Increased compliance in diaphragm muscle of the cardiomyopathic Syrian hamster

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Coirault, Catherine, Jane-Lyse Samuel, Denis Chemla, Jean-Claude Pourny, Francine Lambert, Francoise Marotte, and Yves Lecarpentier. Increased compliance in diaphragm muscle of the cardiomyopathic Syrian hamster. J. Appl. Physiol. 85(5): 1762–1769, 1998.—We investigated the hypothesis that diaphragm compliance was abnormal in cardiomyopathic Syrian hamsters (CSH), an experimental model of myopathy. The passive elastic properties of isolated diaphragm muscles were analyzed at both the muscle and sarcomere levels. We used the following passive exponential relationship between stress ($\sigma$) and strain ($\varepsilon$): $\sigma = E_0/\beta (\varepsilon^n - 1)$, where $E_0$ is the initial elastic modulus and $\beta$ is the stiffness constant. Immunocytochemistry procedures were used to analyze the distribution of two key elastic components of muscle, extracellular collagen and intracellular titin elastic components, as well as the extracellular matrix glycoprotein laminin. Muscle and sarcomere values of $\beta$ were nearly twofold lower in CSH (8.7 ± 1.9 and 8.3 ± 1.4, respectively) than in control animals (19.7 ± 1.7 and 16.8 ± 2.1, respectively) ($P < 0.01$ for each). Compared with controls, $E_0$ was higher in CSH. Sarcomere slack length was significantly longer in CSH than in control animals (2.1 ± 0.1 vs. 1.9 ± 0.1 μm, $P < 0.05$). The surface area of collagen I was significantly larger in CSH (17.4 ± 1.8%) than in control animals (12.4 ± 0.7%, $P < 0.05$). There was no change in the distribution of titin or laminin labelings between the groups. These results demonstrate increased diaphragm compliance in cardiomyopathic hamsters. The increase in CSH diaphragm compliance was observed despite an increase in the surface area of collagen and was not associated with an abnormal distribution of titin or laminin.

Increased active force generation in the myopathic diaphragm. In diaphragm from the mdx mouse, in which degenerative changes resemble those observed in Duchenne muscular dystrophy, a marked reduction in diaphragm muscle compliance has been reported (34). It remains to be determined whether diaphragm compliance is reduced in other myopathies.

The Bio 14.6 cardiomyopathic Syrian hamster (CSH) is a highly reproducible model of progressive skeletal and cardiac muscle disease (5, 35). In CSH, diaphragm dysfunction occurs early in the course of the disease (5, 8, 23) and is associated with muscle hypertrophy and histological signs of myopathy (central nucleation, variation in fiber diameter, necrosis, and fibrosis) (5, 35). The first purpose of our study was to determine whether the passive length-tension relationship was modified in CSH diaphragm. To this end, the elastic properties of isolated costal diaphragm were investigated by analyzing the stress-strain relationships of both muscle and sarcomere over the physiological range of lengths.

The location of the morphological structures responsible for passive tension of striated muscle remains controversial. Passive resting tension has mainly been ascribed to elastic forces generated by collagen (3, 29). More recent studies have highlighted the prominent role of titin, an endosarcomeric protein, in passive tension development (18, 20, 37). Finally, in intact muscle fibers, another possible source of passive tension is the sarcolemma (27, 29). The second purpose of our study was thus to determine whether possible mechanical changes were associated with abnormal distribution of extra- and intracellular elements determining the elastic properties of isolated diaphragm muscle. The distribution of collagen I and titin were investigated by immunostaining. Finally, we also investigated the distribution of laminin, given that laminin deficiency may favor disruption of the linkage between the subsarcolemmal cytoskeleton and the surrounding extracellular matrix (12–14).

**MATERIALS AND METHODS**

**Animals**

Experiments were conducted in 20 six-month-old male Syrian hamsters (Bio 14.6 strain) and 17 age-matched, inbred control golden Syrian hamsters (F1B) obtained from Bio Breeders (Fitchburg, MA). In CSH, the myopathic process is highly reproducible, and impaired diaphragm function is consistently observed in 6-mo-old animals (5, 8, 23). One group of nine cardiomyopathic and nine control animals were...
used for mechanical analysis. Another subgroup of 11 cardiomyopathic hamsters and 8 control hamsters was used for immunolabeling and morphometry. Care of the animals conformed to the Helsinki Declaration, and the study was approved by our institution (Institut National de la Santé et de la Recherche Médicale). After brief administration of ether anesthesia, the animals were laparotomized and then thoracotomized.

**Mechanics**

Diaphragm muscle strips. A strip of the ventral costal diaphragm was carefully removed from the muscle in situ. This diaphragm strip was rapidly mounted in a tissue chamber containing Krebs-Henseleit solution composed of (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.1 KH2PO4, 24 NaHCO3, 2.5 CaCl2, and 4.5 glucose. The solution was maintained at 22°C and equilibrated with a 95% O2-5% CO2 gas mixture, giving a pH of 7.4. The costal extremitiy of the muscle preparation was held by a stationary clip at the bottom of the bath, and the extremity of the central tendon was held by a spring clip attached to an electromagnetic lever system (9, 23). The initial muscle length (L0), corresponding to the length at which twitch active isometric tension is maximum, was determined as follows: muscle strips were electrically stimulated in twitch mode by means of two platinum electrodes delivering 1-ms rectangular pulses at a frequency of 10 pulses/min. Three to four preloads (corresponding to 3–4 initial muscle lengths) were applied to the muscle by means of loading steps. At each initial muscle length, twitch active isometric tension was recorded. Preload is the resting tension that stretches the muscle before electrical stimulation. The cross-sectional area (in mm2) at L0 was calculated from the ratio of muscle weight to muscle length at L0, assuming a muscle density of 1 g/cm3. Characteristics of the studied muscles strips were as follows: cross-sectional area of 1.3 ± 0.1 and 2.4 ± 0.3 mm2 and L0 of 13.4 ± 0.5 and 9.9 ± 0.9 mm in control and myopathic diaphragms, respectively.

Optical technique. The laser diffractrometer used to analyze sarcomere length has been described elsewhere (10). The diaphragm muscle strip was transilluminated with a 5-mW helium-neon laser (laser beam width = 1 mm). Initial sarcomere length at L0 (SL0) was calculated from the relationship θ0 = arc sin l/SL0, where θ0 is the angular separation of the first-order diffraction line at rest relative to the zero-order reference line, and l is the wavelength of the laser beam (0.6328 μm). The precision of sarcomere length measurements corresponded to a spatial resolution of 150–200 Å (10). SL0 were 2.2 ± 0.1 and 2.2 ± 0.1 (SE) μm in control and myopathic diaphragms, respectively.

Muscle and sarcomere stress-strain relationships. Different preloads were successively applied to the muscle (corresponding to different resting sarcomere and muscle lengths) by means of load steps. Mechanical parameters (i.e., muscle and sarcomere lengths and resting tension) were recorded 15 min after each preload change, so as to permit the “creep” phenomenon to occur. The same protocol was used for normal and CSH diaphragms. The elastic properties of the diaphragm were computed by using the exponential relationship between stress (σ) and strain (ε) (7, 18, 24, 26, 36) (Fig. 1)

\[ \sigma = \frac{E_0}{\beta} \left( e^{\beta \varepsilon} - 1 \right) \]

in which σ = stress (resting force/A0), where A0 is cross-sectional area at initial muscle length Li; E0 is the initial elastic modulus of muscle (E0,mus) or sarcomere (E0,s); β is the stiffness constant of muscle (βmus) and sarcomere (βs); and ε is eulerian muscle and sarcomere strains (εmus and εs, respectively). Muscle and sarcomere strains are equal to (Li – Lmin)/Lmin and (SLi – SLmin)/SLmin, respectively, where Lmin is slack muscle length (where σ and εmus equal 0), SLi is resting sarcomere length, and SLmin is slack sarcomere length (where σ and εs equal 0). Eulerian strain was particularly applicable in our study because muscle undergoes deformations >0.2% over the range of load (26). For this reason, strain-stress relationships were analyzed on the basis of instantaneous cross-sectional area, which was calculated at each preload studied, assuming incompressibility of the muscle. Computed-assisted calculations of E0, β, and slack lengths were done in both groups (7). Data were fitted to a personal computer by using the TransERA HTBasic Advanced Math Library (TransERA, Provo, UT). When the stress-strain curve is exponential, the elastic tangent modulus dσ/dε varies linearly according to the equation dσ/dε = βσ + E0 (24, 26) (Fig. 1). The coefficient of correlation r was used to test the accuracy between experimental results and the model estimate.
Antibodies

Collagen immunolabeling was performed by using goat polyclonal antibodies directed against collagen I (Pasteur). Titin immunolabeling was performed by using mouse monoclonal antibodies T11 (Sigma Chemical) directed against the elastic domain of titin near the A-I junction. Rabbit polyclonal antibodies directed against rat laminin (Chemicon International, Temecula, CA) were also used. Anti-mouse Ig conjugated to FITC and anti-rabbit Ig conjugated to FITC were from Amersham. Anti-goat Ig conjugated to Cy 3 was from Sigma Chemical. The specificity of anti-titin and anti-laminin antibodies has been described elsewhere (12, 16).

Immunolabelings

Tissues from left ventral costal hemidiaphragm were snap-frozen in isopentane precooled in liquid nitrogen and stored at -80°C until use. Diaphragm longitudinal cryosections (5 µm thick) were treated with acetone for 20 min at -20°C and air dried before antibody staining. All sections were preincubated for 20 min in PBS (pH 7.2) containing 5% albumin. Serial sections were incubated overnight at 4°C with anti-titin (diluted 1:10 in PBS containing 2% albumin), anti-collagen I (diluted 1:100 in PBS containing 2% albumin), or anti-laminin (diluted 1:100 in PBS containing 2% albumin) antibodies. After washing, sections were incubated with FITC-labeled anti-mouse IgG antibodies (1:10 dilution in PBS containing 5% hamster serum), anti-goat Ig antibodies combined with Cy 3 (1:100 dilution in PBS containing 5% hamster serum), or anti-rabbit Ig antibodies combined with FITC fluorochrome (1:30 dilution in PBS containing 5% hamster serum). Usual control sections included those incubated in the absence of primary antibodies and showed only very low background staining. Appropriate dilutions of each specific antibody were determined in preliminary experiments. Sections were mounted in aqueous medium (Fluoroprep, Biomereux). Fluorescence was visualized under a Leica microscope equipped with epifluorescence optics. All sections were scored by independent observers.

Morphometry

Collagen surface area and fiber diameter were assessed by using a computer-assisted procedure. Sections stained with anti-collagen I were placed in a microscope (magnification, ×20) and blindly studied by a single examiner. The image was calibrated by using the microscopic scale. Each field sent to the image analyzer was transmitted by a video camera connected to the microscope and digitized on a Macintosh II fx. Collagen was quantified by using image-analysis software (Optilab, Grafter). For each section, three to four fields were randomly selected. Segments representing connective tissue and muscle fibers were determined, and the computer was programmed to calculate and sum areas within the stained regions. In myopathic diaphragm, regions with extensive connective tissue and muscle fibers were determined, and the computer was programmed to calculate and sum areas within the stained regions. In control and myopathic diaphragms, the monoexponential relationship between stress and strain was verified at both the muscle and sarcomere levels. In each muscle, the elastic modulus (dr/dεmus) was linearly related to stress (σ) according to the equation dr/dεmus = βmus + Eo, mus, where βmus is the muscle stiffness constant and Eo, mus is the muscle initial elastic modulus (i.e., Eo, is the y-intercept when σ = 0). When the correlation coefficient r indicated a tight clustering of data points around the calculated regression lines (Table 1), the same general behavior was observed at the sarcomere level (Table 1). At both the muscle and sarcomere levels, the stiffness constant β was significantly lower in myopathic hamsters than in control animals (i.e., stiffness was lower) (Table 1). Given that compliance is the reciprocal of stiffness, these results indicated that compliance was higher in myopathic than in control diaphragms. Eo, mus was higher in myopathic than in control diaphragms.

Table 1. Characteristics of muscle and sarcomere stress-strain relationships in the diaphragm of control and myopathic hamsters

<table>
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<th>Muscle</th>
<th>Sarcomere</th>
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<tr>
<td></td>
<td>Control</td>
<td>Myopathic</td>
</tr>
<tr>
<td>β</td>
<td>19.7±1.7</td>
<td>8.7±1.9*</td>
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<tr>
<td>Eo, mus, mN/mm²</td>
<td>10.4±1.9</td>
<td>77.5±23.4*</td>
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<tr>
<td>r</td>
<td>0.997±0.001</td>
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Values are means ± SE; n = 9 animals/group. r, Correlation coefficient. Different preloads were successively applied to muscle corresponding to different resting sarcomere and muscle lengths (Smin and Lmin, respectively). Stress-strain relationships were studied following the equation σ = Eo, βe (e² - 1), where σ is stress; Eo is initial elastic modulus of muscle (Eo, mus) or sarcomere (Eo, s); βe is the stiffness constant of muscle (βmus) and sarcomere (βs), and e is eulerian muscle and sarcomere strains (εmus and εs, respectively). Muscle and sarcomere strains are equal to (L - Lmin)/Lmin and (S - Smin)/Smin, respectively, where Lmin is muscle slack length (i.e., resting muscle length at which σ and εmus equal 0), Smin is sarcomere slack length, (i.e., resting sarcomere length at which σ and εs equal 0). When the stress-strain relationship is exponential, the elastic modulus constant βe vs. stress relationship is linear, i.e., dr/dε = βe + Eo. To test the accuracy of the linear fit between dr/dε and σ, r was used. *P < 0.01 vs. control.

Statistical Analysis

Data are expressed as means ± SE. Fiber diameters and collagen percentage areas were averaged for each section. After ANOVA, comparisons of mechanical parameters, mean values of fiber diameters, and collagen percentage areas between groups were performed by using Student’s unpaired t-test. All comparisons were two-tailed, and P < 0.05 was considered statistically significant.
in myopathic than in control animals (P < 0.01) (Table 1). Sarcomere slack length was significantly longer in myopathic than in control diaphragms (2.1 ± 0.1 vs. 1.9 ± 0.1 µm, respectively, P < 0.05).

Immunolabelings

Collagen I antibodies were detected in the intercellular space surrounding muscle fibers (Fig. 2). Compared with control diaphragms, the collagen I surface area was larger in myopathic animals (17.4 ± 1.8 vs. 12.4 ± 0.7%, P < 0.05). Monoclonal anti-titin T11 yielded striated labeling (Fig. 3). There was no change in the distribution of labeling with anti-titin between control and myopathic animals. Laminin antibodies outlined the basement membrane of muscle fibers (Fig. 4). No changes in labeling distribution with anti-laminin were detected between the groups.

Fiber Diameters

Histograms of fiber diameter in control and myopathic diaphragms are presented in Fig. 5. Both groups had a population within the peak range of between 25 and 35 µm, whereas myopathic diaphragms contained smaller-diameter fibers. Mean fiber diameters were significantly lower in myopathic than in control animals (27 ± 1 vs. 33 ± 2 µm, P < 0.05).

DISCUSSION

These results demonstrate that compliance was higher in cardiomyopathic hamster diaphragm than in control. Modifications in elastic properties were associated with an increased collagen I percentage area and decreased muscle fiber thickness in myopathic diaphragm, whereas both titin and laminin immunostain-
ings were unchanged. This increased compliance, despite moderate but statistically significant increase in collagen percentage area, is consistent with recent studies showing that ultrastructures other than collagen are involved in muscle compliance (24). Possible elastic elements included titin, sarcolemma, and/or laminin.

In our study, the passive elastic properties of diaphragm muscle were expressed in terms of the stress-strain relationship, as previously recommended in other striated muscles (7, 18, 24, 26, 37). Indeed, analysis of stress (σ, force per unit area) and strain (ε, change in length per unit length) has been shown to quantify precisely the passive elastic properties of isolated striated muscles (24, 26). In both control and myopathic diaphragm muscles, we found that stress increased exponentially with strain, as observed in many biological materials (15). This made it possible to express the results in the particularly simple form $d\sigma/d\varepsilon = \beta \sigma + E_0$, where $d\sigma/d\varepsilon$ is the elastic tangent modulus and $\beta$ is the elastic stiffness constant (24, 26). Although both $\beta$ and $E_0$ contribute to the passive elastic properties, the slope $\beta$ of this linear relationship has been proposed as a valuable index to quantify tissue compliance; the lower the value of $\beta$, the greater the tissue compliance. We found that the passive mechanical characteristics of diaphragm muscle and sarcomere differed significantly between myopathic and control hamsters. At the lowest strains, the passive stress of the CSH diaphragms was either higher than or not different from that of control diaphragms (Fig. 1A). However, over the whole range of strain, the increase in passive stress was lower in CSH than in control diaphragms, as shown by the lower stiffness constants $\beta_{\text{mus}}$ and $\beta_S$ in myopathic diaphragms than in controls (Fig. 1, Table 1). This indicated that diaphragm compliance was higher in myopathic hamsters than in control animals. Thus lower tension was required to passively elongate the myopathic diaphragm. Interestingly, muscle and sarcomere values of $\beta$ were on the same order of magnitude,

Fig. 3. Immunocytochemical labeling of titin in normal (A) and myopathic (B) diaphragms. Magnification, ×40.
suggesting that the main source of passive tension was the same in the whole muscle and at the level of the sarcomere (24). The reported value for $SL_0$ in hamster diaphragm muscle has been previously discussed (11). Given that, in vertebrate skeletal muscle, the length of thick myosin filament is 1.6 µm and that of thin filament is 1.0–1.2 µm, the maximal actin-myosin overlap occurs at optimal sarcomere lengths ranging from 2.2 to 2.4 µm (11).

Our results contrast sharply with what has been found in mdx mouse diaphragm, a muscle in which degenerative changes resemble those in skeletal muscles in patients with Duchenne muscular dystrophy (34). Indeed, over a physiological range of muscle length, muscle compliance is markedly lower in mdx than in control diaphragms (34). Thus changes in diaphragm elastic properties seem to depend on the myopathic animal model considered, tissue compliance being reduced in mdx mice (34) and increased in myopathic hamsters (present study). In the mdx diaphragm, compliance has not been analyzed at the sarcomere level (34). Consequently, it was not possible to compare the previous results with ours obtained at a similar sarco-
mure level. In both studies, however, passive stress was investigated over a similar range of muscle strain (±7% of L₀ in mdx mice vs. ±10% of L₀ in our study). Moreover, in physiological conditions, the stiffness constant is independent of initial muscle length. Thus differences in muscle compliance cannot be attributed to differences in the range of initial muscle lengths investigated. In addition, both myopathic hamster and mdx mouse diaphragms exhibit a pattern of muscle degeneration (including wide variations in fiber diameter, necrosis, and fibrosis) and contractile dysfunction (5, 8, 23, 34). Quantitative and/or qualitative differences in histological lesions might contribute to the different changes in compliance between mdx and CSH diaphragms. Nevertheless, the increased compliance observed in CSH diaphragm may have detrimental effects by favoring overstretch, thus further damaging the sarcolemma.

In intact striated muscle, the structural sources responsible for passive sarcomere length-tension properties are still under debate. Connective tissue (collagen), intrasarcomeric elastic elements (titin), and/or sarcolemma have been implicated as important determinants of the stress-strain properties of muscle. Thus, to determine which potential changes among these structures might explain the increased compliance of the myopathic hamster diaphragm, the distribution and intensity of anti-collagen I, anti-titin, and anti-laminin antibodies were successively analyzed.

In viable tissue components, our data indicate that the collagen I surface area was ~50% higher in myopathic than in control muscles. Although the collagen surface area does not directly quantify the amount of collagen, there is a good correlation between collagen measurements obtained by morphometric analysis and those obtained by biochemical techniques (28, 32). Moreover, our results are in entire agreement with the increased collagen content (as estimated from hydroxyproline measurements) previously reported in the diaphragm of the myopathic Syrian hamster (1). Last, fibrosis and increased collagen content are general features of muscle diseases with degenerative and regenerative processes (1, 5, 34). Reduced muscle compliance has generally been attributed to collagen accumulation (3, 17, 22, 29, 34). Accordingly, in mdx mouse diaphragm (34), senescent rat diaphragm (17), and some limb skeletal muscles (2, 22), the decreased muscle compliance has been attributed to an increased collagen density. In contrast, other studies (2, 24) have not shown a correlation between the amount of collagen and the passive mechanical properties of striated muscles. In rat soleus muscle, the increased collagen content during aging is not associated with changes in muscle stiffness (2). Moreover, passive compliance is sometimes normal in the ventricles of patients with aortic stenosis (25), in which collagen content is increased. Thus several reports indicate that compliance is not necessarily associated with collagen content (2, 24, 25), as also observed in our study. This suggests that components other than collagen may contribute to the more compliant stress-strain relationship of myopathic hamster diaphragm. Possible elastic elements involved included titin, sarcolemma, and/or laminin.

Titin, a giant intrasarcomeric protein, has recently been implicated in the elastic properties of resting striated muscles (18, 20, 37). Removal and/or selective destruction of titin has been shown to increase tissue compliance (18, 20). We thus tested the hypothesis that the increased compliance observed in CSH diaphragm was related to modifications in the distribution of titin. We used the monoclonal antibody T11 directed against an elastic domain of titin near the A-I junction (16) and found that the distribution of titin antibodies did not differ between myopathic and control diaphragms (Fig. 3). This indicates that changes in titin distribution are not involved in increased diaphragm compliance in myopathic hamsters. However, because the anti-titin antibody T11 recognizes a specific epitope on the titin molecule, we cannot exclude the possibility that differences in passive length-tension relationships account for either differences in the elastic properties of titin molecules or differences in titin's slack length between myopathic and control hamsters (36).

In intact muscle fibers, another possible determinant of elastic properties resides in the sarcolemma and basal lamina (27, 29). Several muscular dystrophies have been regarded as diseases of muscle cell adhesion, caused by mutations in a number of proteins that link the cell cytoskeleton to the extracellular matrix (6, 13, 19, 30). These proteins include laminin, a noncollagenous protein of the basal lamina that forms with sarcolemmal proteins, a physical link between the subsarcolemmal cytoskeleton and the surrounding extracellular matrix (12, 14, 21). In our study, however, the distribution of laminin did not differ between control and myopathic diaphragms.

Alternatively, one possibility was that the myopathic diaphragm could be more compliant on the basis of less viable tissue per unit cross-sectional area. Indeed, it has been shown that myofibrils bear most of the resting tension in resting muscle (24). In addition, the area occupied by muscle fiber is significantly lower in 6-mo-old cardiomyopathic Syrian hamsters compared with control animals (5). The quantity of myofibrils is thus expected to be lower in myopathic than in control diaphragms, and this might account for the increased compliance observed in myopathic muscles.

In conclusion, we observed a significant increase in diaphragm compliance in cardiomyopathic Syrian hamsters. Increased muscle elasticity was observed despite a significant increase in the extracellular collagen surface area, and this was not associated with abnormal distribution of an intrasarcomeric elastic element, titin, or that of an extrasarcomeric elastic element, laminin.

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