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Effects of Unilateral Phrenic Nerve Denervation on Diaphragm Contractility in Rat

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SHINDOH, C., HIDA, W., KUROSAWA, H., EBIHARA, S., KIKUCHI, Y., TAKISHIMA, T. and SHIRATO, K. *Effects of Unilateral Phrenic Nerve Denervation on Diaphragm Contractility in Rat.* Tohoku J. Exp. Med., 1994, **173** (3), 291-302 —— We examined the early effects of phrenic nerve denervation on the diaphragm muscle 1, 3, 7 and 14 days after unilateral denervation in rats. In the denervated hemidiaphragms, force frequency curves at 3, 7 and 14 days decreased significantly by 51%, 50% and 38% respectively of the peak tension of the force frequency curves of the diaphragms of rats with sham operation. Twitch tensions increased significantly at 14 days, and contraction times and half relaxation times slowed significantly at 3, 7 and 14 days. The tensions of denervated diaphragms at 5 min during the fatigue runs was significantly increased at 14 days. As determined by histological staining, the mean cross sectional area of fast-twitch fibers (type II) decreased significantly from 2,742 (sham) to 1,599 μm^2 (14 days), but that of the slow-twitch fibers (type I) did not change significantly during the same period. These findings suggest that, during the first two weeks of denervation, fast twitch fibers (type II) atrophy more rapidly than slow twitch fatigue resistant fibers (type I), as confirmed by the contractile properties and histological findings. ——
twitch kinetics; muscle atrophy; muscle plasticity

Hemidiaphragmatic paralysis due to acute phrenic nerve injury is well known as a cause of respiratory muscle failure. Concerning the denervation of skeletal muscles, previous studies have shown that denervation of the skeletal muscles induced muscle fiber atrophy, with an increase in cell membrane resistance (Albuquerque et al. 1971), an enhanced extrajunctional acetylcholine (ACh) sensitivity (Axelsson and Thesleff 1959) and a depolarization of the resting membrane potential in the myocyte (Redfern and Thesleff 1971). Furthermore, recent studies have shown that denervation induces changes in the messenger RNA

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(mRNA) expression of a sodium channel (Kallen et al. 1990) and ATP-dependent proteolysis (Medina et al. 1991) at an early period such as 1 day after denervation. Actually, compared to those concerning other skeletal muscles, reports about diaphragm muscle fiber atrophy (Tower 1939; Carraro et al. 1979) are relatively few. Therefore, the change in physiological characteristics of denervated diaphragm and the relationships between muscle contractile properties and fiber changes have not been well characterized.

The purpose of the present study was to examine the effect of unilateral phrenic nerve denervation on the force-frequency curves, twitch kinetics, fatigability in vitro and muscle fiber type compositions as determined by myofibrillar adenosine triphosphatase (ATPase) staining in rat diaphragms at 1, 3, 7 and 14 days after denervation.

Materials and Methods

Animal preparation

Experiments were performed on 80 muscle strips from 40 Wistar rats weighing 250-320 g (Charles River Japan, Kanagawa). The cervical skin was cut with a midline incision while the animals were in the supine position under Ketamine (50 mg/kg) anesthesia. The unilateral phrenic nerve denervation, the side of which was selected at random, was carried out by cutting at each C4-C6 nerve root and removing the insertion (5-8 mm length) of the main phrenic nerve into the thoracic cavity. After recovery from surgery, rats were caged individually, isolated for the duration of the experiment, and maintained on a 12 : 12-hr light-dark cycle at normal ambient temperature. Denervated animals were studied at 1 ($n=8$), 3 ($n=8$), 7 ($n=8$) and 14 days ($n=8$) after denervation. The fifth group was the sham group ($n=8$), operated on in the same way as the denervated group except for unilateral denervation, and studied at 1 day after surgery.

Experimental protocol

The measurements of diaphragm muscle contractility were performed according to the method described by Shindoh et al. (1992). Briefly, two muscle strips (3-4 mm wide) were dissected from the right and left hemidiaphragm, and mounted in separate organ baths containing Krebs-Henseleit solution oxygenated with a 95% O_2-5% CO_2 gas mixture (23.5±0.5°C, pH 7.40±0.05). The composition of the aerated Krebs-Henseleit solution in mEq/liter was as follows: Na^+, 153.8; K^+, 5.0; Ca^{2+}, 5.0; Mg^{2+}, 2.0; Cl^-, 145.0; HCO_3^-, 15.0; HPO_4^{2-}, 1.9; SO_4^{2-}, 2.0; glucose, 110 mg%; 10 μM *d*-tubocurarine; regular crystalline zinc insulin, 50 U/liter. Both muscle strips were simultaneously stimulated with supramaximal currents (i.e., 1.2 to 1.5 times the current required to elicit maximal twitch tension, 200-250 mA, 0.2 ms duration in pulses) by a constant current stimulus isolation unit (SS-302J; Nihon Kohden, Tokyo) driven by a stimulator (SEN-3201; Nihon Kohden, Tokyo). The elicited tensions were measured by a force transducer

(UL-100GR; Minebea Co., Fujisawa). Each muscle strip length was altered by moving the position of the force transducer with a micrometer-controlled rack and pinion gear (accuracy of displacement, 0.05 mm), and measured with a micrometer in close proximity to the muscle. The optimal length of the muscle (Lo) was defined as the muscle length at which twitch tension development was maximal, and this predetermined Lo was maintained in the following measurements.

The diaphragm force-frequency relationship was assessed by sequentially stimulating muscles at 1, 10, 20, 30, 50, 70 and 100 Hz. Each stimulus train was applied for approximately 1-sec, and adjacent trains were applied at approximately 10-sec intervals. The tensions of both muscle strips were recorded by a hot-pen recorder (RECTI-HORIZ-8K; San-ei, Tokyo).

Twitch kinetics were assessed by measuring the time required to develop peak tension (contraction time) and the time required for peak tension to fall by 50% (half relaxation time) during single muscle twitches.

Muscle fatigability was then assessed by examining the rate of fall of tension over 5 min of rhythmic contraction. Rhythmic contraction was induced by applying trains of 20-Hz stimuli (train duration, 0.22 sec; rest duration, 0.44 sec; train: rest ratios, 1 : 2) at a 90 train/min rate.

After completion of this protocol, muscle strips were removed from the bath and weighed. After muscle strips were adjusted to Lo and fixed with pins on a cork plate, samples were immediately embedded in mounting medium (OCT compound, Miles Inc., Elkhart, IN, USA), immersed in isopentane (Wako Pure Chemical Industries Ltd., Osaka) that had been cooled in liquid nitrogen, and stored in a freezer ($-80°C$) for further analysis.

Adenosine triphosphatase (ATPase) stain

Myofibrillar adenosine triphosphatase (ATPase) staining of the diaphragm was performed according to the method of Dubowitz and Brooke (1973). Diaphragm sample tissues were sectioned at 10 μm with a cryostat (BRIGHT Instrument, Huntingdon, UK) kept at $-20°C$. On the basis of their staining reactions for myofibrillar ATPase, after alkaline preincubation (pH 10.4), muscle fibers were classified as either type I or type II (Prezant et al. 1993). Light stained fibers were classified as type I (high oxidative slow-twitch), and dark stained fibers were classified as type II (both high oxidative fast-twitch and low oxidative fast-twitch) (Lieberman et al. 1973). Fiber cross sectional areas were measured by digitalizing with a computerized Personal Image Analyze System (LA-500; PIAS Co., Tokyo). The sample pictures were input using a microscope (OPTIPHOT; Nikon, Tokyo) and a TV camera, and presented on a color display TV (CPV-14CD2; Sony, Tokyo). One fiber cross-sectional area (CSA) ($\times 20$) was determined from the number of pixels within the outlined borders, which were manually traced on the display, with each pixel having an area of 0.676 μm^2 at $\times$ 20 magnification. Fiber type proportions and CSAs were determined from a

sample of 350-400 fibers using nondehydrated sections of each muscle.

Data analysis

The strip cross-sectional area was calculated by dividing muscle mass by the product of strip muscle length and muscle density (1.06 g/cm^3) (Close 1972), and tension was calculated as force per unit area (kg/cm^2). Data among groups were compared by 2-way ANOVA with Newman-Keuls' multiple range test, and the mean values were compared using Duncan's multiple test. Data are presented as means$\pm$S.E. Comparisons with a p value of less than 0.05 were considered statistically significant.

Results

The mean body weights of the sham and denervated groups at each experimental day are summarized in Table 1. In our animal facility, 6 week old Wistar rats ($n=10$) have a body weight of 272.6$\pm$25.9, and thereafter increase to 277$\pm$34.8 (+1 day), 288.4$\pm$31.6 (+3 days), 305.4$\pm$36.3 (+7 days), and 330.0$\pm$32.2 (+14 days) g. The mean weights of sham and denervated groups were not significantly different from those of the normally breeding animals. The muscle strip weight and optimal length at which twitch tension development was maximal were also not significantly different in the intact and denervated sides of the experimental groups.

Denervation effects on muscle contractile properties

Fig. 1A shows the mean force-frequency curves for strips from diaphragms of

Table 1. *Changes of mean body weights, muscle strip weights and optimal lengths of intact and denervated sides in the sham and denervated groups*

		Days after denervation			
	Sham ($n=8$)	1 ($n=8$)	3 ($n=8$)	7 ($n=8$)	14 ($n=8$)
Body weight (g)	281.7$\pm$36.1	276.4$\pm$43.4	281.0$\pm$49.8	294.5$\pm$29.8	322.3$\pm$21.1
Muscle strip weigth (mg)					
Intact side	66.6$\pm$16.3	64.5$\pm$18.1	66.3$\pm$11.5	70.0$\pm$15.9	75.5$\pm$12.4
Denervated side	/	65.6$\pm$18.6	70.3$\pm$11.9	71.2$\pm$15.0	74.8$\pm$12.2
Strip Lo (mm)					
Intact side	20.3$\pm$2.0	19.3$\pm$2.3	19.5$\pm$2.5	19.6$\pm$2.1	20.1$\pm$2.6
Denervated side	/	21.0$\pm$2.0	21.9$\pm$2.0	21.4$\pm$1.8	22.1$\pm$2.5

Values are means$\pm$S.E. Body weigths were measured at experimental days. Lo, optimal length defined as the length at which twitch tension development was maximal.

the sham group and for those from the denervated side of the diaphragm. The mean force-frequency curves for denervated diaphragms at all test periods were significantly lower than those of sham operated diaphragms in the range of 30-100 Hz. On the other hand, the tensions of 1 and 10 Hz of 14 day denervated muscles showed significant increments from those of the sham group. The force-frequency curves of 3 and 7 day denervated diaphragms were not significantly different from each other. The peak tensions of force-frequency curves shown in Fig. 1A were 1.93 ± 0.10 (sham), 1.62 ± 0.24 (1 day), 0.95 ± 0.15 (3 days), 0.97 ± 0.06 (7 days), and 1.19 ± 0.11 kg/cm^2 (14 days), respectively. Fig. 1A shows also that the frequencies of peak tensions of force-frequency curves were shifted leftward from 70 Hz (sham, 1 day), to 50 Hz (3, 7 days) and 20 Hz (14 days). However, the mean force-frequency curves for strips from the intact side of diaphragms of the denervated group did not differ from those of strips from diaphragms of the sham operated group (Fig. 1B).

Fig. 2A summarizes the mean changes of twitch tension, contraction time, and half relaxation time of twitch kinetics in the sham and denervated groups. The twitch tensions of 14 day denervated strips were significantly greater than those of muscle strips from sham animals. Furthermore, the contraction time and half relaxation time of 3, 7 and 14 day denervated strips were significantly longer than those of muscle strips from sham animals. These three parameters for the strips from the intact side of diaphragm did not differ from those of strips from sham operated animals (Fig. 2B).

Fig. 3 shows the tensions at 5 min of repetitive stimulation at 20 Hz for all groups. The data are expressed as a fraction of the tension generated during the

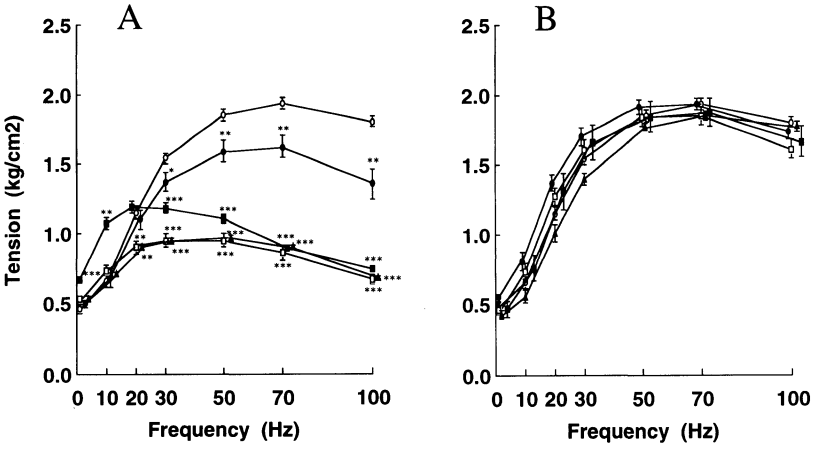

Fig. 1. Force-frequency curves of denervated (A) and intact (B) sides of the diaphragm of sham (○—○), and 1 (●—●), 3 (□—□), 7 (▲—▲) and 14 days (■—■) after denervation ($n=8$ each). Asterisks indicate significant differences at given frequencies compared to sham operated diaphragm ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

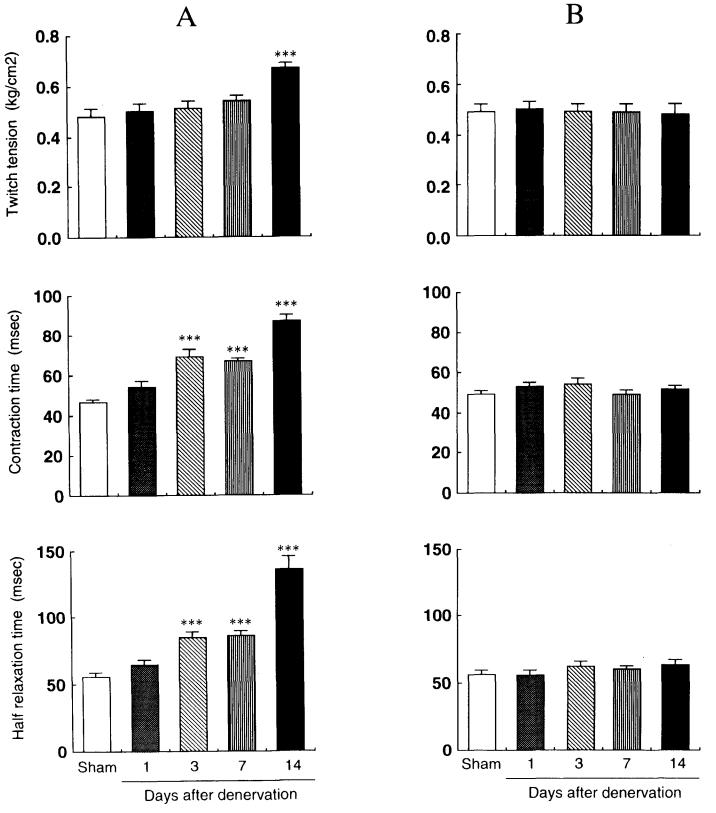

Fig. 2. Mean changes of twitch tension, contraction time and half relaxation time from the diaphragm of sham, and denervated (A) and intact (B) sides of 1, 3, 7 and 14 days after denervation ($n=8$ each). ***($p<0.001$) indicates significant increase compared to sham.

first contraction of the fatigue trial. The tensions of denervated diaphragms at 5 min during fatigue trial increased significantly at 14 days after denervation compared with those of sham animals (Fig. 3A). However, the tensions of nerve intact hemidiaphragm did not differ (Fig. 3B).

Denervation effect on muscle fiber areas and numbers

Fig. 4 shows typical photographs for myofibrillar ATPase staining at an alkaline pH for diaphragms from sham operated (A) and from rats at 14-days after denervation (B). The dark and light stained fibers are fast-twitch (type II) and slow-twitch (type I) muscle fibers, respectively. Table 2 summarizes the changes in mean cross-sectional areas and the percentages in type I and II muscle fibers for all groups. The mean cross sectional areas of type I fibers were not significantly different from control. Those of type II fibers of 3, 7 and 14 day denervated muscle strips decreased significantly compared to those of control. The percent-

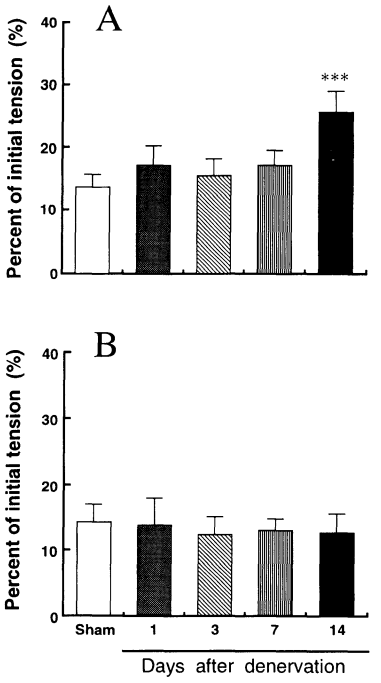

Fig. 3. Mean tensions at 5 min expressed as percentage of the first tension developed in the fatigue trial. The diaphragm muscle of 14 days (A) after denervation showed significantly higher tension than those of other groups ($***p<0.001$). There were no significant changes among the 5 groups from intact side (B).

age of type I fibers of 14-day denervated strips increased and the percentage of type II fibers of 14-day denervated strips decreased compared to that of sham operated diaphragm. The muscle composition of strips from the nerve intact side of the diaphragm in the denervated groups did not differ from that in the sham operated group.

Discussion

Our experiments have shown that the maximal tension of the denervated diaphragm muscle was reduced, and the frequency of maximal tension shifted leftward in the force-frequency relationship, and twitch contraction became slower than that of sham operated diaphragm. The fiber compositions defined by ATPase stain showed a significant decrease in type II fiber cross sectional area, and the total occupied area of type II fibers in the unit area was decreased. Correspondingly, the total area occupied by type I fibers was increased. Muscle contractility and fatigability decreased with changes of the increment of compositions of type I, suggesting that fast fiber (type II) atrophy in diaphragm muscle may be an early step in the degenerative processes of phrenic nerve denervation.

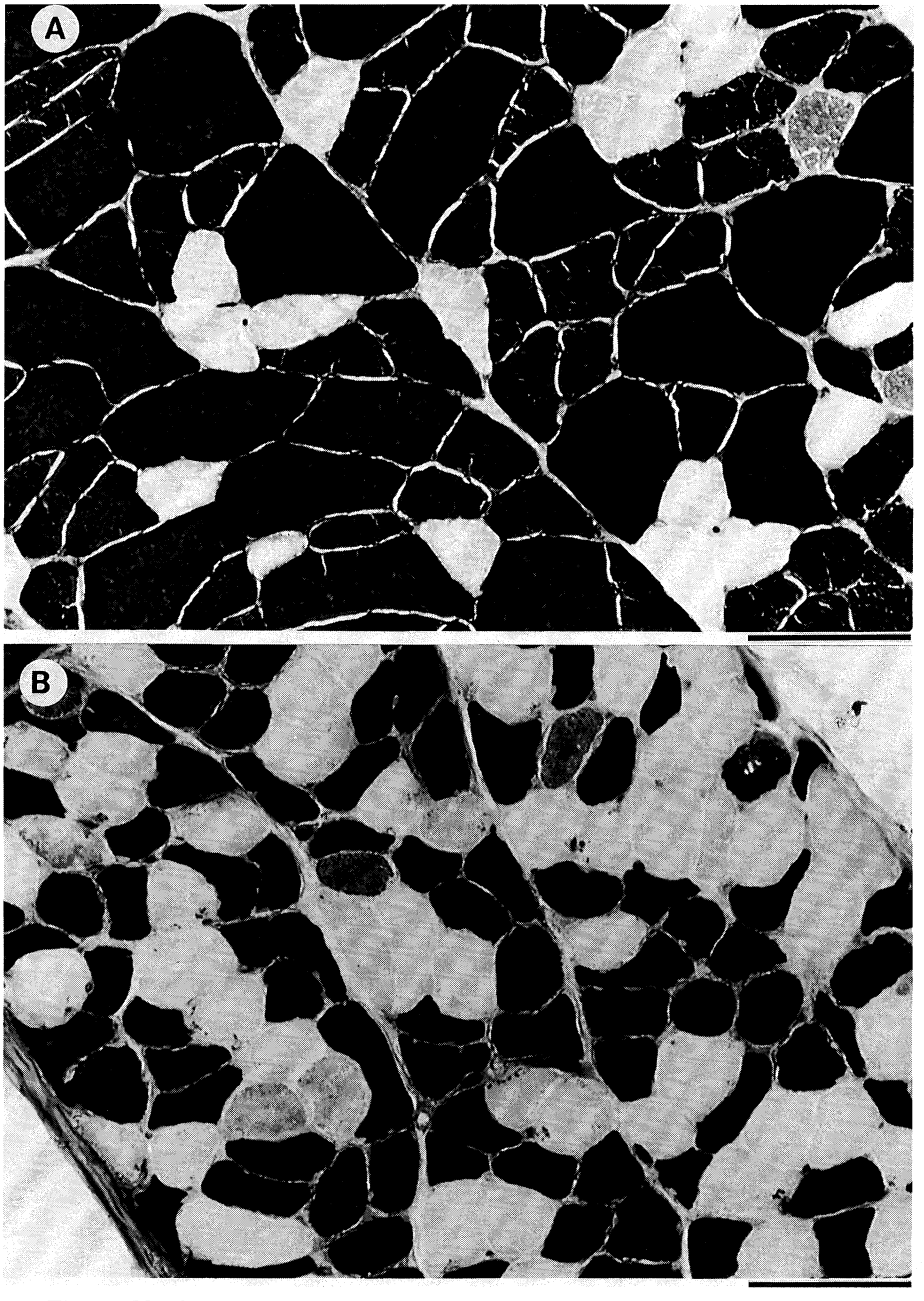

Fig. 4. Myofibrillar ATPase staining of the sham operated (A) and the 14 days denervated (B) diaphragms. Unstained fibers indicate type I and dark stained fibers indicate type II. The horizontal bars indicate 100 μm.

TABLE 2. *Changes of mean cross sectional areas, and percentages of numbers of type I and II muscle fibers in the sham and denervated diaphragm*

	Mean cross sectional area (μm^2)		Percentage of numbers (%)	
	type I	type II	type I	type II
Sham	1700±88	2742±109	29.9±1.2	70.1±1.2
1 Day				
Intact side	1734±172	2684±160	28.0±4.6	72.0±4.6
Denervated side	1767±198	2630±155	31.4±4.7	68.6±4.7
3 Days				
Intact side	1771±214	2734±81	27.4±1.9	73.6±1.9
Denervated side	1839±116	2420±169*	28.2±4.3	71.8±4.3
7 Days				
Intact side	1641±137	2797±182	29.5±2.8	70.5±2.8
Denervated side	1899±105	1903±98**	30.5±1.9	69.5±1.9
14 Days				
Intact side	1746±144	2627±199	26.8±3.0	73.2±3.0
Denervated side	1830±76	1599±164***	35.5±1.8*	64.5±1.8*

Values are means±S.E. $*p<0.05$, $***p<0.001$ compared to sham.

The contractile properties and fiber compositions in the nerve intact hemidiaphragm did not change.

The force-frequency curves in the denervated diaphragm showed a progressive decrease of tension in the high frequencies (50-100 Hz) at 3, 7, and 14 days. At the high frequencies, the force-frequency curves showed rapid decreases by 3 days. There are a number of mechanisms by which denervation may affect the contraction characteristics of diaphragm muscle. As one example, denervation elicits changes in the cell membrane potential of the myocytes. Akaike (1981) reported that the intracellular sodium ion level increases and the potassium ion level decreases in the denervated skeletal muscle. These changes of intracellular sodium and potassium ions may contribute to changes in the resting membrane potential. According to Albuquerque et al. (1971) and Redfern and Thesleff (1971), the resting membrane potential of myocytes becomes less negative at two or three days after denervation, and persists through 7 days of denervation. Such changes in the resting membrane potential may relate to a reduction in the rate of rise of the action potential in the denervated muscle cells, and this reduction in spike generation would possibly contribute to the decreases in the force-frequency curves. In general, the tension decrement at high frequency (i.e., high frequency fatigue) is caused by the failure of excitation of individual muscle fibers resulting from impaired neuromuscular transmission or a failure to generate muscle action

potentials (Edwards 1979). The observed downward shifts of force-frequency curves at 1 and 3 days seem to correspond to high frequency fatigue.

However, some modulation in the denervation effects on the force-frequency curves, that is muscle fiber composition, should be considered. The soleus muscle (slow muscle) can be characterized by more leftward force-frequency curves and less fatigability compared to the extensor digitorum longus muscle (fast muscle) (Burke et al. 1973). We observed that, at 14 days after denervation, the force-frequency curves were more leftward and showed decreased fatigability compared to sham operated diaphragm. This may suggest that the denervated diaphragm muscle became slower than the sham diaphragm muscle.

Although the twitch tensions did not change significantly from 3 days to 7 days after denervation, both contraction times and half relaxation times significantly elongated from 3 days after denervation. Twitch kinetics are thought to be a function of the rate of release and reuptake of calcium from the sarcoplasmic reticulum (Edwards 1979). A previous study (Burke et al. 1973) showed that the rate of muscle contraction was elongated at 7 days after denervation. The present study following 14 days after denervation showed further elongation of both contraction time and half relaxation times, respectively. On the basis of these observations, it is possible that denervation may have affected not only sodium-potassium channels but also the sarcoplasmic reticulum in the early period of denervation. In addition, the significant increase of twitch tension of the 14 day denervated diaphragm may be accounted for by the effects of increment of type I muscle fibers, which have higher tensions at low frequencies than those of type II muscle fibers in the force-frequency curves.

The 14 days denervated diaphragm muscle showed increased tension at 5 min during fatigue trials. This means that the denervated diaphragm muscle became more fatigue resistant compared to the sham group. Generally, the slow muscle (type I) is more fatigue resistant than the fast muscle (type II) (Burke et al. 1973). In the present study, the fatigue resistance at 14 days after denervation may be explained by the changes of muscle fiber compositions such as increased percentages of type I in the denervated diaphragm muscles.

The fiber compositions and the fiber areas of type I and II muscle fibers in this study were almost consistent with the previous studies (Close 1972; Lewis et al 1986). The present data of the decrement of both fiber area and percentage in type II, in other words, the relative increment of type I muscle fiber, seem to be very compatible with muscle contractile profiles after denervation. However, we have to emphasize that the time course of muscle contractile profiles did not perfectly correspond to the muscle fiber composition profiles after denervation. We speculate that the contractile profiles involving contractile proteins and calcium channels may change rapidly (i.e., in hours), but the reconstruction of muscle fibers may need more time, several days, perhaps.

Unilateral phrenic nerve denervation showed considerable changes in the

muscle contractility and the muscle fiber compositions in the denervated side compared to those in the nerve intact side. These changes in denervated diaphragm in rats may be similar to pathophysiological changes in diaphragmatic paralysis induced by acute phrenic nerve injury such as after thoracic surgery, cervical injury or invasive lung cancer. Furthermore, the nerve intact side maintained similar muscle contractility and composition of muscle fibers to sham operated diaphragm muscle during 14 days. From these findings, it seems that the nerve supplying diaphragm muscle is an important determinant factor not only of muscle contractile properties but also of muscle fiber compositions.

In terms of the clinical implications of the present findings, the observed diaphragm muscle changes might cause an overall decrease of inspiratory power, and may result in decreases in the lung volume and also in uneven distribution of inspired gas. Those ventilatory changes may relate to respiratory failure (i.e., hypoxemia and/or hypercapnia) in unilateral phrenic nerve denervated subjects.

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