Response of the diaphragm muscle to electrical stimulation of the phrenic nerve

A histochemical and ultrastructural study

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Electrical stimulation of the phrenic nerve for long-term pacing of the diaphragm has been successful in the treatment of certain cases of chronic ventilatory insufficiency. Concern over the long-term effects of diaphragm pacing on the phrenic nerve and on the diaphragm muscle prompted investigation of the techniques employed and study of the electrophysiological correlates in a canine model. Evaluation of the electrical parameters for diaphragm pacing and studies on the phrenic nerves of these animals have been described. Histochemical and histopathological changes induced in the diaphragm are presented here, and the implications for humans requiring diaphragm pacing and the possible application of the findings to an understanding of human neuromuscular disease are discussed.

Materials and Methods

The diaphragms from 24 adult mongrel dogs were included in this study. Each hemidiaphragm was studied separately at the time of sacrifice of the animal. In addition, the stimulated hemidiaphragms of three animals were biopsied one or two times during the study period. Specimens from unstimulated (control) hemidiaphragms were obtained at the termination of the experiment in 15 animals. In all, 56 specimens of diaphragm muscle were studied for pathological changes. The specimens, whether obtained as a biopsy or at autopsy, were taken from the same general area, midway between the central tendon and the lateral chest wall in the mid-axillary line.

Stimulating Parameters

The technique of electrical stimulation of the phrenic nerve employed in these studies has been described in detail previously. The method involves the implantation in the chest of a platinum electrode which is fixed to the upper third of the phrenic nerve and is attached by multistrand stainless steel wires to a battery-powered stimulator. Pacing of one or both hemidiaphragms was carried out with either high-frequency (27 to 33 Hz) or low-frequency (11 to 13 Hz) electrical stimulation.
Diaphragmatic response to phrenic stimulation

Two types of stimulator waveforms were employed: unidirectional capacitive-coupled (UDC) and bidirectional capacitive-coupled (BDC). Constant-current output amplifiers were used to generate both waveforms. The pulse width was 150 µseconds for both stimulators, with a 1-msecond delay between the cathodal and anodal pulses of the BDC stimulator. The inspiration duration in all experiments was 1.3 seconds and the respiratory rate 20/minute. Typical current applied to the nerve was 1 to 2 mA, which was rarely exceeded. The stimulator was capable of being programmed, after implantation, to a current of 5 mA. The maximal current was never required to stimulate the phrenic nerve but, had it been used, the charge to the nerve would have been 0.75 µC/pulse. As the geometric area of the electrode used is 0.17 sq cm and the real area is 0.2 sq cm, the maximal charge density would have been 3.7 µC/real sq cm.

Pathological studies on the diaphragm and on the phrenic nerves were performed on the same animals whose diaphragms had been chronically stimulated in an earlier study to evaluate the effect of varying electrical parameters on diaphragm fatigue. Most of the animals were stimulated according to a protocol calling for 6 weeks of stimulation of one hemidiaphragm followed by 6 weeks of no stimulation, a program that was repeated three times for a total of 48 weeks. During two of the 6-week periods of stimulation, the current delivered to the phrenic nerve was high enough to cause maximal tidal volume. This level of stimulation was termed "maximal." During the other two 6-week periods of stimulation, a slightly lower current (200 to 300 µA) was used. This level was termed "submaximal." Where additional information was sought on the effects of chronic stimulation on the diaphragm, continuous stimulation of the hemidiaphragm was carried out for as long as 52 weeks at maximal or submaximal levels.

Pathological Examination

When the animals were sacrificed, the diaphragms were totally excised and photographed whole. The area of the diaphragm muscle and of the central tendinous portions was calculated by pantograph. The right and left hemidiaphragms were weighed separately. Squares of muscle, 1.5 cm in diameter or less, were fixed in formalin and cut for routine processing and embedding in paraffin. Transverse and longitudinal sections were prepared and stained with hematoxylin and eosin (H & E), periodic acid-Schiff (PAS), Masson's trichrome, Verhoeff's elastica, and Wilder's reticulin stains. For histochemical studies, cubes of muscle, 0.5 cm in diameter, were oriented and frozen on cryotome chucks in isopentane cooled to −160°C by liquid nitrogen. Transverse sections were cut at 6, 10, and 20 µ in an IEC cryotome at −35°C, picked up and dried on coverslips, and subsequently stained for adenosine triphosphatase (ATPase), with preincubation at pH 9.4 and 4.6, nicotinamide adenine dinucleotide (reduced) (NADH) dehydrogenase, menadione-linked α-glycerophosphate dehydrogenase, and Gomori's trichrome for frozen sections as described previously. Microscopic measurements were obtained with an AO micrometer and microscope† using sections stained with H & E, PAS, or ATPase, or by direct measurement of photographic enlargements with a centimeter rule as described by Dubowitz and Brooke. Minimum diameters were measured, and comparable results were achieved with all methods. Tissue for electron microscopic studies was minced into 1-mm cubes and fixed in 3% glutaraldehyde and phosphate or sodium cacodylate buffer, pH 7.4. The tissue was rinsed in buffer and post-fixed in osmium tetroxide, dehydrated through graded ethanol, and embedded in Epon 812. Sections were cut on a Sorvall MT-2B microtome. For selections of fields, sections 1 µ thick were stained with toluidine blue. Thin sections were placed on 200-mesh copper grids and photographed in a Zeiss EM10B electron microscope.

Results

Gross Appearance

Diaphragms paced at the predetermined high frequencies were markedly different from unpaced controls and from diaphragms paced at the lower frequencies. They were smaller in surface area and had proportionately larger central tendinous areas, but were approximately of the same weight (Table 1). In

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface Area (sq cm)</th>
<th>Weight (gm)</th>
<th>Central Tendon Area (%)</th>
<th>Density (gm/sq cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>stimulated hemidiaphragm</td>
<td>157.7 ± 30.1</td>
<td>56 ± 11.9</td>
<td>21.12</td>
<td>0.363 ± 0.08</td>
</tr>
<tr>
<td>unstimulated hemidiaphragm</td>
<td>196.6 ± 29.0</td>
<td>58 ± 9.1</td>
<td>15.84</td>
<td>0.295 ± 0.00</td>
</tr>
</tbody>
</table>

* The figures represent averages for 14 dogs with one side of the diaphragm paced at high frequency, the other side unstimulated. Eight left and six right diaphragms were paced. None of the paced hemidiaphragms was larger than the control side. Paired t-tests showed p < 0.01 for surface area and density, p < 0.05 for central tendinous area, and no significant difference for the weights.

† AO micrometer and microscope manufactured by American Optical, Eggert and Sugar Roads, Buffalo, New York.

‡ Sorvall MT-2B microtome manufactured by Du Pont Co., Sorvall Biomedical Instruments Division, Newtown, Connecticut.
cross section, the high-frequency-paced diaphragm muscle was thicker, and had larger individual fascicles and a distinct pallor compared to controls (Fig. 1).

**Microscopic Appearance**

On routine histological examination there were remarkable differences between the high-frequency-paced diaphragms and those paced at low frequency. When minimum fiber diameters were measured on 200 or more fibers, and the unstimulated hemidiaphragm was compared to the low-frequency (11 Hz)-stimulated hemidiaphragm, the distribution of muscle fiber diameters was similar (Fig. 2 left). In the high-frequency-stimulated diaphragms there was great variation in myofiber diameter on the stimulated side, with the majority of the fibers appearing much larger than the control or low-frequency-stimulated hemidiaphragm fibers. When both sides of the diaphragm were stimulated at different frequencies, the high-frequency-stimulated side showed a definite overall increase in average fiber diameter (Fig. 2 right).

Other pathological changes were observed in the high-frequency-stimulated diaphragms, including enlarged and increased numbers of sarcolemmal nuclei and the presence of internal nuclei, irregular hyaline cytoplasmic masses, phagocytosis of necrotic fibers, increased fibrous tissue, and, rarely, ring fibers (Fig. 3). The cytoplasmic masses were most prominent in the muscle that had been maximally stimulated. With Gomori's trichrome staining, the cytoplasmic masses appeared as darker green condensations of cytoplasm, but they were not readily distinguishable with the PAS stain. With most other stains, including Masson's trichrome, Verhoeff's elastica, Wilder's reticulin stain, and H & E, cytoplasmic masses were demonstrated.

On routine histochemical staining there was uneven staining of the myofibers of the high-frequency-paced diaphragms, a "moth-eaten" appearance with NADH dehydrogenase stain, and with the acid and alkaline ATPase the fibers stained darkly, revealing irregular enzyme-poor regions (Fig. 4). These enzyme-poor regions undoubtedly correspond to some of the holes in the "moth-eaten" fibers, although not nearly so many affected fibers were seen with the ATPase stain. In occasional tangential cuts, these enzyme-poor regions were observed to be similar to central core-like or targetoid structures, which were seen in longitudinal sections through wavy fibers (Fig. 5). Ultrastructural studies showed focal disruption of myofilaments, increased glycogen, occasional smearing, smudging, and dissolution of Z bands with focal large aggregates of osmophilic Z-band material (Fig. 6). Where biopsies taken during a pacing period using high-frequency stimulation were compared with autopsy specimens many months after cessation of pacing, pathological changes were observed. These changes, although not as prominent as during pacing, included increased variation in fiber diameter, enlarged sarcolemmal nuclei and the presence of internal nuclei, and round-cell infiltrates and necrotic fibers (Fig. 7).
Diaphragmatic response to phrenic stimulation

FIG. 3. Photomicrographs, x 450. Upper left: Experiment 16085. Muscle fibers from a high-frequency (27 Hz)-stimulated canine diaphragm after alternating 6-week periods of stimulation and rest for 1 year, ending with 2 weeks of stimulation. Note the increased variation in fiber diameter, enlarged sarcolemmal and internal nuclei, waxy hyaline inclusion material in sarcoplasm (cytoplasmic masses), increased endomysial connective tissue, and phagocytosis of necrotic fibers. Verhoeff’s elastica. Upper Right: Experiment 18859, showing ring fiber and dark sarcoplasmic masses in a high-frequency (27 Hz)-stimulated diaphragm after alternating 6-week periods of stimulation and rest for 42 weeks. Masson’s trichrome. Lower Left: Experiment 16085. Section of a high-frequency (27 Hz)-stimulated diaphragm showing abundant endomysial connective tissue and reticulin-positive sarcoplasmic masses. Wilder’s reticulin. Lower Right: Experiment 18766, showing a hemidiaphragm after high-frequency (33 Hz) stimulation for 10 weeks (maximal stimulation). Note the prominent dark cytoplasmic masses. Masson’s trichrome.

The simultaneous development of fatigability and pathophysiological changes in the high-frequency-stimulated diaphragms is highly correlated, and several possible mechanisms are suggested by our data. Fatigability in the high-frequency-stimulated diaphragms can be explained simply if we postulate an overall hypercontraction or semitetic contraction of the individual stimulated fibers. There is no obvious physiological hypertrophy of the muscle, since the average weights of stimulated and unstimulated hemi-

average fiber diameter on the high-frequency-stimulated side decreased after cessation of stimulation, while the average fiber diameter on the low-frequency-stimulated side increased (Fig. 8).

Diaphragms paced with the lowest frequency (11 Hz) used in these experiments showed no pathological changes, and did not differ markedly from unstimulated diaphragms, either grossly or microscopically. In some there was an increase in fiber diameter and some enlargement of sarcolemmal nuclei, and the transverse sections showed a rounded profile in the stimulated myofibers compared to the non-paced fibers, which had a more polygonal appearance (Fig. 9). The low-frequency-paced fibers had a normal appearance with NADH dehydrogenase, but stained darkly with both acid and alkaline ATPase stain (Fig. 10), suggesting transformation to Type II fibers. Ultrastructural studies were essentially normal in the low-frequency-stimulated diaphragms except for some increase in mitochondrial activity and glycogen granules.

Discussion

The simultaneous development of fatigability and pathophysiological changes in the high-frequency-stimulated diaphragms is highly correlated, and several possible mechanisms are suggested by our data. Fatigability in the high-frequency-stimulated diaphragms can be explained simply if we postulate an overall hypercontraction or semitetic contraction of the individual stimulated fibers. There is no obvious physiological hypertrophy of the muscle, since the average weights of stimulated and unstimulated hemi-
diaphragms are comparable (Table 1). If the muscle mass remains the same and the fibers shorten, diaphragm fatigability can be explained by diminished stroke length. Increased cross-sectional diameters in the high-frequency-stimulated fibers suggest a contraction of fibers, but some high-frequency-stimulated diaphragms have shown increased numbers of small “atrophic” as well as “hypertrophied” fibers. It is difficult to apply a uniform fiber contraction theory to entirely explain the data, and we are left with the impression that although “hypertrophied” fibers are prominent and may predominate, increased variability of fiber size is the most consistent finding in the high-frequency-stimulated diaphragms.

The observation of other pathological changes in the high-frequency-stimulated diaphragms is particularly interesting since the changes bear some resemblance to those in human disease. The internal myo-
fibrillar changes with the conventional stains suggest necrosis or degenerative changes. On histochemical staining, “moth-eaten” fibers and enzyme-poor cytoplasmic masses with occasional core/targetoid changes indicate disruption of myofiber organization, and somewhat resemble the myopathic changes of muscular dystrophy. Electron microscopic studies confirm disorganization of subcellular structures. Enlarged nuclei suggest activated synthesis and repair. Although the mechanism is unclear, the pathological changes seen in the high-frequency-stimulated diaphragms are common to many neuromuscular disorders. In the experimental animals in which some of these changes persisted even after 6 months of no stimulation (Figs. 7 and 8), the muscle bears a resemblance to myotonic dystrophy or the so-called “myopathic changes” of chronic neurogenic atrophy.6 The severe pathological changes correlated with a poor diaphragmatic response to electrical stimulation.13

The gross transformation of the high-frequency-stimulated diaphragms from red to white muscle appears to be supported by the alteration of histochemical fiber types. Since the color of muscle is determined by the amount of microcirculation,7 it would appear that the high-frequency-stimulated diaphragms had a reduced vasculature. Our microscopic studies were not detailed enough to support decreased vasculature, and it would be difficult on the basis of our studies to rule out anoxia as a cause of the pathological changes.

The fact that fiber transformation occurred with artificial stimulation should not be surprising since transformation of muscle fiber types by electrical stimulation has already been demonstrated, and it is well known that the fiber type is determined by the type of innervation.14,17 With the dog diaphragms the electrical stimulation of phrenic nerve apparently modifies the fibers to produce a fast, strong, tetanic
type of contraction. The histological differences between the high- and the low-frequency-stimulated diaphragms may be explained by the prolonging of the recovery time due to the smaller number of impulses driving the low-frequency-stimulated muscles.

In the high-frequency-stimulated diaphragm, recovery time is shorter since the electrical impulses pass across the neuromuscular junction at a faster rate, resulting in tetanic contractions with depletion of substrates, myofibrillar disarray, and tearing and disruption of fibers. Perhaps the centers of fibers are most susceptible to disintegration, the central regions being the end of the watershed for protein synthesis and repair under nuclear control.

Salmons and associates have reported widening of the Z band in response to chronic electrical stimulation. Although some investigators have reported Z-band smearing in normal individuals, the Z-band smearing and disruption of myofilaments in our cases represents a pathological exaggeration of a normal intramuscular process, possibly caused by the strong force of the contractions or the muscle cells' inability to sustain repair mechanisms. Wavy fibers with central change may represent fibers torn loose from their attachments similar to tenotomized fibers or core/targetoid fibers.

A recent study has shown that tetany induced by tetanus toxin can produce core/targetoid fibers identical to those induced by tenotomy. It is also well known that the induction of target fibers by tenotomy may be prevented by denervation of the muscle.

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**Fig. 7.** Experiment 18766. Photomicrograph showing a high-frequency (33 Hz)-stimulated hemidiaphragm, after 10 weeks of maximal stimulation followed by 24 weeks of rest. Note the great variation in fiber size, increased connective tissue, sarcolemmal nuclei, internal nuclei, and necrotic fibers. PAS, × 450.

**Fig. 8.** Experiment 18766. A: Muscle fiber diameters of high-frequency (36 Hz)-stimulated compared with low-frequency (11 Hz)-stimulated hemidiaphragms after 10 weeks of maximal stimulation. Note the increased fiber diameters of the high-frequency-stimulated fibers. B: Comparison of muscle fiber diameters of high-frequency (36 Hz)-stimulated versus low-frequency (11 Hz)-stimulated hemidiaphragms after 10 weeks of maximal stimulation followed by 24 weeks of rest. Note the great variability of fiber size in the high-frequency-stimulated side and the apparent increase in fiber diameter of the low-frequency-stimulated side. Physiological studies of the high-frequency-stimulated side showed fatigue with poor excursions, even after 24 weeks of rest.
Diaphragmatic response to phrenic stimulation

Although a neural mechanism is likely in our experiments, occasionally observed intramuscular branches of the phrenic nerve appear normal in the canine diaphragms, and extensive studies of the phrenic nerve itself in the same group of animals have failed to demonstrate changes in either high- or low-frequency-stimulated diaphragms when compared with controls. Studies of changes in the end-plate potentials following repetitive stimulation of the phrenic nerve suggest that the neuromuscular junction may be involved. Liu, et al., postulated a “dying back” phenomenon in the axons of an infant they reported who had received phrenic nerve pacing for a congenital hypoventilation syndrome. The “dying back” phenomenon is discussed in more detail in an ongoing study of the effect of electrical stimulation on the phrenic nerves.

We have shown that electrical pacing of the phrenic nerve using high-frequency stimulation can lead to pathological changes in the diaphragm. These findings support the studies on diaphragm function reported earlier. As a result of these experiments demonstrating pathological changes which accompanied functional changes in the diaphragm of the canine, the electrical parameters for pacing the human diaphragm have been modified.

FIG. 9. Photomicrographs in Experiment 18766. Gomori’s trichrome, × 450. Left: Low-frequency (11 Hz)-stimulated hemidiaphragm after 10 weeks of maximal stimulation showing somewhat rounded polygonal-shaped myofibers with abundant mitochondrial granulations but which is otherwise unremarkable. Right: Low-frequency (11 Hz)-stimulated hemidiaphragm after 10 weeks of maximal stimulation, followed by 24 weeks of rest. Note the increased fiber diameter but otherwise unremarkable myofibers (cracking and vacuoles are artifacts).

FIG. 10. Photomicrographs (× 450) in Experiment 18771, showing a hemidiaphragm stimulated at low frequency (12 Hz) for 50 weeks, followed by 2 weeks of rest. All myofibers are stained dark. ATPase, pH 9.4 (left); and ATPase, pH 4.6 (right).
References


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