Passive mechanics of muscle tendinous junction of canine diaphragm

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Hwang, Willy, Neil G. Kelly, and Aladin M. Boriek. Passive mechanics of muscle tendinous junction of canine diaphragm. J Appl Physiol 98: 1328–1333, 2005; doi:10.1152/japplphysiol.00816.2004.—The diaphragmatic muscle tendon is a biaxially loaded junction in vivo. Stress-strain relations along and transverse to the fiber directions are important in understanding its mechanical properties. We hypothesized that 1) the central tendon possesses greater passive stiffness than adjacent muscle, 2) the diaphragm muscle is anisotropic, whereas the central tendon near the junction is essentially isotropic, and 3) a gradient in passive stiffness exists as one approaches the muscletendinous junction (MTJ). To investigate these hypotheses, we conducted uniaxial and biaxial mechanical loading on samples of the MTJ excised from the midcostal region of dog diaphragm. We measured passive length-tension relationships of the muscle, tendon, and MTJ in the direction along the muscle fibers as well as transverse to the fibers. The MTJ was slack in the unloaded state, resulting in a J-shaped passive tension-strain curve. Generally, muscle strain was greater than that of MTJ, which was greater than tendon strain. In the muscular region, stiffness in the direction transverse to the fibers is much greater than that along the fibers. The central tendon is essentially inextensible in the direction transverse to the fibers as well as along the fibers. Our data demonstrate the existence of more pronounced anisotropy in the muscle than in the tendon near the junction. Furthermore, a gradient in muscle stiffness exists as one approaches the MTJ, consistent with the hypothesis of continuous passive stiffness across the MTJ.

The muscle-tendinous junction (MTJ) is a physiologically vital tissue interface. Muscle force is transmitted between the muscle and tendon through the MTJ. Previous MTJ analyses and interpretations considered force applied to the MTJ as parallel to the myofibril direction (11). In vivo, the diaphragm, and therefore the diaphragmatic MTJ, is subjected to complex states of loading not seen with other MTJ of uniaxially loaded skeletal muscles. The effect of biaxial loading on the deformation of the diaphragmatic MTJ remains uncharacterized, because the diaphragmatic MTJ, unlike other skeletal muscle MTJ, is biaxially loaded in vivo. Therefore, the length-tension relationships of the MTJ should be measured not only along the fibers (AF) but also transverse to the fiber direction (TF). Furthermore, comparison of the MTJ loaded biaxially with one loaded uniaxially along the direction of the muscle fibers can help identify structural specialization of force-transmitting junction that experiences more complex loading patterns. Tidball (15) demonstrated the importance of morphological and molecular aspects of force transmission across cell membranes in their elegant paper, but correlation of mechanical and morphological data is necessary to fully understand force transmission at the MTJ.

Our previous study of in vivo mechanics of canine diaphragm showed substantially smaller mechanical strain in the TF compared with AF direction (8). Furthermore, our in vitro mechanics studies of the diaphragm have demonstrated that the muscle of the diaphragm is anisotropic with less muscle compliance in TF than AF (1, 2, 6). Our finite-element membrane model of the passive diaphragm demonstrated that an inextensible cap simulating the central tendon and anisotropic skirt simulating the muscular portion of the diaphragm would change curvature less than a uniform homogeneous isotropic elastic material would when inflated (5). This study suggested but did not prove that both the central tendon inextensibility and muscle anisotropy contribute to restricting changes of diaphragm shape observed in vivo during physiological loading (3, 7).

In this study, we investigated the mechanics of the MTJ of the canine diaphragm in vitro during uniaxial and biaxial mechanical loading conditions. We hypothesized that 1) the central tendon possesses greater passive stiffness than adjacent diaphragmatic muscle, 2) the diaphragm muscle is anisotropic, whereas the central tendon near the MTJ is essentially isotropic, and 3) a gradient in passive stiffness exists as one approaches the MTJ. To test these hypotheses, we measured uniaxial and biaxial passive length-tension relationships of canine diaphragmatic muscle-tendon strips AF and TF. We found that the MTJ sheet is more compliant and considerably more extensible AF than TF. Furthermore, the central tendon was considerably stiffer than adjacent muscle. In addition, the central tendon near the MTJ was essentially isotropic, whereas the adjacent muscle was anisotropic. We also found that passive stiffness increased gradually as one approaches the MTJ.

METHODS

Diaphragmatic muscle strip preparation. Dogs were maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and procedures were approved in advance by the Institutional Review Board of Baylor College of Medicine. Fourteen mongrel dogs (22.4 ± 5.7 kg) were anesthetized with intravenous injections of pentobarbital sodium (30 mg/kg body wt). The left hemidiaphragm was excised from each animal and immediately immersed in a muscle bath containing modified Krebs-Ringer solution (in mM: 137 NaCl, 5 KCl, 1 NaH2PO4, 24 NaHCO3, 2 CaCl2·2H2O, 1 MgSO4·7H2O, pH 7.4) bubbled with 95% O2-5% CO2 and maintained at room temperature throughout the preparation and experimental phases of this study (10). Animals were euthanized using an overdose of pentobarbital. A 5 × 5 cm portion of muscle tendon was carefully dissected near the midcostal region of the left hemidiaphragm. A muscle-tendon strip was cut along the predominant direction of muscle fiber orientation to minimize damage to the tissue. Overhand knotted black sutures (size 3-0 round-tip surgical needle)
were sewn on the thoracic diaphragmatic surface with surgical knot on the opposing abdominal surface to secure each marker in place. These position markers quantified tissue displacement during tissue elongation. To test our first and second hypotheses, we placed eight position markers in a rectangular configuration (~1 cm apart) perpendicular to the axis of the MTJ. To test our third hypothesis, we used a second protocol, whereas the number of position markers was increased to 12. In these configurations depicted in Fig. 1, two rows of markers were aligned AF and multiple pairs were aligned TF, thereby allowing assessment of displacements AF as well as TF. Importantly, all markers were positioned in the central region of the tissue to minimize the edge effects of applied load as described by the St. Venant’s Principle of Mechanics (11).

**Biaxial testing equipment.** The passive elongation of muscle tissue was executed using biaxial testing apparatus described in detail previously (2). Briefly, the apparatus consists of stepper motors aligned directly opposite to force transducers (Grass model FT10) with the two motor-transducer pairs at right angles to each other. A muscle bath was centrally positioned and contained ~1 liter of bubbled Ringer solution, which was continuously circulated over the muscle preparation by an external peristaltic pump. A video camera was located above the bath, and at the end of each experiment a scale was filmed to calibrate the distance of the camera from the muscle. Force distribution carriages with inextensible attachment lines were hooked −0.5 cm apart to all four sides of the muscle strip. Inextensible cable lines from the force distribution carriages were connected to either a stepper motor or a force transducer. The cable line to a transducer was looped back to a motor by means of a nylon pulley, thus allowing a simultaneous tissue displacement on both sides along a given axis. Cable lines from opposite sides of the tissue (i.e., transducer and stepper motor sides) were wrapped around and secured to separate grooved spools connected to the stepper motor shaft. Stretch and release of the tissue occurred by means of clockwise winding and counterclockwise unwinding movements (respectively) of the cable line spools during stepper motor operation. Along each of two orthogonal axes, separately controlled stepper motors with opposing force transducers allowed either uniaxial or biaxial test measurements (tension and muscle length) of passively stretched muscle tendon. Briefly, the length-tension curves were measured by either loading the tissue uniaxially AF, uniaxially TF, or biaxially, i.e., both AF and TF. Although each sample was subjected to all three tissue-stretching protocols, the sequence of these stretching protocols was randomized to avoid possible artifact due to the order of the mechanical test. Before each tissue-stretching protocol, the MTJ was preconditioned by stretching the tissue by 200-g force simultaneously AF and TF and then releasing the tissue to the unstressed length. The process of preconditioning was repeated three times. The same strain rate (1.5 mm/s) was used in both axes of stretch and during all stretch release procedures. This magnitude of strain rate is small enough to simulate quasistatic physiological testing. The force data were simultaneously recorded at a sampling rate of 5 Hz for both axes using a data acquisition platform consisting of an analog-to-digital data acquisition board (National Instruments) and LabView software (version 3.0). Displacement of position markers was recorded on video tape and measured using a spatial assessment function created from image analysis software (Optimas 3.0), which allowed determination of two-dimensional coordinates of the markers at specific time points during passive lengthening and passive shortening.

**Computation of two-dimensional strains.** The strains in the plane of the muscle, MTJ, and central tendon were computed by the following procedure. The marked region is divided into triangles with markers forming the apices. Subsequently, the four sutured markers would define four triangles. For example, triangle A is defined by markers 1–3, whereas triangle B would be defined by markers 1, 2, and 4. The coordinates of these points in that unstressed plane of the diaphragm are denoted $x_i$ and $y_i$ ($i = 1, 2$). The displacement, $u_i$, from the unstressed state to the deformed state is assumed to be a linear function of position and computed as follows:

$$u_i = a_1 + a_2 x_i + a_3 y_i.$$  

This equation with known values of the displacements and the position of three markers substituted for $u_i$, $x_i$, and $y_i$ provided a set of three equations for the three coefficients: $a_1$, $a_2$, and $a_3$. Similarly, the data provided the information required for determining the coefficients $(a_4, a_5, a_6)$ in the following equation:

$$v_i = a_4 + a_5 x_i + a_6 y_i.$$  

The values of the coefficients $a_1$, $a_2$, etc. were used to find the partial derivatives, which were substituted into the following equations:

$$\varepsilon_x = \frac{\delta u}{\delta x}$$

$$\varepsilon_y = \frac{\delta v}{\delta y}$$

$$\varepsilon_{xy} = \frac{\delta u}{\delta y} + \frac{\delta v}{\delta x}$$

where $\delta u$ and $\delta v$ denote the marker’s displacement in the $x$ (AF) and $y$ (TF) directions, defined to calculate strains. The strains $\varepsilon_x$, $\varepsilon_y$, and $\varepsilon_{xy}$ were computed for each triangle. $\varepsilon_x$ is strain in the AF direction, whereas $\varepsilon_y$ is strain in the TF direction, and $\varepsilon_{xy}$ is shear strain.

$$\lambda = 1 + \varepsilon$$

where $\lambda$ is the extension ratio or the ratio of muscle length at a stretched state relative to the unstressed length. The $\varepsilon$ is mechanical strain either in AF or TF. $\varepsilon$ is computed in the tendon, MTJ, and muscle.

**Histological methods.** Three adult mongrel dogs, weighing from 20 to 24 kg each, were killed by intravenous pentobarbital sodium. The diaphragm and ribs of insertion were removed together and placed in a physiological saline solution. We excised portions of the MTJ from the midcostal region of the left hemidiaphragms. Strips ~2 in. wide

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were cut along the muscle fascicles from near the MTJ and through the tendon at their relaxed (unstressed) length. These strips were pinned along their edges to a sheet of cork and placed overnight in a 5% formaldehyde solution. Care was taken to cut the samples ~1 cm wide and 1.5 cm from the area where the muscles were pinned. These samples were placed in histological cassettes, cut into longitudinal sections, and stained using a trichrome staining technique. This technique allowed intracellular structures of different architecture and composition to be visibly varied: the muscle fibers and connective tissue of our samples stained red and blue, respectively.

RESULTS

In the first protocol, eight position markers were placed in a rectangular configuration (~1 cm apart) perpendicular to the axis of the MTJ muscle-tendon interface and defined strain in the muscle, MTJ, and tendon. A representative set of data in Fig. 2 shows the length-tension relationship of muscle tendon uniaxially loaded AF and demonstrates muscle strain > MTJ strain > tendon strain. For example, at tension of 200 g/cm, the strain in these regions was 50, 15, and 5%, respectively. We computed the extension ratio or muscle length as a fraction of unstressed length at tension of 200 g/cm AF and found that the extension ratio to be 1.50, 1.15, and 1.05 for the muscle, MTJ, and tendon, respectively. A representative set of data in Fig. 3 shows the length-tension relationship of biaxially loaded dia-phragm and also demonstrates muscle strain > MTJ strain > tendon strain. At tension of 200 g/cm, mechanical strains in these regions were 55, 25, and 5%, respectively. The extension ratio at tension of 200 g/cm AF was found to be 1.55, 1.25, and 1.05 for the muscle, MTJ, and tendon, respectively. At low tension, the muscle is more compliant during uniaxial loading (Fig. 2) than during biaxial loading (Fig. 3). For example, at tension of 100 g/cm, the muscle region near the MTJ is passively stretched by ~50% during uniaxial stretch, whereas the muscle region is stretched to only ~40% at the same level of applied tension. The MTJ however, appears to exhibit similar stress-strain relationships during uniaxial and biaxial loading. For example, at tension of 100 g/cm, the MTJ is stretched to ~14% during either uniaxial or biaxial loading. Surprisingly, the tendon near the MTJ exhibits greater strain in response to biaxial loading than in response to uniaxial loading.

In the second protocol, 12 position markers were placed in a rectangular configuration (~1 cm apart) perpendicular to the axis of the MTJ muscle-tendon interface and defined three regions in the muscle, one region at the MTJ, and one region at the tendon. A representative set of data in Fig. 4 shows a representative set of data from uniaxially loaded muscle tendon strips. During lengthening, there is a slow and continuous increase in tension over the range of imposed strains. Muscle extensibility in the direction of the muscle fibers ranges from ~33% at the interface with the tendon to ~77% at ~1.5 cm away from the junction. For all five regions, extension ratios were computed at a tension of 200 g/cm in the AF (extension ratio 1 = 1.1, extension ratio 2 = 1.35, extension ratio 3 = 1.65, extension ratio 4 = 1.7, extension ratio 5 = 1.75). The central tendon demonstrated the lowest extension ratio of all regions. Compliance ratios in the AF calculated for comparison of adjacent regions demonstrate a gradient of progressively increasing stiffness in the transition from muscle to central tendon. At a tension of 200 g/cm, the compliance ratios in the AF were as follows: 0.97 between regions 4 and 5, 0.97 between regions 3 and 4, 0.82 between regions 2 and 3, and 0.81 between regions 1 and 2. It appears that the diaphragmatic muscle has a gradient in stiffness near the MTJ with the greatest muscle stiffness at the junction. The tendon is relatively inextensible in the direction of the muscle fibers.
Tendon near the MTJ is essentially isotropic, and the diaphragm muscle is anisotropic, whereas the central greater passive stiffness than adjacent diaphragmatic muscle, increased at a strain of imposed. However, tension transverse to the muscle abruptly increased continuously over the range of strains that were instantaneous stretch AF and TF. Tension along the muscle fibers uniaxial stretch in the muscle fiber direction and during simultaneous stretch AF and TF (Fig. 5) during diaphragm we measured the length-tension relationship of numbers on graph correspond to labeled regions of muscle-tendon strip.

To confirm the anisotropic behavior of the muscle of the diaphragm we measured the length-tension relationship of costal muscle sheet in the directions AF and TF (Fig. 5) during uniaxial stretch in the muscle fiber direction and during simultaneous stretch AF and TF. Tension along the muscle fibers increased continuously over the range of strains that were imposed. However, tension transverse to the muscle abruptly increased at a strain of ~0.32 and 0.25 during uniaxial and biaxial stretch, respectively. During passive lengthening in the direction transverse to the muscle, there is a very compliant region with transition to a stop of almost infinite stiffness. This stop occurs at a lower extension during biaxial stretch and at a stress that approximates that at FRC in supine anesthetized dogs.

These data support the MTJ in Fig. 3 and confirm that the diaphragm muscle is anisotropic with greater stiffness TF than AF.

**DISCUSSION**

Our data demonstrated that 1) the central tendon possesses greater passive stiffness than adjacent diaphragmatic muscle, 2) the diaphragm muscle is anisotropic, whereas the central tendon near the MTJ is essentially isotropic, and 3) a gradient in passive stiffness exists as one approaches the MTJ. Furthermore, our data show that the diaphragm is stiffer during biaxial than during uniaxial loading. Nonlinear length-tension relationships exist both in the AF and in the TF. In the AF, the diaphragm becomes progressively stiffer with increasing extension ratio (the ratio of stressed muscle length to unstressed length). In the TF, the diaphragm becomes inextensible at a relatively low extension ratio.

It is important to note that the data on the muscle length-tension relationships in Fig. 3 are for the muscular region of the MTJ strip, whereas the data in Fig. 5 are for the central region of the muscle between the central tendon and the rib cage. At least in TF, the muscle near the MTJ exhibits less compliance compared with the central region of the diaphragm. During stretching of the diaphragm, an abrupt stop in the lengthening occurs at ~25% during biaxial loading. The corresponding stretch of the muscle region near the MTJ occurs at ~22%. This confirms a stiffness gradient near the MTJ in TF.

Central tendon possesses greater stiffness than adjacent muscle. During physiological loads, the central tendon is extremely stiff relative to muscular stiffness along the direction of muscle fibers. Our current results show an increase of costal diaphragmatic muscle stiffness near the MTJ. A sharp transition in stiffness between an inextensible tendon and elastic muscle tissue would result in a condition that could promote muscle-tendon injury at the junction under severe loads. Therefore, in the TF, and relative to the unstressed length of the tissue, one would anticipate essentially zero mechanical strain at the MTJ and almost zero mechanical strain in the muscle fibers near the central tendon. As illustrated in Fig. 6, the individual muscle fibers do not all terminate at a single distinct boundary between muscle and tendon but progress through a zone of transition with gradually increasing proportions of connective tissue. As the muscle transitions into tendon, the increasing proportion of connective tissue establishes a stiffness gradient, thus preventing muscle-tendon injury at the MTJ. The connective tissue near the MTJ is slack in unloaded muscle; therefore, the length-tension relations are a J-shaped curve. Presumably, during muscle contraction in vivo, the low modulus region of the MTJ stress-strain curve could prevent muscle injury at the MTJ during muscle contraction. During contraction, the muscle exerts force along its longitudinal axes. If the diaphragm were extensible in the TF at the MTJ, then as
transdiaphragmatic pressure and stress in both directions increase during muscle contraction, the diaphragm would tend to expand in the TF as it contracts along the myofiber direction, and this would create stress concentrations at the MTJ due to tendon inextensibility. The high transverse stiffness of the muscle at the MTJ and inextensibility of the central tendon demonstrated in our data combine a mechanism to eliminate stress concentrations at the muscle-tendon interface, and this mechanism may play an important role in preventing muscle-tendon injury.

**Anisotropic muscle vs. isotropic central tendon at the MTJ.**

If one considers the diaphragm to behave mechanically as an isotropic elastic membrane, i.e., a membrane with elastic properties independent of direction of loading with uniform tension, then a reduction of tension should cause uniform strain. More specifically, during passive inflation when transdiaphragmatic pressure decreases, the diaphragm would contract uniformly and principal strains in the plane of the diaphragm would be identical. Our previous results have shown that, relative to FRC, mechanical strains of the diaphragm muscle in TF are substantially smaller than strains in the AF (8). This is consistent with anisotropic properties of the diaphragmatic muscle. However, this low strain may result from either high stiffness or low tension in the TF. Our pressurized finite-element membrane model of the passive diaphragm with a hemispherical shape shows that anisotropic material properties limit the repertoire of shapes available to the diaphragm regardless of pressure distribution within the physiological range (5). The results of our diaphragm model demonstrate that the anisotropic muscular portion of the diaphragm changes curvature less than a uniform homogeneous isotropic elastic material would when inflated and is therefore crucial in restricting changes of diaphragm shape. Our data support the inextensibility of the central tendon and anisotropy of the muscle in our model (5).

Although Chuong et al. demonstrated anisotropy of the central tendon canine diaphragm, they did not specifically investigate the properties of the MTJ (9). Analysis of data from current passive mechanical responses to the various uniaxial and biaxial mechanical loading conditions demonstrates that MTJ behaves as a nonlinear anisotropic tissue with a greater stiffness in the TF than in the AF. Furthermore, the muscle-tendon strip exhibits a nonlinear passive length-tension relationship along the direction of the muscle fibers and becomes progressively stiffer with increasing extension from the unstressed length. As noted by Smith and Loring (14), this nonlinearity is reflected in disproportionate increases in transdiaphragmatic pressure with myofiber elongation at low lung volumes. They reasoned that such nonlinear elastic behavior is presumably related to recruitment phenomena: as extension increases, unstressed fibrous elements at low extension ratio are progressively recruited, contributing their elastic stiffness in parallel at high stiffness. The J-shaped curve of the diaphragmatic MTJ is also consistent with previous data on the passive stress-strain relationship of the frog semitendinosus muscle (11). Furthermore, the Lieber data also showed aponeurosis strain > bone-tendon junction > tendon strain, consistent with the corresponding data of our study. Our data from biaxially loaded muscle-tendon strips show that stiffness in the TF is greater than that in the AF, and the anisotropy is more pronounced in the muscle than in the tendon. Consistent with the results of previous studies on skeletal muscle and tendon, the results of this study specifically establish the anisotropic properties of MTJ.

**Stiffness gradient.** Our previous data have shown that the diaphragmatic muscle is relatively inextensible in the TF during physiological loading in supine dogs with average principal strains of $0.015 \pm 0.033$, $0.004 \pm 0.042$, and $0.005 \pm 0.023$ near the central tendon, midway between the insertion, and near the chest wall, respectively (8). The data in the present study demonstrate greater stiffness in the TF of the costal diaphragmatic muscle near the MTJ, and such a large stiffness may result from an increase in the fraction of connective tissue to muscle fibers in that region. As the muscle transitions into tendon near the MTJ, the stiffness increases until it approaches that of the central tendon. This is consistent with the finding of Scott and Loeb (13) in the distal aponeurosis. One would expect a continuity of transverse stiffness across the interface of the MTJ. The relative inextensibility of the costal diaphragmatic muscle in the TF should not cause a discontinuity in the microstress across the interface between the muscle and the tendon, at least in the TF. Our gross anatomic data have shown an average thickness of 0.256 ± 0.037 cm for the dog midcostal costal diaphragm and a thickness of 0.03 ± 0.004 cm for the central tendon (4). The costal diaphragmatic muscle therefore tapers near the MTJ to provide a mechanism for reducing stress concentration that may be generated at the MTJ during physiological or high-resistive mechanical loading. Given the tapering of muscle near the MTJ, one would expect continuous microstresses across the MTJ. This is supported by our data that demonstrate the existence of a stiffness gradient in the transition from muscle to central tendon.

**Challenges of in vitro material testing.** Nielsen et al. (12) identified a number of difficulties with in vitro biaxial testing of biological tissue. Among them are 1) the complexity in loading the tissue with hooks, sutures, or support needles, 2) the nonuniform distribution of stress and strain throughout the tissue due to the application of point forces along the edges of the tissue, and 3) the difficulty in creating physiological strain rates and loading conditions. All of these limitations affect the accuracy of in vitro stress-strain analyses. Our biaxial test method is modeled after similar devices with modifications...
intended to circumvent these problems as best as possible. Biaxial loading by hooks and sutures, as opposed to support needles, reduces any undesirable constraints imposed on the tissue (12). The Saint-Venant’s Principle of Mechanics states that two different distributions of force acting on the same portion of a body have essentially the same effects on parts of the body that are sufficiently far from the region of application, provided that these force distributions have the same result. We designed our experiments such that mechanical strains were measured in a small, central region of the tissue, and that renders edge effects negligible (10, 12, 16).

In summary, our study is the first to provide data on physiologically relevant mechanical loading of the diaphragmatic MTJ. Our finding that the central tendon is essentially inextensible in the AF and TF directions is consistent with a mechanism to eliminate stress concentrations at the muscle-tendon interface. Furthermore, this finding supports the possibility that muscle energy expended by fiber shortening during muscle contraction will be utilized in displacing the diaphragm rather than being wasted in deforming the central tendon. In addition, our finding that the diaphragm muscle is anisotropic, whereas the central tendon near the junction is essentially isotropic, provides insight to the understanding of the mechanical behavior of this specialized junction under complex loading conditions. Finally, our finding that there is gradient in passive stiffness that exists AF and TF as one approaches the MTJ provides supporting data that stiffness continuity from the muscle continues through the tendon across the MTJ, a requirement that is fundamental to prevent muscle injury at the MTJ of the diaphragm, possibly during forceful breathing maneuvers.

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