

DEPENDENCE OF FATIGUE PROPERTIES ON THE PATTERN OF STIMULATION IN THE RAT DIAPHRAGM MUSCLE

ASUMAN GÖLGELI⁺, ÇİGDEM ÖZESMI^{*} AND MUSTAFA ÖZESMI^{**}

Departments of ^{*}Physiology and ^{**}Lung Medicine,
Faculty of Medicine, Erciyes University,
38039 Kayseri, Turkey

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Abstract: Diaphragm fatigue was studied in isolated phrenic nerve diaphragm strips from 28 Swiss Albino rats. Three procedures were used to estimate the isometric twitch characters and force frequency responses to fatigue of the rat diaphragm at different rates of phrenic nerve stimulation. Diaphragm fatigue was induced by using low frequency stimulation (0.2 ms pulse duration, at 5 Hz frequency, 3 min), high frequency stimulation (0.2 ms pulse duration, at 50 Hz frequency, 3 min), and by producing brief submaximal contraction (25 Hz, for 160 ms at the rate of 1/s for 45 contractions). Tension was reduced to $26.67 \pm 5.10\%$ and $6.59 \pm 2.64\%$ and $68.69 \pm 2.45\%$ of the initial value at the end of the low and high frequency and brief submaximal stimulation, respectively. In all the fatigue experiments, twitch tension and tetanic tension decreased, contraction and 1/2 relaxation time prolonged and force-frequency curves shifted to the right. The most significant changes were observed in low frequency fatigue whereas the most moderate ones were recorded in brief submaximal fatigue. It was concluded that fatigue in the rat diaphragm depended on the frequency and duration of stimulation as well as on the number of stimuli delivered to the muscle. Various mechanisms of muscle fatigue are described in the discussion to explain the observations made in the present investigation.

Key words: fatigue isometric contractile properties
force-frequency curve rat diaphragm *in vitro*

INTRODUCTION

Weakness and fatigue in the skeletal muscle of respiration compromise their ability to generate normal levels of contractile tension. Like other skeletal muscles, the isolated diaphragm fatigues (1, 2) and, because of its unique role in respiration, contributes to ventilatory distress under experimental conditions (3). In patients with severe pulmonary disease, this process may contribute to ventilatory failure.

There have been a lot of documents in isolated rat (4-8), mouse (9) and hamster (10, 11) diaphragm preparations that are useful for the exploration of the pathophysiology of fatigue because contractile tension can be measured directly and because the preparation can be

isolated from possible neural and humoral influences.

There exist a great diversity of fatigue models in literature (6-13). They regard 20 Hz and below as low frequency stimulation (8, 12), 50 Hz and above as high frequency stimulation (13, 14). In clinical practice low-frequency fatigue has been demonstrated in the respiratory muscles of patients with chronic airways obstruction. For brief submaximal fatigue 25 Hz, 160 ms trains of 0.2 ms pulses duration, 1/s rate, 45 contractions has been used. This pattern is chosen as a closer approximation to the physiological contraction pattern of the diaphragm (which is repeated submaximal contractions).

Most investigators have found that fatigue

*Corresponding Author

prolongs muscle contraction and relaxation times (8, 13), decreases twitch tension (6, 8, 14), decreases tetanic tension (4, 7, 9, 13), and shifts the force-frequency relationship to the right (4, 6, 7, 11, 13). Although there are studies comparing high and low frequency fatigue (13, 14). We have not come across any study comparing these three fatigue models (high, low frequency and brief submaximal). We have chosen 5 Hz, 0.2 ms pulse duration as low frequency fatigue, and 50 Hz, 0.2 ms pulse duration, as high frequency fatigue and compared them with brief submaximal fatigue. We decided on 3 min of stimulation period in which glycogen stores are maintained (8, 12). In our preliminary work, we applied a 3 min stimulation for brief submaximal contraction, and observed that no significant difference occurred between 3 min and 45 stimulations. Therefore, we used the same stimulation length, in conformity with the one in literature (1/s rate, 45 contractions) in our consequent studies of brief submaximal fatigue. In the present study we aim at comparing more realistically the effects of the three fatigue models on the isolated muscle contraction properties using the animal and preparation of the same kind.

METHODS

General procedures : Twenty eight adult male Swiss albino rats weighing 180-230 g were used in the present study. Experimental animals maintained according to "Guide to the care and use of experimental animals" by the Canadian Council on Animal Care (15). Rats were quickly killed by cervical dislocation, under light ether anaesthesia, and the left hemidiaphragms removed after the procedure Kelsen and Nochomovitz (6). The left hemidiaphragm was isolated and a triangular piece of muscle with central tendon, ribs, and phrenic nerve was removed and quickly placed in a dissecting dish containing Krebs solution (oxygenated 95% O₂, 5% CO₂). The Krebs solution was similar to that used by Segal and Faulkner (16) and contained (in mM) NaCl, 137; KCl, 5; CaCl₂, 2; MgSO₄, 1; NaH₂PO₄, 1; NaHCO₃, 24; and glucose, 11. A single costal strip of muscle was isolated by

making two longitudinal cuts parallel to the fibres ~ 5 mm on either side of the point of entry at the phrenic nerve. A small section of ribs was left attached to the muscle at one end, while a sheet of central tendon was left attached at the other. This nerve/muscle, strip preparations was mounted vertically in a glass chamber containing Krebs solution. The muscle strip was secured to a phrenic-nerve electrode (Harvard Phrenic Nerve Electrode with Oxygen Bubbled) by means of a silk thread (6-0) attached to the ribs cage. A force-displacement transducer (Nihon Kohden Force Displacement Transducer TB 611 T) was attached to the central tendon of the diaphragm by a silk ligature (6-0). Phrenic nerve was placed at a sliding jaw in the same electrode. The chamber was then heated to 32°C by circulating water and maintained at the temperature ($\pm 0.5^\circ\text{C}$) throughout the experimental period. A temperature of 32°C instead of 37°C was selected because of the better stability that occurs with the lower temperature (16, 17). Tissues were bathed in Krebs solution (pH: 7.30-7.40) through which a gas mixture of 95% O₂-5% CO₂ was continuously bubbled. Fifteen min of equilibration time were allowed after each change before making measurements.

Stimulation protocols : Muscle contraction was induced via supramaximal electrical stimulation to the central end of the attached phrenic nerve via a phrenic nerve electrode. To obtain supramaximal stimulation conditions, single electrical pulses (square wave pulses of 0.2 ms duration) were delivered to the muscles once every 5s (Nihon Kohden SEN 3201 Stimulator and SS-201 J Isolation Unit) at increasing voltages and the resulting force was measured. The signal from the force transducer was amplified and recorded simultaneously on a Digi-Scope Converter 500 (Volf Craff) which was connected an oscilloscope (Trio Digital Memory Scope MS 1650 B) and a paper recorder (Nihon Kohden W1 681 G).

Muscle length was altered by raising or lowering the force transducer with a micrometer clamp. Muscle length was measured with a

scale positioned next to the muscle strip inside the bath and resting muscle length was then varied to determine the length (L_0) at which twitch force was maximal. Generally 8 to 10 stimulations were necessary for this determination; all subsequent studies were performed at this length. The strip was then stimulated with single pulses (2 per min) until the shape and size of the twitch had stabilized (approximately 20 min).

Measurement of mechanical properties :
Isometric Twitch Characteristics : For the measurement of isometric twitch characteristics, supramaximal voltages (0.2 ms square wave pulse) were delivered via phrenic nerve electrode. The force transducer output was amplified and recorded on pen recorder (Nihon Kohden WI 681 G) with the paper speed set at 200 mm/s. From these records, peak twitch tension (Pt), twitch contraction time (CT), one-half relaxation time ($1/2$ RT) were determined using the average of three contractions. The CT was defined as the time from the start contraction to the point where peak tension was achieved. The $1/2$ RT was defined as the time from peak tension to the point where twitch tension dropped by 50%. Twitch contraction time and one-half relaxation time were also determined by Digi-Scope converter and were found similar.

Force-frequency responses : Measurement of isometric force-frequency response for nerve stimulation was determined by stimulating the nerve with 0.2 ms pulses at 10 ($P_{10\text{Hz}}$), 20 ($P_{20\text{Hz}}$), 50 ($P_{50\text{Hz}}$) and 100 ($P_{100\text{Hz}}$) Hz in random order and with a 30-s delay between excitations. At each tetanic frequency, stimulation was maintained until tension plateaued (approx 1 s). Peak P_0 was measured at $P_{100\text{Hz}}$. No further significant increase in force was found with frequencies in excess of 100 Hz.

Fatigue studies : In this study, the diaphragms were subjected to three types of fatiguing stimulations: 1) Low frequency fatigue (5 Hz, 0.2 ms, 3 min, $n=7$), 2) High frequency fatigue (50 Hz, 0.2 ms, 3 min, $n=7$), 3) Brief submaximal contractions (25 Hz, for 160 ms at

the rate of 1/s for 45 contractions, $n=7$). Immediately after the fatigue run, single twitch and tetanic tension measurements were repeated. Only the first fatigue trial performed on any muscle strip was analyzed. The cross-sectional area of the muscle was calculated by dividing the volume of the muscle (muscle density assumed to be 1.06 g/cm³) by the length of the muscle (16). The muscle was removed from the bath, blotted dry with a paper towel, and then air dried for ~ 1 min before it was weighed.

Statistical analysis : It was performed with the use of the Student's "t" test (18). Comparison within a single strip (i.e., pre-fatigue vs post-fatigue) was made with use of a paired "t" test. Comparison between runs in control and fatigue use of an unpaired "t" test. Data in the text are mean \pm SD. Significant difference was defined at $P<0.05$.

RESULTS

There was no significant difference in body weight (205 ± 16.3 g), length (1.82 ± 0.13 cm) and weight of the diaphragm strips (134 ± 10.9 mg) between the experimental and control groups ($P>0.05$).

Tension changes with fatigue : Fig. 1 shows a typical muscle response during the development of isometric fatigue under various conditions. The effect of prolonged stimulations (5 Hz, 0.2 ms, 3 min and 50 Hz, 0.2 ms, 3 min) on the tension-generating ability of a single diaphragm muscle strip are shown in Fig. 1A and B. Tension fell to $73.33\pm 5.10\%$ and $93.41\pm 2.64\%$ of the pre-fatigue tension after low and high frequency stimulations respectively. At low frequency fatigue in 71% of the experiments tension fell transiently within the 1st min of repeated stimulation before increasing to a maximum value. This transient decrease in tension is a general property, both *in vivo* and *in vitro*, of contracting skeletal muscle and appears to be due of transient alterations in uptake and release of calcium by the sarcoplasmic reticulum (19). With continued stimulations, tension fell progressively from the

maximum value in a curvilinear fashion in all experiments. However, increase in the stimulation frequency was associated with more rapid falls in tension (Fig. 1B). The muscle was fatigued by stimulation at 25 Hz for 160 ms trains at the rate of 1/s for 45 contractions and the fall in tension with repeated contractions (fatigue run) is shown in Fig. 1C.

Tension fell more rapidly in the first five contractions, then remained fairly stable over the next 25 contractions, before starting to fall again. Tension fell to $68.29 \pm 2.45\%$ of the prefatigue tension after 45 contractions. In our preliminary work, we applied a 3 min stimulation for brief submaximal contractions, and observed that no significant difference occurred between 3 min and 45 stimulations.

Isometric contractile properties : The isometric contractile properties of the rat diaphragm and the effects of time course and fatigue protocols are shown in Table I and II respectively. All fatigue protocols produced a significant prolongation in isometric twitch contraction and one-half relaxation times when the significant reductions were observed in peak twitch tension.

Force-frequency response : Effect of fatigue on the force-frequency relationship between force and stimulation frequency before and after a fatigue trial is shown in Fig. 2. Force at each frequency is expressed as a percentage of the maximal tension, achieved with 100 Hz stimulation. In both the rested and fatigued diaphragm, increases in isometric diaphragmatic force were curvilinearly related to the increases

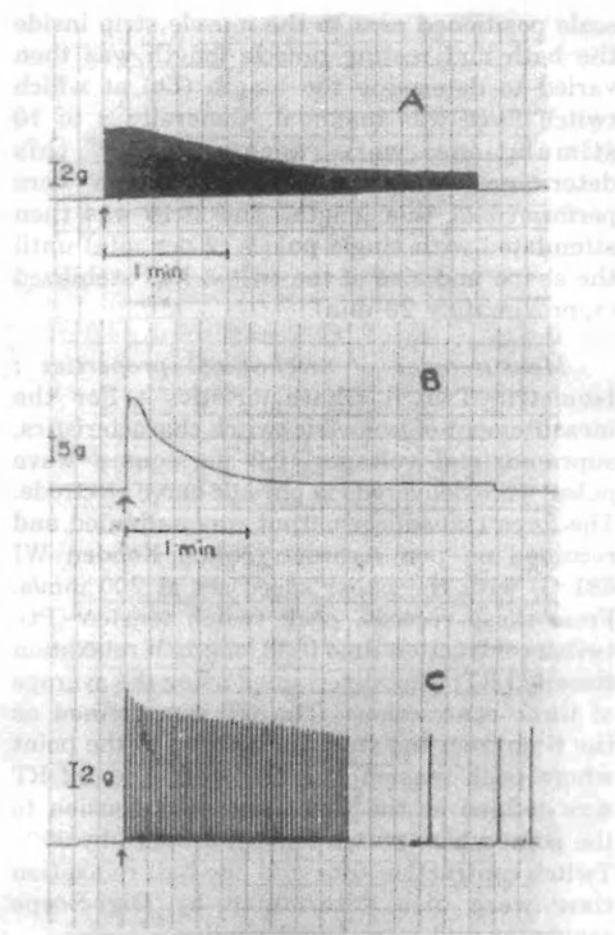


Fig. 1 : Isometric fatigue of isolated rat diaphragm muscle. Muscle was repetitively subjected to electrical stimulation in a fashion described in text. In A; low frequency fatigue (5 Hz, 0.2 ms, 3 min), B; high frequency fatigue (50 Hz, 0.2 ms, 3 min) and C; brief submaximal contraction (25 Hz, for 160 ms at the rate of 1/s for 45 contractions).

TABLE I : Contractile properties of isolated rat diaphragm at the time control group.

	Pt, kg.cm-2	Po, kg.cm-2	Pt/Po	CT, ms	1/2 RT, ms
Pre-experimental (n=7)	550.00±11.90	1814.28±76.38	0.306±0.02	29.43±1.51	28.29±2.14
After 2 hours (n=7)	546.14±13.38	1757.14±60.75	0.311±0.01	30.29±2.14	29.43±1.90

Values represent means \pm SD (n=7). Pt; peak twitch tension, Po; maximum tetanic tension, Pt/Po; twitch-to-tetanus ratio, CT; twitch contraction time, 1/2 RT; twitch one-half relaxation time. Muscle was stimulated supramaximal electrical stimulation at 1 Hz, 0.2 ms pulse duration, every 2 min for 2 hour via phrenic nerve electrode. None of them was different from preexperimental values $P > 0.05$.

TABLE II : Alterations in the twitch characteristics in the response to repeated phrenic nerve stimulation.

	Prefatigue (n=7)	Postfatigue (n=7)
Pt, kg. cm⁻²		
Low frequency	544.28 ± 8.38	278.57 ± 19.52 ^{a,c}
High frequency	547.85 ± 10.75	224.28 ± 17.18 ^a
Brief submaximal	538.57 ± 21.35	398.57 ± 35.79 ^a
Po. kg. cm⁻²		
Low frequency	1764.287 ± 149.20	1178.14 ± 50.07 ^{a,c}
High frequency	1807.14 ± 171.83	1395.71 ± 35.05 ^{a,d}
Brief submaximal	1792.85 ± 178.49	1601.43 ± 42.59 ^a
Pt/Po		
Low frequency	0.311 ± 0.02	0.236 ± 0.01 ^a
High frequency	0.306 ± 0.03	0.161 ± 0.01 ^{a,d}
Brief submaximal	0.303 ± 0.03	0.249 ± 0.02 ^a
CT, ms		
Low frequency	28.86 ± 1.07	47.14 ± 2.67 ^a
High frequency	29.71 ± 1.79	38.57 ± 3.78 ^{a, d}
Brief submaximal	29.14 ± 1.57	32.71 ± 3.68 ^b
1/2 RT, ms		
Low frequency	28.00 ± 2.31	50.7 ± 3.45 ^{a, c}
High frequency	28.57 ± 2.23	44.29 ± 1.89 ^{a, d}
Brief submaximal	28.29 ± 2.43	39.29 ± 3.45 ^a

Values are mean±SD,

- a. Significantly different from prefatigue P<0.01
- b. Significantly different from prefatigue P<0.05
- c. Significantly different from postfatigue values of high frequency and brief submaximal fatigue P<0.01
- d. Significantly different from postfatigue values of low and brief submaximal fatigue P<0.01.

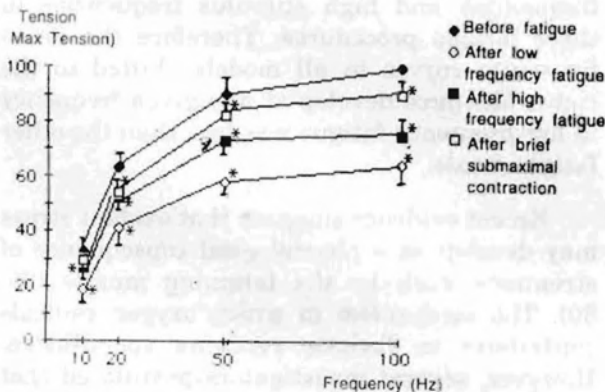


Fig. 2 : Relationship between isometric tension and frequency of stimulation. Muscle stimulated at optimal length with pulse duration of 0.2 ms, train duration sufficient for development of maximum tension (approx 1 s). Data obtained from 7 strips. Values are mean±SD.

in frequency. After completion of a fatigue trial, the force developed at any given frequency was less than that was produced prior to the fatigue trial.

DISCUSSION

Muscular fatigue is generally defined in terms of failure of a muscle to continue to develop a certain level of tension (3). Indeed, fatigue of the diaphragm and other respiratory muscles has been defined in these terms and has been implicated as a cause of respiratory failure (1, 2). When the diaphragm was made to contract rhythmically *in vitro* by cyclically stimulating the phrenic nerve, increases in the stimulation frequency was observed to increase the rate of fatigue. We studied both low and high frequency stimulation (5 Hz, 0.2 ms, 3 min and 50 Hz, 0.2 ms, 3 min) and brief submaximal contractions (25 Hz, 160 ms pulse trains, 1/s rate 45 contractions). The main findings of this study we have carried out, are; 1) time had no effect on twitch characteristics at the time control group, 2) muscle response and twitch tension decreased, contraction and 1/2 relaxation time prolonged, 3) force-frequency curves shifted to the right. The most significant changes were observed in low frequency fatigue whereas in brief submaximal fatigue there were only mild changes.

The preparation used in the present experiments has several advantages over studies performed *in vivo*. First, the isometric force developed by the contracting diaphragm can be measured directly rather than inferred from pressure volume of thoracic configuration (20). Second, the stimulus delivered to the muscle can be controlled, which is not possible under conditions in which phrenic nerve activity is determined reflexly or volitionally. The studies were performed at 32°C, because studying the *in vitro* muscle at 37°C (approximate physiologic conditions) has some drawbacks (16, 17, 21). The main problem is that although metabolism within the muscle goes on at the usual rate, oxygen transport is limited as it relies on diffusion. Therefore, the most central fibers

become, anoxic and damaged (16). Even though the rat diaphragm muscle strip is quite thin and the area of significant anoxia is small, any loss of functioning fibers will decrease the tension reached with a given stimulation. To control for the tension loss due to this effect, the time control studies were carried out. These studies are helpful as the effect of the various interventions would have been overestimated without the time control measurements.

When subjected to repeated 5 Hz stimulations the diaphragms of rat undergo a relatively slow decrease in contractility. The relatively long time required for this pattern of fatigue to develop makes it likely that intracellular milieu changes are involved (22). Indeed, there is evidence that stimulation that initiates low frequency slow-onset fatigue does so by initiating contractile events in which energy demand on the muscle exceeds the aerobic capacity of the muscle, causing a high level of anaerobic metabolism (23). The rapidity of onset makes it unlikely that changes in the intracellular milieu are responsible for this fatigue pattern. It is also unlikely that the rapid fatigue is the result of a reversible K^+ build up in the extracellular space in that such build ups occur only at higher frequencies of stimulation (24). In our study, high-frequency fatigue resulted in rapid loss of force which was also found in human fibialis anterior muscle by Sacco et al (25). The most marked alterations of CT and 1/2 RT are seen in low frequency stimulation pattern.

The prolongation of relaxation time is thought to be related to decrease in the rate of Ca^{2+} uptake by the sarcoplasmic reticulum (SR). Increases in CT have also been observed earlier (26) and may reflect impairment in the rate and duration of release of Ca^{2+} from the SR or changes in contractile characteristics. In this regard, it should be emphasised that because the diaphragm is a mixed muscle composed of different fiber types (17), it is not possible to determine to what degree the specific fiber types were responsible for the observed changes. There appears to be a good

correlation between the CT and 1/2 RT of a muscle and the rate of Ca^{2+} uptake by SR isolated from the muscle (27). In this context, it seems possible that the slowing of CT and 1/2 RT may be due in part to a reduction in the rate of Ca^{2+} uptake by the SR in fatigued muscle.

The present study does not elucidate the site(s) involved in neuromuscular transmission affected by the fatigue process. It does indicate, however, that in the fatiguing diaphragm the decline in tension is due both to the failure of mechanisms involved in the excitation of diaphragmatic muscle cells and to the failure of the mechanisms within the cell involved in force generation. It has been suggested (that the site(s) of fatigue in skeletal) muscle can be determined by measuring force output at different stimulus frequencies (28). Decrease in force generated by fatigued muscle during high stimulus frequencies (approx. 50 Hz or more) indicate impairment in neural mechanisms involved in exciting the muscle. On the other hand, reductions in force at lower stimulus frequencies (approx. 20 Hz or less) suggest impairment in the coupling of excitation and contraction or impaired function of the intrinsic contractile machinery. In this study, we also observed reductions in force both at low stimulus frequencies and high stimulus frequencies in three fatigue procedures. Therefore the force-frequency curves in all models shifted to the right. The force develop at any given frequency in low frequency fatigue was less than the other fatigue trials.

Recent evidence suggests that oxidant stress may develop as a physiological consequence of strenuous work by the fatiguing muscle (29, 30). The mechanism in which oxygen radicals contribute to fatigue remains speculative. However, several investigators postulated that oxygen-derived free radicals produced during fatigue either damage subcellular organelles or injure cellular membranes (29, 31, 32).

We suggested that the production of free radicals may represent an important potential

for cellular injury that may mediate diaphragmatic dysfunction during heavy work. It is also possible to study the free oxygen radical levels or to apply antioxidant substances

in these three fatigue models, and this will probably make the role of free-oxygen damage in the mechanisms of diaphragmatic fatigue more clear.

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