Diaphragm muscle fiber function and structure in humans with hemidiaphragm paralysis


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The diaphragm is the main muscle of inspiration and contracts during each breathing cycle. Thus, throughout life, the diaphragm is constantly contractile active. Several studies have suggested that the diaphragm is remarkably sensitive to changes in contractile activity. For instance, the elevated contractile activity of the diaphragm that accompanies pulmonary hypertension or chronic heart failure has been associated with significant diaphragm atrophy and weakness (4, 35). Recently, the response of the diaphragm to the contractile inactivity that is associated with mechanical ventilation in the intensive care unit has gained much attention, since it has been suggested to play an important role in the development of weaning failure. A landmark study by Levine and colleagues (18) revealed >50% atrophy of both slow and fast diaphragm fibers in brain-dead patients being mechanically ventilated for only 1–3 days. The nonrespiratory pectoralis muscle, which was unloaded as well, showed no signs of atrophy. This rapid and selective diaphragm muscle fiber atrophy was likely caused by activation of proteolytic pathways, such as the ubiquitin-proteasome (11, 12, 15, 18) and autophagy-lysosome pathways (11).

Another condition that is associated with contractile inactivity of the diaphragm is hemidiaphragm paralysis. The diaphragm muscle is separated by the union of its central tendon and the pericardium into a left and a right hemidiaphragm, each with its own phrenic nerve supply. Typically, hemidiaphragm paralysis is an acquired condition that can result from a number of abnormalities that affect the neuromuscular axis between the cervical spinal cord and the diaphragm. Although the most common causes are idiopathic, paralysis can occur through tumor encroachment on one of the two phrenic nerves and through phrenic nerve trauma from surgery.

Studying hemidiaphragm paralysis allows to investigate the effect of contractile inactivity on diaphragm muscle fiber function and structure, independent of confounding effects related to other major illness or systemic effects. Previous extensive work by Sieck and colleagues (19, 39) revealed that 2 wk of hemidiaphragm paralysis in rats, thus completely abrogating diaphragm contractile activity as well as myoneural interactions, caused atrophy, activation of proteolytic pathways, and weakness of the paralyzed diaphragm (1, 6, 19). So far, it is unknown how hemidiaphragm paralysis affects function and structure of the paralyzed diaphragm in humans. Here we studied muscle fiber function and structure in the paralyzed diaphragm of humans with hemidiaphragm paralysis. Our findings reveal that diaphragm muscle fiber atrophy and weakness following hemidiaphragm paralysis develops slowly and takes months to occur. This might indicate that the human diaphragm is resistant to contractile inactivity.

METHODS

Patients. Biopsies were obtained from the midcostal region of the diaphragm from eight patients with hemidiaphragm paralysis (HDP) during thoracotomy for diaphragm plication. All patients had unilateral HDP of known duration, caused by en bloc resection of the phrenic nerve (4–5 cm) with a tumor (thymoma or non-small cell lung carcinoma; see Table 1). Elevation of the paralyzed hemidiaphragm was determined to evaluate activation of the ubiquitin-proteasome pathway. The force-generating capacity, as well as muscle fibers was determined, as well as fiber ultrastructure and morphology. Finally, expression of E3 ligases and proteasome activity was not significantly upregulated in diaphragm fibers following paralysis; not even after 72 and 88 wk of paralysis, at which time marked atrophy of slow and fast diaphragm fibers had occurred. Diaphragm muscle fiber atrophy and weakness following hemidiaphragm paralysis develops slowly and takes months to occur.

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was performed in a total reaction volume of 25 μl with 50 ng of total RNA in a 20-μl reaction volume. The primers for MAFbx and MuRF-1 expression were as reported previously (28). Total RNA was extracted from diaphragm samples by using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. The extracted RNA was homogenized in ice-cold buffer (pH 7.5) containing (in mM) 50 Tris·HCl, 5 MgCl₂, 250 sucrose, 1 dithiothreitol, 0.2 PMSF, and protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, the Netherlands) by means of a Dounce homogenizer. Subsequently, the proteasomes were isolated from the homogenates by three sequential centrifugation steps; the first centrifugation was at 10,000 g for 20 min. The supernatant was centrifuged at 100,000 g for 1 h. The obtained supernatant was then centrifuged at 100,000 g for 5 h. The final pellet, containing the 20S proteasomes, was resuspended in buffer (pH 7.5) containing 50 mM Tris·HCl, 5 mM MgCl₂, and 20% glycerol. The protein content of the proteasome preparation was determined on the basis of Bio-Rad Protein Assay (Bio-Rad, Veenendaal, the Netherlands). The proteolytic activity of the 20S proteasomes was determined by measuring the activity against the fluorogenic substrate succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (LLVY, Sigma-Aldrich, Zwijndrecht, the Netherlands). This substrate is preferentially hydrolyzed by the chymotrypsin-like peptidase activities of the 20S proteasome. To measure proteolytic activity, 15 μg of proteasome extract was added to 60 μl of medium containing 62.5 mM Tris·HCl, 12.5 mM MgCl₂, 1.25 mM 1,4-dithiothreitol, 0.01 U apyrase, and 100 μM of LLVY. The reaction took place at 37°C for 45 min. The peptidase activity was determined by measuring the generation of the fluorogenic cleavage product (methylcoumarylamide) at 380-nm excitation wavelength and 440-nm emission wavelength continuously with a spectrophotometer. Standard curves were established for the fluorogenic product, and peptidase activity was expressed as picomoles per microgram protein per minute. Addition of the proteasome inhibitor MG132 to the reaction resulted in complete inhibition of proteasomal activity. The patients with hemidiaphragm paralysis were subdivided into two groups: one with paralysis of short duration (up to 8 wk) and one with paralysis of long duration (longer than 8 wk). Differences between HDP groups and controls were analyzed by one-way ANOVA. A value of \( P < 0.05 \) was considered significant. Data are presented as means ± SE.

RESULTS

The patients with HDP were divided into two subgroups: patients with HDP of short duration (2, 3, 6, and 8 wk, henceforth referred to as Short-HDP) and patients with HDP of longer duration (40, 52, 72, and 88 wk, henceforth referred to as Long-HDP). Note that for clarity Figs. 2–5 show data from 4 weeks of paralysis.
individual HDP patients, but that for statistical analyses the averaged data from the Short-HDP and Long-HDP subgroups were used.

**Diaphragm muscle fiber function.** We determined the contractile performance of 25 diaphragm fibers from three control subjects and of 36 diaphragm fibers from the four Short-HDP patients (per subject 7–12 fibers were analyzed). Because of fragility and atrophy of muscle fibers we were unable to dissect muscle fiber segments from the diaphragm biopsies from Long-HDP patients. Maximum force was normalized to fiber cross-sectional area. Since we found no differences in force generation between muscle fibers expressing slow vs. fast myosin heavy chain isoforms (in line with previous work; Ref. 30), data from both fiber types were pooled. As shown in Fig. 2, the average maximum force generated by diaphragm fibers from Short-HDP patients was comparable to that generated by the control subjects. Thus the force-generating capacity of diaphragm muscle fibers from HDP patients is preserved up to at least 8 wk of paralysis.

**Diaphragm myofibrillar ultrastructure.** To study whether, in line with the preserved myofibrillar contractile function, myofibrillar ultrastructure is preserved during the first months of diaphragm paralysis, we performed electronmicroscopy on diaphragm myofibrils. These experiments revealed well-aligned z disks and regular myofibrillar structure in control subjects (Fig. 3). In diaphragm fibers from Short-HDP patients myofibrillar structure was preserved, with small accumulations of glycogen between myofibrils at 8 wk (see Fig. 3A). In the Long-HDP patients myofibrillar structure progressively deteriorated, with dissolution of contractile material (for example see Fig. 3A). For quantification of abnormal myofibrillar structure, see Fig. 3B. (Note that due to limited biopsy size we were not able to perform electronmicroscopic analyses on the patient with 52 wk of HDP).

**Diaphragm muscle fiber cross-sectional area.** Next, we determined the cross-sectional area of diaphragm muscle fibers. As shown in Fig. 4, the cross-sectional area of fast diaphragm fibers was not significantly different in the Short-HDP patients as a group compared with control subjects. However, note that within the Short-HDP group, the patient with 8 wk of HDP displayed ~50% smaller fiber cross-sectional area compared with controls. In the Long-HDP group, the cross-sectional area of fast fibers was significantly reduced compared with control subjects. The cross-sectional area of slow diaphragm fibers was significantly smaller in both the Short-HDP and the Long-HDP patients compared with controls, with a more pronounced reduction of slow fiber size in the Long-HDP group (\( P = 0.03 \), Short-HDP vs. Long-HDP). For typical examples of diaphragm cross sections stained for slow and fast myosin heavy chain, see Fig. 4A. The proportion of fast fibers was higher in the Long-HDP group compared with Short-HDP patients and controls (Fig. 4C), indicating a fiber-type shift toward more glycolytic fibers. Fiber-type area fraction, however, did not differ significantly between groups (Fig. 4D).

**Expression of E3 ligases and proteasome activity in diaphragm muscle fibers.** The majority of protein degradation during conditions of muscle wasting occurs via the ubiquitin-proteasome pathway (21, 23). To test whether the ubiquitin-proteasome pathway is activated during HDP, the mRNA levels of the E3 ligases MAFbx and MuRF-1 were determined in diaphragm muscle fiber samples by means of real-time quantitative PCR. As shown in Fig. 5A, the mRNA levels of MAFbx and MuRF-1 were not different in Short-HDP and Long-HDP patients compared with those in controls. Furthermore, to study whether proteasome activity was elevated in the diaphragm of patients with HDP, we measured the activity of isolated proteasomes against the substrate LLVY (a measure of chymotrypsin-like activity). As shown in Fig. 5B, proteasome activity was not significantly different between controls and Short-HDP or Long-HDP patients. (Note that because of limited biopsy size we pooled the tissues of the three controls.
subjects and we were not able to determine proteasome activity in the patient with 40 wk of HDP).

DISCUSSION

This study is the first to investigate the effect of hemidiaphragm paralysis on diaphragm muscle fiber function and structure in humans. It was found that both diaphragm muscle fiber function and structure are preserved up to 8 wk of paralysis, followed by a gradual loss of muscle fiber size and structure during the course of paralysis. These findings indicate that diaphragm muscle atrophy and weakness following hemidiaphragm paralysis develops slowly and takes months to occur, suggesting that the diaphragm might be relatively resistant to contractile inactivity.

Study limitations. We investigated the effects of diaphragm unloading following hemidiaphragm paralysis in eight patients. This number was limited to eight for the following reasons. First, since the aim of the present study was to investigate the changes in diaphragm muscle fiber function and structure during the course of hemidiaphragm paralysis, it was important to know the exact duration of paralysis at biopsy. In general, the cause of hemidiaphragm paralysis is idiopathic (9). Therefore, in the vast majority of patients it is not possible to determine the duration of paralysis, leaving only a very limited number of patients with hemidiaphragm paralysis of exactly known duration. Second, HDP patients who also suffered from diseases known to impact diaphragm function, such as chronic obstructive pulmonary disease (24, 29, 30), chronic heart failure (35), and neuromuscular disorders were excluded. Together, this approach precluded the inclusion of a large number of patients, but, importantly, it provided the means to study a homogenous patient group without potential confounding effects of other illness.

The control subjects had stage I lung cancer. Although we cannot completely rule out that the cancer impacted diaphragm muscle fiber function, there is no published evidence of such impact. As shown in Table 1, the control subjects were not cachectic and had normal body mass index, suggesting no
overt muscle wasting. Because studying healthy controls is impossible owing to ethical reasons, we feel that patients with stage I lung cancer provide a plausible alternative. The use of such control group is widely accepted as witnessed by many recent publications (4, 5, 15–18, 28–30).

**Diaphragm muscle fiber function is preserved following 8 wk of paralysis.** The patients with HDP studied here all underwent resection of 4–5 cm of phrenic nerve tissue during resection of thymoma or of non-small cell lung carcinoma (Table 1). Following this procedure, some patients developed severe dyspnea within weeks, whereas others did not until after several years. In line with diaphragm paralysis, chest X-ray showed an elevated hemidiaphragm in all patients (for an example, see Fig. 1). Surgical treatment of diaphragm paralysis includes plication to reduce excursions during inspiration, thereby decreasing lung compression. This surgical intervention provided the rare opportunity to gain access to paralyzed human diaphragm tissue and to obtain diaphragm biopsies. Note that the biopsy procedure damages the muscle fibers’ sarcolemmata, which precludes the isolation and study of intact muscle fibers in these biopsies. An elegant alternative to investigate diaphragm function is the use of demembranated muscle fibers isolated from patient biopsies (see Refs. 29, 30). In demembranated muscle fibers, the membranous structures, such as the sarcolemma and sarcoplasmic reticulum, are made permeable, while leaving the contractile machinery (i.e., myofilaments) intact. By attaching these demembranated fibers to a force transducer and exposing them to exogenous calcium, their contractile performance was evaluated. As shown in Fig. 2, the maximum force-generating capacity of diaphragm muscle fibers from patients with 2, 3, 6, and 8 wk of paralysis was comparable to that of diaphragm fibers from patients with no paralysis. Note that we were unable to dissect single muscle fiber segments from the biopsies of patients with HDP longer than 8 wk (most likely this was the result of an increased susceptibility to rupture during fiber dissection due to loss of the structural integrity of myofilaments after 8 wk of HDP). Because in demembranated muscle fibers only the myofilaments are left intact, the unaffected contractile performance of these diaphragm fibers from patients with HDP suggests that myofilibrillar structure is preserved. Indeed, electronmicroscopy revealed well preserved myofilibrillar structure up to 8 wk of HDP (Fig. 3). Interestingly, the preserved contractile performance of diaphragm muscle fibers after 8 wk of paralysis is in contrast

with previous work on rats with unilateral diaphragm denervation that showed significant diaphragm muscle fiber weakness already after 1–2 wk of paralysis (6, 37). Perhaps the resistance of the human diaphragm to contractile inactivity-induced loss of contractile performance is related to its relatively low contractile activity during normal breathing. Normal breathing frequency in humans is ~14 breaths/min vs. ~100 breaths/min in rats (31). Hence, the transition from normal diaphragm activity to complete loss of activity during paralysis is less drastic in humans than in rats. We speculate that this might attenuate its deleterious effects on human diaphragm function.

**Retarded muscle fiber atrophy following paralysis-induced diaphragm contractile inactivity.** The preserved contractile performance (normalized to cross-sectional area) of diaphragm muscle fibers up to 8 wk of HDP was accompanied by preservation of the cross-sectional area of fast fibers and a modest atrophy of slow fibers (Fig. 4). Only little is known in humans on the effects of short-term denervation on muscle structure. Gregory et al. (8) studied quadriceps muscle adaptations to 11 wk of spinal cord injury. That study indicated that after 11 wk of denervation the cross-sectional area of fast fibers was reduced by ~50%, a reduction that is not observed in fast diaphragm fibers following 8 wk of paralysis (Fig. 4). Similarly, the cross-sectional area of slow muscle fibers of quadriceps is reduced by more than 50% after 11 wk of paralysis, a magnitude of reduction that is not observed in slow diaphragm fibers. Although we do not have data from patients with 11 wk of HDP, these results suggest that fiber atrophy in response to denervation likely occurs on a slower time scale in fibers from the diaphragm compared with peripheral muscle fibers. Thus these findings do not support the notion that the human diaphragm is very sensitive to contractile inactivity (11, 18).

**Muscle fiber cross-sectional area depends on the balance between protein synthesis and degradation.** The bulk of sarcromere protein degradation occurs via the ubiquitin–proteasome pathway (20, 22). In this pathway, muscle-specific E3-ligases, such as MAFbx and MuRF-1, ubiquitinate proteins that are subsequently degraded by the proteasome. Hence MAFbx and MuRF-1 are considered key markers of proteolytic activity in muscle (3). The marked diaphragm atrophy that is observed in mechanically ventilated brain-dead patients (11, 12, 15, 18) and in animal models for mechanical ventilation (34), as well as the diaphragm atrophy that occurs in rats with unilateral
Diaphragm denervation (2), is accompanied by marked increases of MuRF-1 and MAFbx, suggesting that the diaphragm atrophy is, at least partly, caused by activation of the ubiquitin-proteasome pathway. To our surprise, in the present study we found no evidence for significant upregulation of E3-ligases or for elevated proteasome activity in diaphragm muscle fibers following paralysis, not even after 72 and 88 wk of paralysis, at which time marked atrophy of both slow and fast diaphragm fibers had occurred (Fig. 5). Thus, in humans, diaphragm muscle fiber atrophy following hemidiaphragm paralysis is not caused by activation of the ubiquitin-proteasome pathway. Future studies should address whether diaphragm atrophy is the result of activation of other proteolytic pathways, such as autophagy (11), or of changes in protein synthesis rate.

Passive stretching of diaphragm muscle fibers during paralysis. The mechanism(s) underlying the human diaphragm’s low sensitivity to contractile inactivity following paralysis is unclear. In contrast to the cyclic passive shortening of the diaphragm during mechanical ventilation, the paralyzed hemidiaphragm undergoes cyclic stretch due to the persistent contractions of the normally innervated contralateral hemidiaphragm. This paradoxical, cranial, movement of the paralyzed diaphragm during inspiration is a hallmark feature of patients with HDP (see Fig. 1). Work on diaphragm paralysis in rabbits (38) showed that fibers in the midcostal region of the paralyzed hemidiaphragm were passively stretched by 3–5% during inspiration. Although the magnitude of diaphragm fiber stretch in humans with hemidiaphragm paralysis has not been determined, chest X-ray suggests that this is higher than only 3–5% (see Fig. 1 and Ref. 36). Passive stretch is a known stimulus for muscle protein synthesis and growth (10) and plays an important role in the maintenance of muscle mass. For example, repetitive passive stretch suppressed denervation-induced atrophy of soleus muscle in rats through upregulation of protein synthesis (36). Within the muscle’s sarcomere, titin is considered to play an important role in the stress-response machinery (7, 13), by acting as a mechanosensor that regulates muscle protein expression in a sarcomere strain-dependent fashion (14, 32). Thus titin is an attractive candidate for sensing the passive stretch imposed on paralyzed diaphragm muscle fibers and might be involved in the initial preservation of muscle fiber size and contractile function. Clearly, future studies are needed to identify the mechanical and molecular mechanisms that underlie the human diaphragm’s resistance to weakness and atrophy following paralysis.

In conclusion, the present study indicates that where diaphragm muscle fiber atrophy following hemidiaphragm paralysis develops slowly, contractile performance is preserved up to at least 8 wk of paralysis. We propose that the resistance of the paralyzed diaphragm to contractile inactivity might lie in the passive cyclic stretch due to the persistent contractions of the normally innervated contralateral hemidiaphragm.

REFERENCES


GRANTS

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