the mid-costal region of the diaphragm muscle. One muscle segment was dissected with one centimeter of phrenic nerve attached to it and used for in vitro transmission failure assessment at NMJ. After assessment, the muscle segment was stretched to optimal length and rapidly frozen until assay for myofibrillar ATPase.

Assessment of neuromuscular transmission failure

A muscle segment with phrenic nerve was mounted vertically in a glass chamber in which Ringer solution was oxygenated at 95% O_2, 5% CO_2 and maintained at pH 7.4 and 25±1℃. The muscle was attached at one end to a force transducer and at the other end was fixed to be immobile. Muscle fiber length was adjusted where maximal isometric twitch responses were obtained (optimal length). Intensity was set at 125% of this value where maximal intensity can be obtained. The phrenic nerve and diaphragm muscle were stimulated through a suction and plate electrodes, respectively. First, initial forces generated by the phrenic nerve and muscle stimulation were compared. According to the procedure of Burke et al. (4), the phrenic nerve was stimulated repetitively at 40Hz in 330 ms duration, and continued for a 2-min period. Then, direct muscle stimulation was performed two times.

According to the method described by Aldrich et al. (1), the difference between the force generated by nerve and direct muscle stimulation was used to estimate the neuromuscular transmission failure to muscle fatigue, using the following formula: (F − MF) / (1 − MF)

Where F is the percent decrement in force during repetitive nerve stimulation and MF is the percent force decrement during direct muscle stimulation.

Histochemistry for endplate and muscle

The muscle segments were washed and immersion fixed in 2% paraformaldehyde. The fixed samples were blocked for nonspecific staining by using 10% donkey serum in 0.1M Tris-buffered saline containing 1% Triton. The tissue was washed and incubated in 5ug/mL α-bungarotoxin-tetramethylrhodamine (SIGMA, Tokyo). In each animal, at least 25 endplates were sampled. In total, more than 200 images in each group were analyzed. Images were obtained using a laser-scanning confocal system (C1, Nikon, Tokyo) mounted on a microscope (E600, Nikon, Tokyo) and equipped with a HeNe-Green laser (543nm excitation wave length). Sets of optical sections were transferred to a comprehensive image-manipulation and -analysis software package (3D-Doctor, Solution System, Tokyo). Two-dimensional (2D) stack images for each labeled endplate were reconstructed and the areas were measured.

The frozen muscle segment was cut using a frozen microtome (CM510, Leica, Tokyo) into two transverse sections of 10μm thickness. The sections were histochemically stained to detect myofibrillar ATPase after prior incubation at pH 4.6 and 10.3, based on the technique of Brooke and Kaiser (3). On the basis of histochemical staining image examination, muscle fibers were classified as type I, IIA and IIB fibers. To calculate the relative contribution of each fiber type to total cross sectional area (CSA), fiber type population (%) and type-specific CSA were measured.

Statistics

The results obtained in this study were analyzed by two-way ANOVA for all data with experimental groups (CTL or CAC) and age groups (young, adult or old) as grouping variables. Post-hoc analysis was performed using a T-test with the Bonferroni adjustment method. In all cases, statistical significance was set at $p<0.05$. All values are reported as the mean ± standard deviation.

Results

Neuromuscular transmission failure (NF)

Typical force decline by repetitive stimulation of young and old control (CTL) are shown in Fig. 1. The ratios of force generated by nerve to force generated by muscle stimulation at the beginning of the experiment were 96.8±2.0% in young CTL, 97.0±1.7% in young CAC, 96.3±0.7% in adult CTL, 97.2±1.1% in adult CAC, 95.8±0.9% in old CTL, 96.6±1.3% in old CAC groups, indicating that no damage was induced by setting up muscle specimens onto the experimental system. Mean value of NF was 67.8±4.0% in young CTL, 60.1±3.8% in young CAC, 64.7±5.2% in adult CTL, 51.1±8.4% in adult CAC, 41.8±7.2% in old CTL, 36.3±5.2% in old CAC groups (Fig. 2). In CTL group comparison, NF in old group was less than those observed in both young and adult groups, whereas there was no difference between young and adult groups. The NF in CAC group was less than those observed in age-matched CTL groups in both young and adult groups, whereas there was no difference in the old group.

Contribution of each muscle fiber type to total cross sectional area (CSA)

Relative contributions of each muscle fiber type to total CSA (relative area) are shown in Fig. 3. In CTL groups, the relative area of type I, IIA, and IIB fibers were 24.9±3.0, 31.5±3.3% and 43.6±4.1% in young, 24.1±3.4%, 27.1±4.7% and 48.8±7.5% in adult, and 33.7±6.1%, 29.8±6.8% and 36.5±7.1% in old groups, respectively. In CAC groups, the relative area of type I, IIA and IIB fibers were 30.1±7.1%, 32.5±9.4% and 37.4±9.8% in young, 25.4±3.5%, 28.1±7.5% and 46.5±6.9% in adult, and 27.0±5.1%, 24.9±7.6% and 48.1±9.9% in old groups, respectively. In CTL groups, as compared to young and adult, there were significantly higher values of type I fiber and concomitant lower values of type IIB fiber in old groups. As compared to the old CTL group, significantly higher value in type IIB fiber was observed with concomitant lower values of type I fiber in the old CAC group.

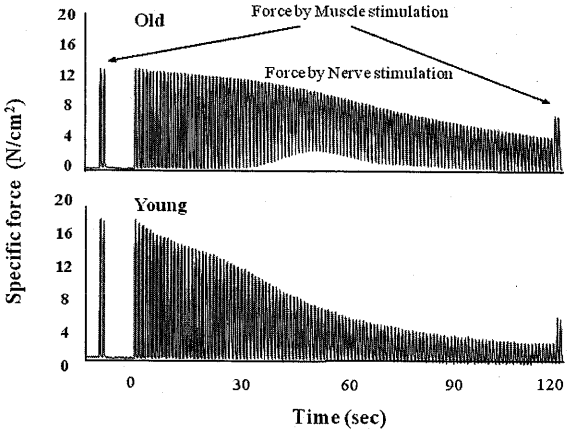

Fig. 1 Typical force generations (N/cm^2) by repetitive stimulation of young (lower) and old (upper) groups in control animals
Before and after nerve stimulation for 2 min, two direct stimulations for diaphragm muscle segment were performed. Basically, the difference between the force generated by last nerve stimulation and following muscle stimulation represents transmission failure in neuromuscular junction.

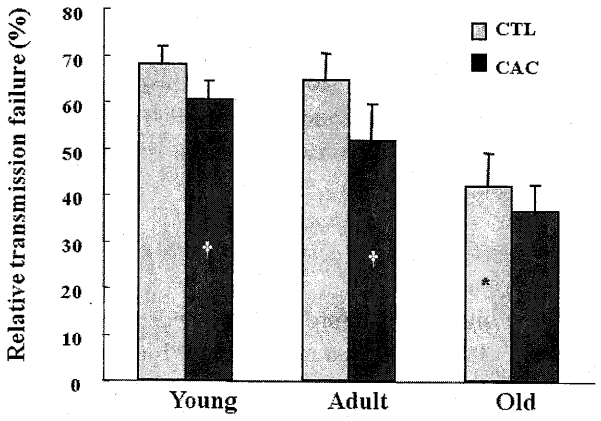

Fig.2 Comparison of mean values in neuromuscular transmission failure to muscle fatigue between control (CTL) and compensatory activation (CAC) animals in each age group
*; significant difference between the old group and the other two groups in CTL animals
+; significant difference between CTL and CAC animals in each age group

Endplate Morphology

Immunohistochemically labeled NMJ images are shown in Fig. 4. The labeled endplates in young diaphragms were uniform in shape, but this was not observed in old diaphragms. In pooled data analysis, the endplate area distribution in old CTL varied more extensively between 200 to 2000 μm^2, whereas the variation in young CTL was only between 200 to 700 μm^2 (Fig. 5). These findings indicate that the endplate tends to be diversified with aging. In addition, there were many endplates with ab-

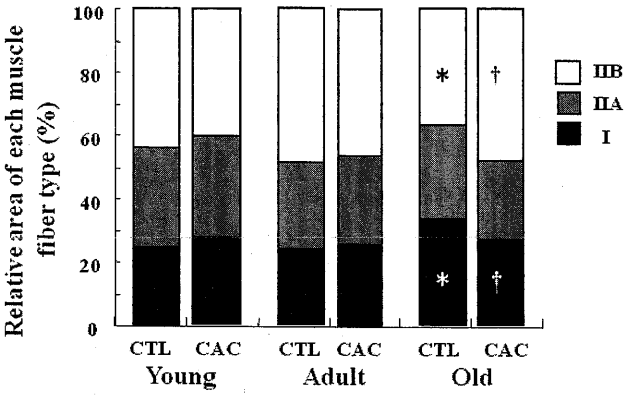

Fig. 3 Comparison of mean values in relative contributions of each muscle fiber type to total cross sectional area (relative area)
*; significant difference between the old group and the other two groups in CTL animals
+; significant difference between CTL and CAC animals in old group

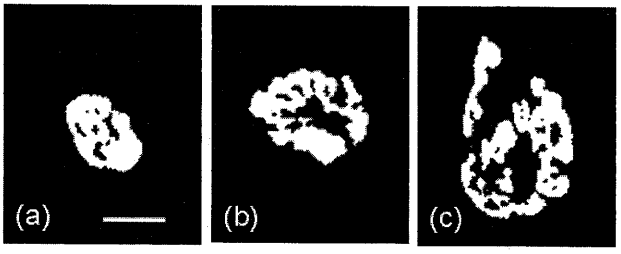

Fig. 4 Labeled endplates with α-bungarotoxin-tetramethylrhodamine in young (a), adult (b) and old (c) diaphragm muscle
Two-dimensional stack images for each labeled endplate were reconstructed at the same magnification. Bar indicates 20 μm

normal shape in the old group, but not in the young and adult groups. Mean endplate areas of 7 animals in each group were 406±40 μm^2 in young CTL, 491±29 μm^2 in young CAC, 614±34 μm^2 in adult CTL, 656±34 μm^2 in adult CAC, 648±103 μm^2 in old CTL, 692±81 μm^2 in old CAC groups (Fig. 6). In comparison among age groups, endplate area was significantly smaller in the young group than the other two groups in both CTL and CAC groups. In young and adult animals, the endplates were significantly larger in CAC as compared to age-matched CTL, but not in the old group.

Discussion

Age-related changes in neuromuscular junction

In this study, the muscle fiber type was determined by histochemical analysis for myofibrillar ATPase. Therefore, as demonstrated in a previous study using histochemical and electrophoretic methods (29), it should be mentioned that the type IIB fibers in this study contained a large amount of type IID/X fibers.

The present data showed that the improvement in neuromuscular transmission with aging is attributed to the changes in relative area of each muscle fiber type and its endplate area. The most remarkable age-related changes in muscle fiber was the decrease in the relative area of type IIB fibers with concomitant increase in that of type I fibers. Previous studies (17) on type-specific neuromuscular transmission indicated that NF in slow-twitch fibers is modest compared to fast-twitch muscle fibers. In addition, a recent study demonstrated that synaptic vesicle density was greater at type I/IIA than IIX/IIB fibers in rat diaphragm in an electron-microscopic observation (21). Therefore, significant higher relative area of type I fiber and lower relative area of type IIB fiber was considered to be a main reason for reduced NF in old CTL group. Although age-related fiber type remodeling was not significant in old CAC group, reduced NF was still found. We speculate that endplate structural change may have a closer relationship to neuromuscular transmission than fiber type modification. Prakash and Sieck (26) demonstrated a significant expansion in endplate area on type IIX and IIB fibers in rat diaphragm with aging. These adaptations were appropriate for securing the safety factor because when nerve terminal and endplate enlargement occurs, the subsequent increase in ACh release and an increase in number of its receptors may result. The age-related modification in the ability to supply acetylcholine and acetylcholinesterase activity leading to increased safety factor resulted in less NF to continuous stimuli.

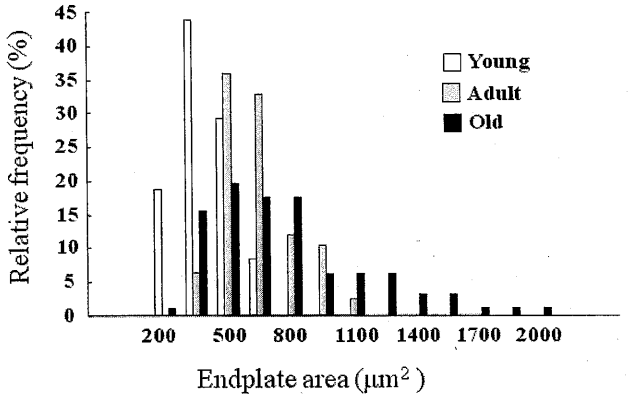

Fig. 5 Relative frequency of endplate area in each age group in CTL animals

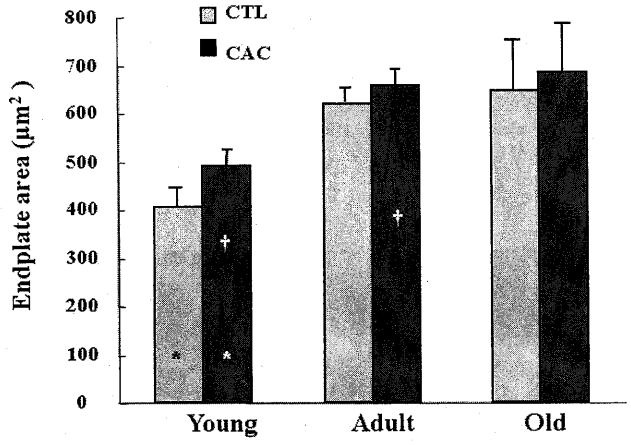

Fig. 6 Comparison of mean values in endplate area between CTL and CAC animals in each age group
*; significant difference between young and the other two group in each experimental group
+; significant difference between CTL and CAC animals in each age group

In this study, wide distribution of endplate area and abnormal endplate shape were seen in the old group, but not in the young and adult groups. As mentioned in a previous study (14), the age-related fragmentation of the endplate might lead to a loss of muscle fiber and motor unit remodeling. Further studies are needed to evaluate the functional and biochemical properties of enlarged and/or fragmented endplate.

Diaphragm muscle plasticity to compensatory increased activity

The significant improvement in neuromuscular transmission after compensatory increased activity was observed in young and adult rats, but not in old rats. We considered that increased activation could attenuate age-related selective atrophy of type II fibers, then the significant increase in relative area of type I fiber was not found as younger rats. Furthermore, the significant enlargement of endplate was not induced in old CAC group, indicating that differential adaptations to CAC occurred between young and old groups. The activity-induced effect on NMJ

as mentioned below may be not induced in old age rats.

In young rats, training effects at NMJ have been introduced previously, such as increased safety factor by increased neurotransmitter release when given active training stimuli (7), nerve terminal enlargement (6, 2, 31), and increased acetylcholinesterase activity (11, 12, 16). From these observations, it was thought that the nerve terminal enlargement, increased acetylcholine release, and increased acetylcholinesterase activity were attributed to increased activity level. As stated earlier, these factors contributed to increased safety factor and then influenced neurotransmission.

However, there are some studies equivocal to our speculation in regard to acetylcholinesterase in muscle groups chronically active by nature. Washio et al. (30) showed that age-related higher acetylcholinesterase activity at EDL was observed, but not in diaphragm and soleus muscle, which usually have higher activity. Consistent with this study, Sketelj et al. (27, 28) showed that the acetylcholinesterase activity of fast twitch muscle (EDL) was decreased when the stimuli pattern, like soleus muscle, was administered continuously, and conversely, inhibiting slow twitch fiber activity, the acetylcholinesterase activity was increased.

Although the mechanisms of activity-induced increased expression of acetylcholine receptor and acetylcholinesterase activity are still unclear, many studies indicate that the plasticity in motoneuron and muscle fiber were influenced by motoneuron or muscle derived trophic factors. For example, Keller-Peck et al. (18) has reported that excessive expression of glial cell line-derived neurotrophic factor (GDNF) induced multiple neural innervations to a skeletal muscle fiber. Alteration in endogenous neurotrophin-4 expression that occurred with changing activity level has been reported (10). It is possible to consider that the changes in the activity level triggers alteration in neuron or muscle derived trophic factors, and concomitant improvement in NMJ, leading to improvement in neuromuscular transmission. In fact, Mantilla et al. (20) demonstrated direct evidence that brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NT-4), could improve transmission function in adult rat diaphragm. Therefore, future study needs to focus on age-related expression of trophic factors, along with alteration in aged NMJ structure.

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