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Age-related changes in contraction and relaxation of rat diaphragm

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ABSTRACT

Age-related changes of physiological and biochemical properties were examined in the diaphragm muscle, which has particularly high activation compared to that of other skeletal muscles. The diaphragm from 10-week-, 50-week- and 100-week-old male Wistar rats were used to measure *in vitro* isometric contractile properties, sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activity, and myosin heavy chain (MHC) isoform composition. Although there were no significant differences in specific twitch tension of the diaphragm among the groups, there was significant reduction in specific tetanic tension in the 50-week to 100-week groups. The contraction time and 1/2 relaxation time of twitch contraction extended with aging, and significant differences were found between 10-week-old and 100-week-old diaphragms. Regarding the activity of SR Ca^{2+}-ATPase, the pattern of age-related change was similar to that in the 1/2 relaxation time and there was a significant difference between 10-week-old and 100-week-old diaphragms. There was a significant increase in the relative composition of the MHC I isoform in 100-week-diaphragms compared to that in 10-week-old diaphragms and a concomitant decrease in the relative composition of fast myosin was noted. These findings demonstrated that older diaphragms have slower contraction and relaxation speeds, and these alterations were attributed to changes in SR Ca^{2+}-ATPase activity and MHC isoform composition.

Common age-related changes in physical functions include decreases in balance, visual acuity and hearing. With regard to skeletal muscles, changes such as muscle mass loss (18), lower muscle tension (9, 15), selective atrophy of fast-twitch fibers (16), and reduced mitochondrial enzyme and glycolytic enzyme activities (6) have been reported. These age-related changes may cause falls, fractures and bed confinement, which are important social problems. Age-related changes in skeletal muscles have also been reported to affect not only the muscle fibers themselves, but also the oxygen supply to the cardiovascular system (22) and a decline in the number of spinal cord axons and nerve conduction velocity involved in contraction. Suppressing such age-related changes is considered important for successful aging.

In rehabilitation, the diaphragm plays a wide variety of roles, including respiratory rehabilitation before and after surgery, home oxygen therapy for chronic obstructive pulmonary disease and cardiorespiratory responses in sports. Many rehabilitation therapists select abdominal breathing (diaphragmatic respiration) as the first-choice respiratory muscle exercise in acute and chronic patients. The diaphragm is the main action muscle of diaphragmatic respiration, and is always active in life maintenance; thus, when compared with other skeletal muscles, the diaphragm is more active. Hence, investigating chrono-

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logical changes in the functional and morphological characteristics of the highly active diaphragm is important for ascertaining the plasticity of skeletal muscles in old age. In other words, if activity can be consciously maintained, then it may be possible to suppress age-related changes in muscles.

In the present study, we analyzed diaphragms from rats in three different age groups (10, 50 and 100 weeks) in terms of contractile force, contraction/relaxation time, SR Ca^{2+} ATPase activity, and myosin (contractile protein) species composition.

MATERIALS AND METHODS

Animals. Twenty-four male Wistar rats in the following age groups were used: 10 weeks (n = 8), 50 weeks (n = 8) and 100 weeks (n = 8). After reaching the target age, body weight was measured, and animals were administered 50 mg/kg pentobarbital sodium intraperitoneally to induce anesthesia. We then excised the diaphragm, and divided it into three sections along the muscle fiber alignment. The section with phrenic nerve attachment was used to analyze isotonic contraction, the ventral section was used to measure SR Ca^{2+} ATPase activity, and the dorsal section was used to determine myosin heavy chain (MHC) isoform compositions.

Temperature and humidity of the animal rearing room were maintained at 23°C and 50%, respectively, with a 12-hour light cycle. Throughout the rearing period, rats had free access to food and water. The present study conformed to the animal study guidelines compiled by the Department of Agricultural Sciences of Yamaguchi University, based on the "Standards for rearing and maintenance of laboratory animals" (March 1980, Notification No. 6 issued by the General Administrative Agency of the Cabinet).

In vitro isometric contraction measurement. Each diaphragm sample had tendons at both ends, and the costal-side tendon was fixed using a clip, while the central side was attached to a transducer. The specimen was fixed in Ringer's solution composed of (in mM): 115 NaCl, 5 $KHCO_3$, 1 $MgCl_2$, 20 $NaHCO_3$, 2 $CaCl_2$, 5*N*,*N*-bis(2-hydroxyethl)-2-amino-ethanesulfonic acid, 11 glucose, 0.3 glutamic acid, and 0.38 glutamine. Ringer's solution was continuously aerated with 95% O_2 and 5% CO_2, and its pH and temperature were maintained at 7.4 and 25°C, respectively. After connecting and fixing the diaphragm to the transducer (45196A; NIHON KODEN SANEI, Tokyo, Japan), the muscle was directly excited by electric stimulation using a plate electrode, and the optimal muscle length achieving the maximal switch tension was determined. Stimulation intensity was set to 125% of the intensity that induced maximal switch tension. Based on a twitch curve obtained using a digital oscilloscope, the maximum potential difference, twitch time, half relaxation time and tetanic tension were measured at stimulation frequencies of 50 or 100 Hz. After measuring tension, muscle cross-sectional areas were determined based on optimal muscle length and muscle mass according to the formula proposed by Mendez *et al.* (12), and the muscle force per unit area was determined at each stimulation frequency.

Sarcoplasmic reticulum Ca^{2+} ATPase activity. SR Ca^{2+} ATPase activity was measured using a partial modification of the method described by Simonides *et al.* (17). After trimming each muscle sample to 50–100 mg, the samples were homogenized in test tubes, followed by centrifugation at 3000 rpm for 10 min at 4°C. Then the supernatants were used for measurement. The measurement reaction was initiated by adding 8 mM of ATP at 37°C. At 30 and 90 s after the start of the reaction, a spectrophotometer was used to measure the nicotinamide adenine dinucleotide (NADH) concentration (V-530; Nihon Bunko, Tokyo, Japan) at a wavelength of 340 nm. Next, at a Ca^{2+} concentration of 2 mM, we measured NADH concentration, and the difference in the rate of decrease was defined as SR Ca^{2+} ATPase activity.

Myosin heavy chain (MHC) composition. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to isolate MHC isoforms in cryopreserved muscle samples. After trimming to 3–18 mg, each muscle sample was homogenized in a microtube to prepare a 40-fold extract. Using incubation medium, this extract was diluted 75-fold and electrophoresis was performed. Using the resulting myofibrillar protein extract, we electrophoresed 5 μL/lane at 4°C for 44–48 h with a constant voltage of 160 V. After electrophoresis, silver staining was performed and the composition ratio of each band (MHC I, IIa, IId and IIb) was calculated using the image analysis software Scion Image version 1.62 (Scion co., Maryland, USA). To improve reproducibility, SDS-PAGE was performed three times for each sample, and average values were calculated.

Statistical analysis. All numerical results are expressed as means ± standard deviation. One-way

ANOVA was used to analyze age-related changes in contractile properties and MHC composition ratios, and Scheffe method was used for multiple comparisons. In all analyses, $P < 0.05$ was accepted as significant.

RESULTS

Body weight changes

From 10 to 50 weeks of age, average body weight increased by about 2.5-fold from 264 ± 11 g to 604 ± 40 g and then continued to increase slightly for about 24 more weeks. Thereafter, body weight began decreasing. Average body weight for the 100-week-old group (597 ± 94 g) was comparable to that for the 50-week-old group, while average body weights for the 50- and 100-week-old groups were significantly higher than that for the 10-week-old group. As indicated by the standard deviations, individual differences were particularly large for the 100-week-old group (Fig. 1).

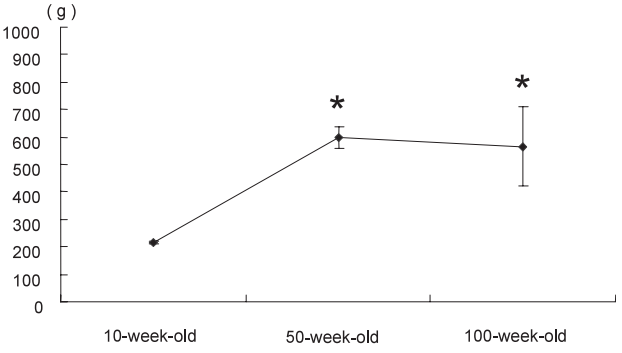


Fig. 1 Age-related changes in body weight. Average body weight for the 100-week-old group was comparable to that for the 50-week-old group, while average body weights for the 50- and 100-week-old groups were significantly higher than that for the 10-week-old group. All data are shown as mean ± SD. *Significantly different from 10-week-old ($P < 0.05$).

In vitro contraction

Table 1 shows the mean and standard deviation for diaphragm contraction properties in each age group. While there were no significant differences in twitch tension among the groups, twitch tension in the 100-week-old group was about 80% of that in the 50-week-old group. In addition, for stimulation at 50 or 100 Hz, tetanic tension per unit area was highest in the 50-week-old group, while the tetanic tension per unit area was significantly lower in the 100-week-old group than in the 50-week-old group.

Twitch time in the 10-week-old group was significantly shorter than those for the 50- and 100-week-old groups. There were no significant differences between the 50- and 100-week-old groups. The half relaxation time in the 100-week-old group was significantly longer than that in the 10-week-old group.

Sarcoplasmic reticulum Ca^{2+} ATPase activity

Figure 2 shows the SR Ca^{2+} ATPase activity in the 10, 50 and 100-week-old groups. The average SR Ca^{2+} ATPase activity in the 10-week-old group was 43.1 ± 7.8 (μmol/min/g), in the 50-week-old group was 37.8 ± 7.3 (μmol/min/g), and in the 100-week-old group was 29.1 ± 8.5 (μmol/min/g). While there were no significant differences between the 10- and 50-week-old groups, the SR Ca^{2+} ATPase activity in the 100-week-old group was significantly lower than that in the 10-week-old group. Age-related decreases in SR Ca^{2+} ATPase activity closely resembled those for half relaxation time.

Myosin heavy chain isoform composition

Figure 3 shows the MHC isoform composition of the diaphragm in each age group. For all age groups, MHC IId accounted for about 45–50% of the total, while MHC IIb accounted for less than 10%. For the 10- and 50-week-old groups, MHC IIa was the second most common component, accounting for about 25%, but for the 100-week-old group, there was more MHC I (about 28%) than MHC IIa.

Table 1 *Age-related changes in contractile properties of diaphragm*

	10-week-old	50-week-old	100-week-old
Twitch tension (N/cm^2)	6.5 ± 1.4	7.8 ± 1.6	6.2 ± 1.6
Twitch time (ms)	52.9 ± 5.6	75.0 ± 11.0*	70.0 ± 12.0*
Half relaxation time (ms)	56.6 ± 8.8	66.7 ± 13.3	73.5 ± 13.4*
50 Hz tetanic tension (N/cm^2)	15.6 ± 3.3	19.4 ± 2.6	15.2 ± 3.2†
100 Hz tetanic tension (N/cm^2)	15.5 ± 3.7	18.8 ± 2.3	14.3 ± 2.9†

Age-related changes in contractile properties of diaphragm. All data are shown as mean ± SD. *Significantly different from 10-week-old ($P < 0.05$). †Significantly different from 50-week-old ($P < 0.05$).

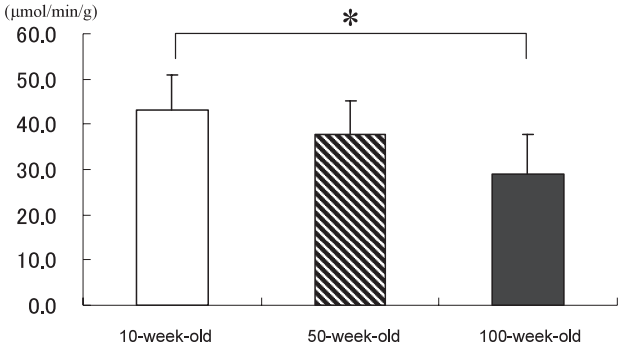
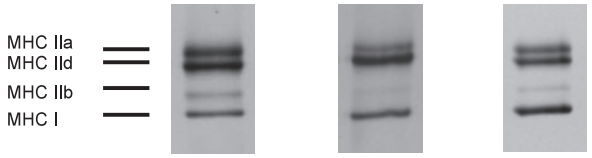


Fig. 2 Age-related changes in sarcoplasmic reticulum Ca^{2+} ATPase activity of the diaphragm. The SR Ca^{2+} ATPase activity in the 100-week-old group was significantly lower than that in the 10-week-old group. Age-related decreases in SR Ca^{2+} ATPase activity closely resembled those in half relaxation time. *Significantly different from 10-week-old ($P < 0.05$).



	10-week-old	50-week-old	100-week-old
MHC I	19.4 ± 2.0	20.9 ± 2.2	27.7 ± 5.3*
MHC IIa	23.4 ± 2.0	25.9 ± 3.6	21.6 ± 2.9
MHC IId	48.7 ± 4.8	49.5 ± 4.4	44.8 ± 6.6
MHC IIb	8.5 ± 7.0	3.7 ± 4.5	5.8 ± 5.3

Fig. 3 Age-related changes in MHC isoforms in rat diaphragm. The relative ratio of MHC I was significantly greater in the 100-week-old group. All data are shown as mean ± SD. *Significantly different from 10-week-old ($P < 0.05$).

Compared to that in the 10-week-old group, the relative ratio of MHC I was significantly greater in the 100-week-old group. There were no significant age-related changes in other isoforms.

DISCUSSION

The present study investigated age-related changes in the contractile properties of rat diaphragm and in the structural function as determined by biochemical tests. The results showed decreased tetanic tension and elongated relaxation time for the 100-week-old group, and at the same time, changes such as relative increases in MHC I and decreases in SR Ca^{2+} ATPase activity were observed.

Age-related changes in contractile force per unit cross-sectional area

In the present study, rat body weight in the 50-week-old group was higher than those in the 10-week-old group and the 100-week-old group. Previous age-related changing studies have shown that rat body weight peaks at age 60–72 weeks, and decreases thereafter (8). Studies have also found that the ratio of MHC I increases by about 20% in the rat soleus muscle at age 80–96 weeks (8), and that at the ages of 96–104 weeks, marked age-related changes occur in the remodeling of motor units, which appeared to involve denervation of fast muscle fibers followed by reinnervation of denervated fibers by axonal sprouting from slow fibers in the hindlimb muscles (7). The findings of this study suggest that structural changes occur at the muscle level around 100 weeks of age.

Brooks *et al.* (1) reported decreases in age-related tension per unit area in the murine extensor digitorum longus muscle and suggested that structural changes in contractile proteins limit cross-bridge movements. In the same study, they found that there was no decrease in the tension per unit area for the soleus muscle, suggesting that there are no marked age-related changes in either slow-twitch fibers or highly active muscles. Thompson *et al.* (21) measured the tension per unit area of individual muscle fibers using the rat soleus muscle and reported that the average tension at the age of 100 weeks was about 20% lower than that at the age of 50 weeks. In addition, Gosselin *et al.* (4) evaluated tension per unit area using similar methods as the present study, and reported that the tetanic tension at age 100 weeks was 15–18% lower than that at age 24 weeks. They concluded that this was due to something other than changes in MHC composition. In the present study, the tetanic and twitch tension per unit area of rat diaphragm was the lowest in the 100-week-old group—about 75–80% of that in the 50-week-old group. Although the results of this study supported Gosselin's report, there were contradictory findings for MHC isoform composition. In this study, we conclude that a change in the contraction protein as theorized by Brookes, *i.e.* MHC composition, affects muscle contraction. The present findings support the thesis that structural changes occur at the muscle level around age 100 weeks.

In recent years, Lowe *et al.* (10) also performed electron paramagnetic resonance spectroscopy to evaluate myosin heads in the process of contraction and demonstrated that in the semimembranosus muscle fibers of aged rats with low tension per unit area (−27%), the ratio of strong-binding myosin

heads was low. Furthermore, Lowe *et al.* (11) analyzed the effects of denervation on activity and showed that changes in the ratio of strong-binding myosin heads (16–35%) corresponded to those in tetanic tension per unit area (23–34%). Their findings indicate that changes in myosin microstructures are the direct cause of low tension due to aging and denervation, and that the myosin microstructure changes and adapts in relation to the amount of activity. The results of this study suggest that the same adaptation process involving changes in myosin microstructure occurred in the diaphragm.

Age-related changes in contraction and relaxation velocities

In the present study, when compared to the 10-week-old group, the MHC I ratio for the 100-week-old group was significantly greater—the increase was 8.3%. However, Li and Larsson (8) found that the ratios of MHC I and IIa in the soleus muscle and extensor digitorum longus muscle increased by more than 10% at age 80–96 weeks. In addition, Gosselin *et al.* (4) reported that the ratios of MHC I and IIa in the diaphragms of 96-week-old rats were comparable to those for young rats. The soleus muscle is a typical slow-twitch muscle and the extensor digitorum longus muscle is a typical fast-twitch muscle; however, the diaphragm includes both muscle types. Therefore, the differences between the present study and previous studies may be due to differences in muscle contraction activity.

According to previous studies (5, 14), the duty times (activity time/total time) for the soleus muscle and extensor digitorum longus muscle are about 13.9 and 1.9%, respectively, while that for the diaphragm is markedly higher at 40%. The results of the present study suggest that age-related changes in the contractile proteins of skeletal muscles can be partially suppressed by maintaining high activity levels. However, in terms of functional changes, *i.e.*, contractile properties, about 30% increases in contraction and relaxation times were seen, and these changes are comparable to the results of studies investigating the soleus muscle and extensor digitorum longus muscle at comparable ages.

In the present study, SR Ca^{2+} ATPase activity in the 100-week-old group was about 30% lower when compared to that in the 10- or 50-week-old groups, and this decrease was comparable to the decrease in relaxation time. Previous studies of SR Ca^{2+} ATPase activity in rat skeletal muscles have reported that the SR Ca^{2+} ATPase activity for fast-twitch fibers was at least six times higher than that for slow-twitch fibers (3). This suggests that as the ratio of fast myosin decreases with age, SR Ca^{2+} ATPase activity decreases. However, decrease in the SR Ca^{2+} ATPase activity (30%) was markedly greater than that in the ratio of fast myosin (8%), suggesting that age-related changes in SR Ca^{2+} ATPase activity are not dependent on the expression of muscle contractile proteins.

The results of previous studies on denervation have suggested that nutritional factors from motor neurons are indirectly involved in maintaining the morphological and functional properties of myoplasmic reticula (13, 20). Decreases in SR Ca^{2+} ATPase activity are directly linked to increases in intracellular calcium ion concentration (19). This facilitates the production of superoxide, a reactive oxygen species, and causes lipid hyperoxidation and protein denaturation. Therefore, decreases in SR Ca^{2+} ATPase activity, along with selective atrophy and disappearance of fast-twitch fibers, are important age-related changes that markedly affect overall skeletal muscle function.

In the future, it will be necessary to investigate factors suppressing age-related changes in SR Ca^{2+} ATPase activity, including the effects of nerve-derived nutritional factors.

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