Diaphragm remodeling and compensatory respiratory mechanics in a canine model of Duchenne muscular dystrophy

A. F. Mead,1 M. Petrov,1 A. S. Malik,1 M. A. Mitchell,1 M. K. Childers,2 J. R. Bogan,3 G. Seidner,1 J. N. Kornegay,3 and H. H. Stedman1

1Department of Surgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; 2Institute for Regenerative Medicine, School of Medicine, Wake Forest University, Winston-Salem, North Carolina; and 3Department of Pathology and Laboratory Medicine, School of Medicine, University of North Carolina, Chapel Hill, North Carolina

Submitted 16 July 2013; accepted in final form 1 January 2014

Duchenne muscular dystrophy (DMD) is a severe, childhood-onset muscle wasting disease with an approximate incidence of 1 in 3,500 boys caused by mutations in the X-linked dystrophin gene (28). As with less common childhood-onset muscular dystrophies, progressive weakness in muscles of locomotion dominates the clinical course to loss of ambulation, but the leading cause of late-stage morbidity and mortality is progressive failure of the respiratory pump (4, 21, 25). Patient-oriented studies have defined the rate of decline in standardized indices from pulmonary function tests (4, 21, 27), and studies in mdx mice have provided mechanistic information about some of the molecular and cellular correlates of muscle degeneration (9, 42). However, a reliable model of the effect of dystrophin’s absence on respiratory mechanics and loss of ventilatory capacity has been elusive. Whereas the diaphragm of the mdx mouse is its most severely affected muscle, studies of respiratory mechanics have been limited to whole-body plethysmography, limiting the extent to which the contribution of different respiratory muscle groups can be determined (29, 30). Therefore, there is a compelling need for a model of respiratory mechanics in DMD to improve our understanding of the disease process in man, and to provide a platform for therapeutic development. Golden Retriever muscular dystrophy (GRMD) is an important translational model of DMD that exhibits the major hallmarks of the human disease on a greatly accelerated time scale (33, 47), but its potential as a model of respiratory pathobiology in DMD has not been fully exploited.

To address these needs, we applied both novel and classical experimental approaches to assess the age-related loss of ventilatory capacity in GRMD and the accompanying changes in respiratory mechanics. In DMD, exertional increase in metabolic rate is limited by global weakness of the muscles of locomotion, rendering the progressive decline of ventilatory capacity early in DMD clinically silent (4). Consequently, assessment of respiratory mechanics at higher ventilatory rates has been challenging. In a canine model, we used doxapram to exploit the pharmacodynamics of direct channel activation in the sensory afferents controlling respiratory drive, thereby dissociating respiratory mechanics from metabolic loading. This allowed us to test hypotheses related to changes in the mechanics of tidal breathing in the setting of elevated respiratory drive in relatively young dogs with the GRMD mutation and with no overt clinical signs of ventilatory insufficiency.

In DMD, the diaphragm is affected earlier and more severely than other skeletal muscles. This is possibly due to heavy use as the primary driver of ventilation at rest. We used esophageal and gastric balloon manometry to test the hypothesis that doxapram stimulation would uncover deficits in transdiaphragmatic pressure (Pab) caused by loss of diaphragm contractile capacity in GRMD during tidal breathing.

The two-compartment chest wall model of respiration first established by Konno and Mead allows for the relative contribution of opposing inspiratory muscles to be estimated noninvasively by comparing volume changes in the abdomen (Vab) and rib cage (Vrc) approximated by respiratory inductance plethysmography (RIP) (11, 31). Because positive change in Vab during inspiration is caused largely by the shortening and caudal motion of the diaphragm, the diaphragm’s contribution to inspiration can be estimated by measuring ΔVab expressed as a percentage of total tidal volume (VT) during normal breathing. In DMD, clinical studies suggest that the diaphragm’s contribution to inspiration at rest progressively diminishes during the first two decades of life, with ΔVab...
that a similar shift in VRC/VAB partitioning would be observed
cles overwhelms the diaphragm (11, 23, 39). We hypothesized
wall pressure developed by the intercostal and auxiliary mus-
diaphragm weakness sometimes results in abdominal paradox:
oxygen desaturation (37, 44). In very advanced DMD, severe
vention would limit increases in Vt under stimulated conditions,
in GRMD at rest, that loss of abdominal volumetric contribu-
phragm.

under load. To test this, we developed and utilized a novel
cases, would affect the muscle’s passive mechanical properties,
morphometry of the GRMD diaphragm itself, in particular the
ventilatory insufficiency.

Simultaneous recording of compartmental volumes and
pressures can be used to assess the contribution of nondia-
phragm inspiratory and expiratory muscles. We predicted that
during periods of stimulated respiratory drive, dogs with the
GRMD mutation would rely more heavily on inspiratory and
expiratory intercostal and abdominal muscles than normal dogs
to achieve similar rates of ventilation.

Dystrophin-deficient muscles undergo progressive changes
in passive as well as active properties (49), and radiographic
and NMR studies have reported thickening of the GRMD
phragm (7, 51). We tested the hypothesis that changes to the
morphometry of the GRMD diaphragm itself, in particular the
presence of additional interstitial collagen resulting from fibro-
sis, would affect the muscle’s passive mechanical properties,
potentially impacting overall respiratory mechanics at rest and
under load. To test this, we developed and utilized a novel
system for oxygenated perfusion of the isolated hemidia-
phragm.

MATERIALS AND METHODS

Animals. This study included 34 dogs with the GRMD mutation; 4
dystrophin-expressing GRMD-carrier dogs; and 17 unaffected, nor-
mal dogs, either from the GRMD breeding colony or mongrel, ranging
from 4 to 18 mo of age. Seven dogs with the GRMD mutation and five
normal control dogs were included in doxapram studies under anes-
thesia at the time of euthanasia. All animals used in this study were
cared for and used humanely in accordance with the requirements
specified by the Institutional Animal Care and Use Committees at the
University of Pennsylvania, the University of North Carolina-Chapel
Hill, and Wake Forest University, and in accordance with the Guide
for the Care and Use of Laboratory Animals (revised 1996; National
Research Council, Washington, DC). RIP studies of awake dogs were
conducted at the Perelman School of Medicine, University of Penn-
sylvania, University of North Carolina-Chapel Hill, and Wake Forest
University. All doxapram studies were conducted at the University
of Pennsylvania. Although all dogs were a part of other research proj-
ects, no intervention was expected to affect parameters assessed in the
present study.

Respiratory inductance plethysmography. RIP traces were gener-
ated by inductance bands embedded in the Lifeshirt jacketed telemetry
system (Vivometrics) and analyzed using Vivologic software (Vivo-
metrics). Shirts from a range of sizes were fitted to dogs with the
primary aim being proper location of rib cage (RC) and abdominal
(AB) inductance bands. The RC band was located at the level of the
fourth rib, and the AB band just caudal to the rib cage. Measurements
of awake and anesthetized dogs were made in the laterally recumbent
posture. The standard method of calibrating RIP traces involves
performing an isovolume maneuver to establish the relative contribu-
tion of RC and AB signals to Vt (31). Because this method is
inapplicable to dogs, we used the quantitative diagnostic calibration
(QDC) technique first described by Sackner et al. for use in humans,
and subsequently applied to dogs (41, 42, 45). QDC compares
standard deviations of raw RC and AB signals collected over ~5
min of regular, quiet breathing to calculate a proportionality constant, K,
which relates the relative contributions of those signals to Vt. Subse-
sequently, Vt as measured by spirometry over the same period, is used
to calculate the scaling factor, M, which scales the proportionally
calibrated sum of RC and AB signals to real Vt. Our calibration
procedures differed slightly between dogs studied during wakeful
breathing and those included in the anesthetized doxapram study. In
awake dogs, when we were interested in the proportional contribution
of AB and RC compartments, we calculated K alone without spirom-
etry. These animals were fitted with the Lifeshirt as described above,
placed in lateral recumbency, and allowed to breath quietly for ~5
min. QDC calibration was performed on RC and AB traces from this
period. For the doxapram study, dogs were intubated and anesthetized
(2% isoflurane), and placed in lateral recumbency before performing
the QDC calibration as described above. In addition, we calculated the
scaling factor, M, to establish approximate, RIP-derived values for Vt,
VAB, and VRC by using spirometry data from a Datex Omeland
anesthesia machine, which integrates signals from syringe-calibrated
flow sensors to determine Vt.

We measured respiratory phase angle from RIP tracing as an index
of abdominal paradox. Phase angle is a measure of the degree to
which RC and AB movements are synchronized during tidal breath-
ing. A phase angle of zero indicates fully synchronous motion,
whereas 180° indicates a state in which RC and AB are exactly
asynchronous, Values from 1° to 180° reflect AB lagging behind RC
by a proportional amount of the breathing cycle. Vivologic software
calculates phase angle on a breath-by-breath basis as described (3).
We averaged breath-by-breath phase angle measurements over ap-
proximately 30-s periods of regular quiet breathing or, in the case of
doxapram, 30 s beginning at peak Vt following doxapram adminis-
tration.

Pressures. Gastric pressure (Pgas) and esophageal pressure (Pes)
were measured as described (40) using balloons constructed from an
intraaortic balloon pump coronary perfusion support system (Fi-
delity; Datascpe). These polyethylene balloons have the advantage
of being mounted on long, flexible cannulae. The balloon material is
inelastic, and therefore does not contribute to Pgas or Pes as a result of
any inherent recoil. The balloons were connected to calibrated
TSD104A pressure transducers (Biopac Systems), the output digitized
at 500 Hz with an MP150c data acquisition system (Biopac Systems).
Inter-

breath pressures were set to zero during restful breathing. Transdia-
phragmatic pressure (Pd) was calculated by subtracting Pgas from Pgas.
Anesthesia and doxapram studies. Dogs were induced with dext-
medetomidine hydrochloride (375 µg/kg) (Pfizer Animal Health)
before being mask down with isoflurane (Baxter Healthcare), intu-
minated, and maintained at 2% isoflurane continuously throughout the
study in a laterally recumbent position. Gastric and esophageal bal-
loon catheters were placed, and the Lifeshirt jacket was fitted to the
dog as described above. Doxapram hydrochloride (Baxter Healthcare)
bolus was administered via the saphenous vein at 1 mg/kg, followed by
a second dose of 2 mg/kg 5 min later.

Histology. The length of the costal muscular diaphragm was mea-
sured in vivo at three standardized places through an incision in the
abdominal wall at necropsy. To determine sarcomere length at these
dimensions, strips were clamped with two-headed biopsy forceps,
divided by 10.220.33.6 on October 23, 2017 http://jap.physiology.org/ Downloaded from
Regenerating the GRMD mutation measured at two time points.

Henseleit buffer containing, in mmol/l: CaCl₂, 1.5; glucose, 5.5; KCl, with perfusate warmed to 37°C. Perfusate was a modified Krebs-

internal thoracic artery was cannulated, and the muscle was perfused was removed at the time of euthanasia and rapidly cooled on ice. The

function in GRMD.

expressing carrier dogs (H9004), and 12 normal dogs (o) shows a loss of diaphragm

Table 1. Major ventilatory parameters of 7 dogs with the GRMD mutation and 5 normal control dogs included in the
doxapram study.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Dystrophin,</th>
<th>Age, months</th>
<th>Weight, kg</th>
<th>Ventilation, ml/kg · 1 min⁻¹ (baseline)</th>
<th>Ventilation, ml/kg · 1 min⁻¹ (1 mg/kg doxapram)</th>
<th>Tidal volume, ml/kg (baseline)</th>
<th>Tidal volume, ml/kg (1 mg/kg doxapram)</th>
<th>Respiratory rate, breaths/min (baseline)</th>
<th>Respiratory rate, breaths/min (1 mg/kg doxapram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g892</td>
<td>–</td>
<td>10</td>
<td>16.6</td>
<td>17.5</td>
<td>246.5</td>
<td>5.3</td>
<td>12.1</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>g692</td>
<td>–</td>
<td>13</td>
<td>13.6</td>
<td>142</td>
<td>375.3</td>
<td>10.9</td>
<td>22.1</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>g124</td>
<td>–</td>
<td>6</td>
<td>9.8</td>
<td>58.2</td>
<td>189.8</td>
<td>8.2</td>
<td>18.6</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>g547</td>
<td>–</td>
<td>17</td>
<td>16.3</td>
<td>37.5</td>
<td>372.3</td>
<td>9</td>
<td>17.3</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>g603</td>
<td>–</td>
<td>16</td>
<td>13.8</td>
<td>8.8</td>
<td>333.8</td>
<td>7.8</td>
<td>19.6</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>g890</td>
<td>–</td>
<td>17</td>
<td>20</td>
<td>10.8</td>
<td>308.1</td>
<td>5.7</td>
<td>10.6</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>n290</td>
<td>+</td>
<td>7</td>
<td>21.5</td>
<td>109.8</td>
<td>304.4</td>
<td>11</td>
<td>23.3</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>n559</td>
<td>+</td>
<td>7</td>
<td>20.3</td>
<td>83.1</td>
<td>463.7</td>
<td>13.8</td>
<td>31.3</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>n530</td>
<td>+</td>
<td>9</td>
<td>23</td>
<td>82.8</td>
<td>346.4</td>
<td>10.3</td>
<td>22.6</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>n256</td>
<td>+</td>
<td>13</td>
<td>27.8</td>
<td>135.1</td>
<td>330.9</td>
<td>11.3</td>
<td>21.6</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>n616</td>
<td>+</td>
<td>7</td>
<td>20</td>
<td>115</td>
<td>576.3</td>
<td>10.5</td>
<td>24</td>
<td>10</td>
<td>25</td>
</tr>
</tbody>
</table>

Ex vivo diaphragm mechanics. A section of costal hemidiaphragm was removed at the time of euthanasia and rapidly cooled on ice. The internal thoracic artery was cannulated, and the muscle was perfused with perfusate warmed to 37°C. Perfusate was a modified Krebs-Henseleit buffer containing, in mmol/l: CaCl₂, 1.5; glucose, 5.5; KCl, 4.7; MgSO₄, 1.66; NaCl, 118.0; NaH₂PO₄, 1.18; NaHCO₃, 24.88; and Na-pyruvate, 2.0 in H₂O, to which was added 20% heparinized whole

collagen cross-sectional area (CSA) was quantified for each by averaging three 500-μm², nonoverlapping images. 

RESULTS

Diaphragms in animals with the GRMD mutation are dysfunctional during quiet breathing. We used RIP to measure ∆V₂, in 22 dogs with the GRMD mutation, 12 normal dogs, and 3 dogs that were dystrophin-expressing GRMD carriers that were laterally recumbent, during periods of wakeful, quiet breathing. Dogs with the GRMD mutation showed reduced ∆V₂ compared with normal and carrier dogs (20.2 ± 15.8% vs. 54.2 ± 16.3%; P < 0.01) indicating reduced diaphragm range of motion and contribution to inspired volume. The difference was larger in older dogs (Fig. 1A). Seven dogs with the GRMD mutation from this group were studied at two time points, and all showed an age-related decline in ∆V₂ (Fig. 1B) at rest. Although normal dogs were not measured at multiple time points, no normal (or carrier) dog over 1 year of age showed a ∆V₂ of less than 10%.

P₂O₂ in dogs with the GRMD mutation is reduced at rest and during direct stimulus of respiratory drive. To address whether doxapram would uncover deficits in P₂O₂ caused by loss of diaphragm contractile capacity in GRMD we used balloon manometry to trace P₂O₂ throughout the hyperventilatory response to doxapram bolus injection. In anesthetized and instrumented dogs with the GRMD mutation (n = 7) and normal (n = 5) dogs, we administered doxapram intravenously to temporarily stimulate an abrupt increase in ventilation while monitoring respiratory performance. Table 1 shows ages, weights, and basic respiratory parameters of dogs included in the doxapram study. End-inspiratory P₂O₂ in dogs with the GRMD mutation was lower than in normal dogs at rest (5.45 ± 2.39 mmHg vs. 7.8 ± 1.11 mmHg; P < 0.05) and at the peak...
Increasing Vt, then respiratory rate in a manner consistent with groups, dogs responded to the onset of doxapram first by capacity in the GRMD animals. In both cases and in both A, indicating a loss of ventilatory GRMD mutation (Fig. 2).

A subsequent higher dose (2 mg/kg) further increased ventilation in normal dogs but not in dogs with the GRMD mutation employed higher respiratory rates earlier to achieve equivalent increases in ventilation. D: respiratory phase angle was not different between groups before or during maximal doxapram-mediated ventilation.

However, at equal rates of ventilation, Vt was consistently lower in the dogs with the GRMD mutation, which compensated with higher respiratory rates (Fig. 2B). Neither anesthesia nor doxapram had an appreciable effect on ΔVAB in individual dogs from either group (data not shown). At rest and at peak ventilation following doxapram administration (1 mg/kg), we observed no difference in phase angle between dogs with the GRMD mutation and normal dogs, indicating an absence of abdominal paradox (Fig. 2D).

Expiratory muscles compensate for diaphragm dysfunction in GRMD. DMD is associated with reduced functional residual capacity (FRC). We were interested in whether loss of volumetric reserve observed in GRMD would be accompanied by abnormal end-inspiratory chest wall volume (EIVcw) and end-expiratory chest wall volume (EEVcw). Figure 3 shows relative maximum EIVcw and minimum EEVcw by compartment in dogs with GRMD and normal dogs before and after a doxapram bolus (1 mg/kg). We found that the increases in Vt that dogs with the GRMD mutation achieved were almost entirely the result of lowered EEVcw and not increased EIVcw (Fig. 3A). In contrast, normal dogs achieved larger increases in Vt primarily by tapping into inspiratory reserve. Furthermore, RIP allowed us to differentiate between changes in rib cage and abdominal contributions to these volumes. The reduction in EEVcw observed in dogs with the GRMD mutation upon doxapram administration was primarily caused by lower end-expiratory VAB rather than VRC, indicating increased cranial motion of the diaphragm at end-expiration (Fig. 3, B and C).

If abdominal expiratory muscles drive VAB and chest wall volume below its equilibrium point during expiration, we would expect to observe a consequent increase in abdominal pressure (Pgas) (18, 27). Therefore, we performed a detailed analysis of gastric and esophageal pressures (Pgas and Pes, respectively) before and after doxapram administration. Indeed, dogs with the GRMD mutation demonstrated a pattern of transient increases in Pgas over two time scales following the initial dose of doxapram (1 mg/kg) (Fig. 4). The first was repeated each breath at end-expiration, observed as a rightward
spike in $P_{es}$/Pgas loops. The second was a longer-term increase occurring over several breaths during peak doxapram effect, observed as a rightward shift of the entire loop. The end-expiratory Pgas spike emerged only at the higher dose in normal dogs, and then to a lesser degree than in dogs with the GRMD mutation, whereas there was no long-term increase in Pgas after either dose in normal dogs.

Remodeling increases the passive stiffness of the GRMD diaphragm. We examined the gross morphometry, histopathology, and passive viscoelastic properties of the GRMD diaphragm. When measured at equal sarcomere lengths, the muscular diaphragms of dogs with the GRMD mutation were shorter in the direction of contraction compared with normal dogs (31.7 ± 7.2 mm vs. 75.6 ± 15.6 mm; $P < 0.01$) and thicker in the orthogonal plane (8.06 ± 3.4 mm vs. 2.4 ± 0.4 mm; $P < 0.01$). These findings indicate a process of remodeling that involves the deletion of sarcomeres rather than hypercontraction (Fig. 5).

The dynamic elastic modulus between 100% and 107% of $L_0$ was 21.4 g/mm² in dogs with the GRMD mutation vs. 2.4 g/mm² in a carrier dog.

DISCUSSION

In this study we combined noninvasive, invasive, ex vivo, and histological approaches to improve our understanding of the mechanics of breathing during the progressive loss of ventilatory capacity in a translational model of DMD. Our data indicate that respiratory decline in dogs with the GRMD mutation is accompanied by changes in passive and active properties of the respiratory pump. In both DMD and GRMD, widespread muscle disease severely limits the loading of the diaphragm for potential transplantation (34), we found that the collateral connections between the internal thoracic and intercostal arteries afforded adequate delivery of oxygen to a large volume of tissue (Fig. 7A) (5). These studies showed that the specific passive stiffness of the GRMD diaphragm strip was much higher and invariant at all physiologically relevant cyclic shortening rates than in a dystrophin-expressing carrier diaphragm at muscle lengths above 100% of $L_0$ (Fig. 7B). The dynamic elastic modulus between 100% and 107% of $L_0$ was 21.4 g/mm² in dogs with the GRMD mutation vs. 2.4 g/mm² in a carrier dog.

![Figure 4](http://jap.physiology.org/)

**Fig. 4.** A and B: representative traces of esophageal vs. gastric pressure throughout the breathing cycle before and at peak doxapram (1 mg/kg) effect in a dog with the GRMD mutation and a normal dog. Gray arrows indicate direction of loop. Gray circles indicate end-expiration. Dogs with the GRMD mutation showed transient increases in gastric pressure ($P_{gas}$) throughout the breathing cycle consistent with a postexpiratory recoil (PER) breathing strategy. C: representative synchronized traces of pressures and compartmental volumes in the time domain during two complete breathing cycles before and at peak doxapram (1 mg/kg) effect in a dog with the GRMD mutation and a normal dog.

![Figure 5](http://jap.physiology.org/)

**Fig. 5.** The GRMD diaphragm undergoes a dramatic remodeling that alters passive properties of the muscle. A: schematic cranial view of typical diaphragms from 1-year-old dogs viewed cranially (GRMD, red; normal, blue) showing reduced muscular length in the direction of shortening and expansion of central tendon area in GRMD. B: representative photographs of longitudinal sections of costal diaphragm (location indicated in A) showing reduced muscle length and increased thickness in GRMD. White arrowheads indicate central tendon, orange arrowheads indicate insertion on rib. C: plot of muscular diaphragm thickness (um) vs. length (mm) in six dogs with the GRMD mutation and five normal dogs. D: sarcomere length at these muscle lengths do not differ between dogs with the GRMD mutation and normal dogs, indicating that the reduced diaphragm length in GRMD results from removal of sarcomeres in series.
respiratory system by way of an exercise-induced increase in metabolic demand. To circumvent this limitation and to simulate the effects of moderate exercise, we uncoupled these processes using the respiratory stimulant doxapram (13, 14).

Normally, increasing demand for ventilation through exercise results in a progressive and sequential recruitment of additional inspiratory and expiratory contractile reserve that follows a well-characterized pattern. In graded exercise, increased respiratory drive caused by metabolic loading leads first to the recruitment of additional diaphragmatic motor units, followed by intercostal and auxiliary muscles to increase Vt, followed in turn by an increase in respiratory rate (1). Ultimately, at high rates of exercise, expiratory muscles are recruited first, to the recruitment of additional diaphragmatic motor units, increased respiratory drive caused by metabolic loading leads.

Recruitment of the expiratory musculature in GRMD is further facilitated by a remodeling of the diaphragm, which affects the passive mechanical properties of the muscle. Although the GRMD diaphragm becomes weaker, as evidenced by reduced Pdi, it also becomes shorter, thicker, and severely fibrotic, with a resulting increase in passive resistance to distension at three levels. First, series deletion of sarcomeres alone increases passive stiffness as individual sarcomeres are stretched further for a given absolute muscle length change. Second, increased collagen in the extracellular matrix increases muscle length-specific and CSA-specific stiffness. Finally, the increase in muscle length-specific stiffness is further multiplied by the increase in overall CSA of the thickened muscle. It should be noted that due to animal availability, passive stiffness in the GRMD diaphragm was compared with that from a heterozygous carrier animal. Although carrier diaphragms appeared grossly normal, and respiratory disease has not been reported in human or canine carriers, further comparisons to

**Fig. 6.** A: representative photomicrographs of muscle sections stained with picrosirius red from one dog with the GRMD mutation and one normal dog. Scale bar = 100 μm. Collagen (stained red) is increased in GRMD. B: collagen cross-sectional area (CSA) quantified in muscle sections stained with picrosirius red from dogs with the GRMD mutation and normal dogs. The number of dogs in each group is indicated in white. GRMD diaphragms had significantly higher collagen CSA than other GRMD muscles.

The observation that doxapram reduces EEVAB together with increased end-expiratory Pgas in GRMD is consistent with the compensatory adoption of a respiratory muscle recruitment sequence, termed postexpiratory recoil (PER), which normally emerges only during heavy exercise or under pathological conditions such as diaphragm paralysis. Grimby et al. first showed, in healthy subjects during a period of heavy exercise, that contraction of abdominal wall muscles at end-expiration functions not only to increase expiratory flow and Vt, but also to store elastic and gravitational energy in the diaphragm for the next breath (26, 19, 35). PER appears to be further augmented in dogs with the GRMD mutation because the entire range over which the diaphragm moves is shifted cranially under increased respiratory drive, likely increasing passive energy storage in the diaphragm’s elastic elements.

**Fig. 7.** Perfusion via the interior thoracic artery enables study of ex vivo diaphragm mechanics. A: schematic of a region of the costal diaphragm viewed cranially [see refs. (5, 34)]. A section of costal diaphragm is removed (region outlined in light blue) and the interior thoracic artery is cannulated (1) and perfused with a mixture of modified Krebs-Hanselite and heparinized whole blood at 37°C. A wide strip (~1 cm) of costal diaphragm is excised (pink shaded area) but left attached at the rib (2), whereas the central tendon end is attached to a muscle ergometer (3). Perfusate is collected and pumped (4) through a heat exchanger (5) and membrane oxygenator (6) before being reperfused. B: lissajous loops of specific passive tension vs. length in viable, perfused ex vivo diaphragm muscle strips from a dog with the GRMD mutation and a dystrophin-expressing carrier dog showing increased stiffness at lengths over 100% of L0 in GRMD.
normal diaphragms are needed. Regardless, the reliance on PER combined with the absence of abdominal paradox observed in this study suggests a possible compensatory role for a stiff diaphragm.

Two clinical examples lend support to this interpretation. Abdominal paradox resulting from a weak or nonfunctional but compliant diaphragm is an energetically and volumetrically unfavorable scenario for gas exchange (53). An example of severe abdominal paradox in the clinic is acute diaphragmatic paralysis following iatrogenic phrenic nerve injury during cardiac surgery. Surgical therapy for refractory cases involves plication (reduction of the area and excursion of the nonfunctional diaphragm), which serves to eliminate abdominal paradox, increase maximum lung volumes, and reduce the work of breathing for a given \( V_t \) (16, 22, 46, 50). In other words, it is better by these important metrics to have a short and therefore stiff diaphragm with little or no range of motion, than a compliant but weak or nonfunctioning diaphragm.

Second, dogs with the GRMD mutation are not a unique example of compensatory PER in the setting of diaphragm sarcomere deletion. Hyperinflation of the lungs and increased FRC in chronic obstructive pulmonary disease leads to a remodeling of diaphragmatic muscle fibers, whereby sarcomeres are deleted in series in such a way that ideal resting sarcomere length is maintained in the shortened muscle (10). This allows \( P_{aw} \) to be more effectively generated around a higher chest wall equilibrium volume and enables compensatory PER breathing (10, 19, 35). Patients with DMD (and dogs with the GRMD mutation in this study) breathe at a lower than normal FRC (38), suggesting that the stimulus and mechanism for diaphragm remodeling are likely different.

There are important caveats in ascribing a compensatory role to the observed diaphragm remodeling in GRMD. Abdominal paradox is typically observed in scenarios of diaphragm dysfunction where other respiratory muscles are normal. Because abdominal paradox requires inspiratory intercostal muscles to be strong enough to overpower a weak diaphragm and all respiratory muscles are affected in GRMD, the absence of abdominal paradox could be a function of insufficient intercostal strength. A similar argument could be made against PER, because clinical examples typically involve normal abdominal muscles. We view changes to the mechanics of breathing in GRMD not so much as compensation for a failing diaphragm per se, but as a redistribution of the work of breathing to a larger volume of muscle, thus minimizing peak force on any individual sarcomere. In this context, the augmentation of diaphragmatic elastance could play an important role by further enabling the muscles of the abdomen to contribute to inspiration. This interpretation recalls the significance of the Gowers’ sign in the clinical assessment of locomotory muscle performance in DMD (8, 23). Gower first observed that when asked to stand from a seated position without the aid of elevated structures, children with body-wide neuromuscular disease follow a stereotyped sequence of motions, the effect of which is to spread the work of standing among as many muscles as possible. Although the combined effect of diaphragm remodeling in GRMD appears to be beneficial, further study is needed to determine processes underlying the observed changes, and whether any component of diaphragm stiffness is maladaptive.

There is indirect evidence that a similar process of remodeling and compensatory recruitment of the respiratory musculature takes place in DMD, but there has been no previous opportunity to integrate the individual components as facilitated in this translational model. One study reported sonographically detectable thickening of the muscular diaphragm in boys with DMD (17), and progressive loss of diaphragm range-of-motion beginning early in the disease without abdominal paradox has been reported in DMD (30, 35). And, although boys with DMD breathe at a lower FRC, PER has not been reported. The interpretation of adaptive diaphragm function put forward here attributes a possible positive functional role to fibrosis, a process usually considered pathologic. Therefore, these findings could be relevant to therapies that target this process. Moreover, the diaphragm has an essential role in anatomical partition, which might be compromised by necrotizing myocytolysis in the absence of fibrosis, leading to a high incidence of additional morbidity from transdiaphragmatic herniation of abdominal viscera (7, 12).

The observation that dogs with the GRMD mutation recruit a broader range of respiratory muscles than normal control dogs to achieve the same rates of ventilation suggests that the lack of a doxapram dose-response in this population reflects a functional limitation of the respiratory pump. We cannot, however, rule out the possibility that dystrophin expression in the central nervous system (CNS) directly affects neuronal pathways involved in the integration of afferent and efferent signals responsive to doxapram-receptor binding. Although the functions of dystrophin and the dystrophin-associated glycoprotein complex in the CNS are not well understood, cognitive impairment has been identified in some patients with DMD, and progress has been made in identifying potential biochemical pathways that might be implicated (2, 15, 52).

Although the phenotype is severe in dystrophin-deficient dogs, quantifiable biomarkers of disease progression have been difficult to standardize. The ratio of diaphragmatic length to thickness reported here is arguably the most extreme morphometric abnormality noted in GRMD to date, and holds promise as an endpoint measure in translational research. Furthermore, having established quantifiable changes in respiratory mechanics and capacity in a group of dogs with the GRMD mutation and with relatively severe disease, we anticipate that longitudinal studies consisting of serial measurements of individual animals will further enhance the value of this translational model in view of the inherent phenotypic variability of the outbred GRMD line. This variability cannot be addressed by inbreeding without compromising the viability of the overall colony (32). Noninvasive or minimally invasive biomarkers of skeletal muscle function in GRMD are currently limited to analyses of gait, passive range of motion, magnetic resonance imaging (MRI) analysis of muscle volume and composition, and of proximal and distal hindlimb muscle force during nerve electrostimulation (31). Because MRI and electrostimulation require general anesthesia in dogs, we have recently added doxapram respiratory studies before anesthesia reversal, without observable detriment to animals undergoing serial studies.

ACKNOWLEDGMENTS

We thank D. Bogan and S. Chakkalakal for excellent technical assistance; and M. Cereda, D. Cooper, G. Guild, T. Khurana, S. Levine, D. Ren, S. Singhal, and T. Svitkina for insightful discussions.
REFERENCES

38. Lo Mauro A, D’Angelo MG, Romei M, Motta F, Colombo D, Comi GP, Pedotti A, Marchi E, Turconi AC, Bresolin N, Aliverti A. Ab- dominal volume contribution to tidal volume as an early indicator of...


