Hypoxemia-induced modification of troponin I and T in canine diaphragm

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Simpson, Jeremy A., Jennifer E. Van Eyk, and Steve Iscoe. Hypoxemia-induced modification of troponin I and T in canine diaphragm. J. Appl. Physiol. 88: 753–760, 2000.—Impaired muscle function (fatigue) may result, in part, from modification of contractile proteins due to inadequate O2 delivery. We hypothesized that severe hypoxemia would modify skeletal troponin I (TnI) and T (TnT), two regulatory contractile proteins, in respiratory muscles. Severe isocapnic hypoxemia (arterial partial pressure of O2 of ~25 Torr) in six pentobarbital sodium-anaesthetized spontaneously breathing dogs increased respiratory frequency and electromyographic activity of the diaphragm and internal and external obliques, with death occurring after 131–285 min. Western blot analysis revealed proteolysis of TnI and TnT, 17.5- and 28-kDa fragments, respectively, and higher molecular mass covalent complexes, one of which (42 kDa) contained TnI (or some fragment of it) and probably TnT in the costal and crural diaphragms but not the intercostal or abdominal muscles. These modifications of myofilibrillar proteins may provide a molecular basis for contractile dysfunction, including respiratory failure, under conditions of limited O2 delivery.

RESPIRATORY FATIGUE IS ASSOCIATED with the decreased ability of respiratory muscles to generate the pressures required to maintain ventilation. Rather than maintaining ventilation, individuals will tolerate the fall in arterial partial pressure of O2 (PaO2) and rise in arterial partial pressure of CO2 (PaCO2), which accompany hypventilation. In individuals with acute exacerbations of obstructive diseases, hyperinflation also ensues; the resulting decrease in diaphragmatic length and increased radius of curvature place it at a mechanical disadvantage. Any decrease in contractile efficiency combined with decreased energy supply (reduced PaO2) only makes the diaphragm (and other respiratory muscles) more susceptible to eventual failure (for reviews, see Refs. 24 and 32).

Diagnosis of respiratory muscle dysfunction typically involves measurements of either electrical activity of the diaphragm or pressures generated during maximal voluntary efforts, sniffs, or magnetic stimulation of the phrenic nerves (see Refs. 11, 16, 24, 32). Although some of these methods have distinct advantages (e.g., the simplicity of the sniff test), others are either technically demanding (e.g., magnetic stimulation, computation of power spectrum of the electromyogram) and/or are invasive (placement of catheters in the esophagus or abdomen). Moreover, they all share one major limitation: insensitivity; all require that enough damage be present to result in a significant reduction in, for example, pressure. Plasma levels of creatine kinase (CK), a marker of cellular necrosis, have yet to be established as a useful marker of respiratory failure (1, 10).

Although plasma levels of the fast and, possibly, slow skeletal isoforms of troponin I (sTnI) increase following severe exercise (22, 27, 28), it is unclear if intact sTnI or some unidentified modification product(s) was measured. Indeed, in one study (28), only the fast isoform was measured. Hence, we do not know the extent of cell damage, including necrosis, responsible for leakage of the protein, intact or modified, into the plasma. In contrast, plasma levels of two myocardial proteins, cardiac troponin I and troponin T (cTnI and cTnT), can now be rapidly measured, with their levels indicating the extent of infarction (see Ref. 7). Myocardial ischemia-reperfusion injury in isolated rat hearts has recently been shown to be associated with modification of cTnI (12, 20, 31); in fact, the extent and nature of cTnI modification are related directly to the severity of the ischemia-reperfusion injury and the fall in force generation (12, 31). Even under conditions that involve little or no loss of protein, indicating minimal cellular necrosis, cTnI undergoes degradation and covalent complex formation (20). Thus cTnI modification within the myocyte may therefore be the earliest marker of myocardial damage. With more severe injury, other myofilament proteins, including cTnT, are also modified (e.g., Refs. 14, 20, 31). Thus modification products of myofilament proteins may provide useful indexes of the severity of injury.

Plasma levels of sTnI and sTnT cannot, however, be used to diagnose respiratory muscle dysfunction until confirmation of both their modification within tissues and their release into the plasma. In this study, we addressed the first issue by testing the hypothesis that severe hypoxemia would modify myofibrillar proteins of respiratory muscles of anesthetized, spontaneously breathing dogs. Our results show that both TnI and TnT were modified but only in diaphragm; these modifications consisted of lower molecular mass degradation...
products and higher molecular mass covalent complexes consisting of TnI, TnT, and possibly troponin C (TnC).

METHODS

Experiments were conducted according to procedures established by the Canadian Council on Animal Care and after approval by the Animal Care Committee of Queen’s University. Six mongrel dogs (2 males, 4 females; weight of 16–24.4 kg, mean 19.8 kg) were anesthetized with an intravenous injection of pentobarbital sodium (35 mg/kg), which was supplemented if the animals displayed a brisk response to noxious stimulation of a toe pad or blinked in response to stimulation of the cornea. In brief, after instrumentation and collection of control tissue samples (see below), they breathed a hypoxic gas mixture; measurements of cardiorespiratory parameters were made at 20-min intervals until signs of impending death appeared. They were then mechanically ventilated, and biopsies were taken from a limb muscle and various respiratory muscles for subsequent analysis of tropinin modification.

Surgical preparation included insertion of an endotracheal tube to which a heat and moisture exchanger was attached, a venous “butterfly” cannula into a forelimb vein for administration of supplemental anesthetic, and a cannula into the carotid artery for measurement of arterial blood pressure and withdrawal of blood for measurements of arterial blood gases. Two Swan-Ganz catheters (131HF7, Baxter Edwards, Deerfield, IL) were inserted via the right jugular vein, one into the pulmonary artery and the other into the right atrium, according to measurements of pressure at the tip of each catheter. The former was used for sampling mixed venous blood, and both were used for thermal dilution measurements of cardiac output (Qt) when cold saline was injected into the latter (Edwards 9520 cardiac output computer, Santa Ana, CA).

Tissue Samples

Before imposition of hypoxemia, control biopsies were taken from the quadriceps and internal oblique (IO) muscles. To gain access to the IO, the aponeurosis of the overlying external oblique (EO) was incised and the muscle was reflected laterally. Samples at the end of the experiment were taken from quadriceps, EO, and IO as well as the transverse abdominalis, costal and crural diaphragms, and caudal and midthoracic (intercostal spaces 9–10 and 4–6, respectively) external and internal intercostals. Tissue samples were obtained before the heart stopped or within 4 min of death (cessation of the heart beat). They were immediately washed in cold saline, frozen in liquid N₂, and stored at −70°C until later biochemical analysis (see Biochemistry, below).

Electromyographic Recordings

After the first biopsies were taken, pairs of fine wires that were insulated except for the tips, which were bent back over the outside of 23-gauge needles, were inserted under direct observation ~15 mm apart into the IO on the side opposite to that from which the biopsy had been made. The needles were then withdrawn, leaving the wires in place. Similar electrodes were placed into the ipsilateral EO. The overlying skin was then closed. Recordings of the diaphragmatic electromyograph (EMG) were made by inserting identical electrodes percutaneously at the sixth or seventh right interspace. All signals were amplified and filtered (Grass P511J, Quincy, MA); signals were recorded on videotape after pulse code modulation (NeuroCorder DR886, New York, NY) and recorded on paper (Gould TA2000, Cleveland, OH), either as raw signals or after “integration” (Paynter filter, time constant of 100 ms).

Hypoxemia

Severe “isocapnic” hypoxemia was instituted by having the dogs breathe through a circuit that maintains normal capnia regardless of the level of hyperpnea (26). In brief, dogs inhaled a gas mixture of 9.5% O₂-balance N₂ delivered at ~2 l/min to a balloon attached to a one-way valve on the inspiratory side of the breathing circuit. The remaining gas inspired during hypoxia-induced hyperpnea came from a cylinder containing 9.5% O₂-6.5% CO₂-balance N₂ and connected to a demand valve (Dacor, Northfield, IL). Thus any hypoxia-induced demand for ventilation greater than the basal flow was supplied by gas from the second cylinder, which, because of its CO₂ content, prevented hypocapnia.

Protocol

Two sets of control measurements, 20 min apart, were taken of arterial and mixed venous blood gases and pH (Radiometer ABL30, Copenhagen, Denmark) and Qt. The dogs were then placed on the breathing circuit. Once the PaO₂ had fallen to the desired range (24–28 Torr), measurements were repeated at 20-min intervals until the dogs died. Death was always preceded by a sudden slowing of respiratory frequency, at which time dogs were ventilated with room air and biopsies were taken as described.

Biochemistry

Plasma levels of CK before and at the end of hypoxia exposure were determined (Beckman Synchron CX, Fullerton, CA). For SDS-PAGE analysis, frozen tissue samples were homogenized in 50 mM Tris, pH 7.8, plus a cocktail of protease inhibitors (50 μM phenylmethylsulfonyl fluoride, 3.6 μM leupeptin, 2.1 μM pepstatin A, and 10 mM EDTA). Total protein concentration was determined by the Lowry assay before preparation of protein samples in Laemmli buffer and 1 mM dithiothreitol (DTT) and storage at −20°C (20). SDS-PAGE (12.5%) and Western immunoblots were performed as previously described (20) using mini-Protein II and wet transfer mini-systems (Bio-Rad, Hercules, CA). Gels were stained with Coomassie blue or transferred to nitrocellulose (27 V for 16 h). Nitrocellulose blots were transiently stained with ponceau S to identify molecular mass markers and then incubated in blocking solution [50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (vol/vol) Nonidet P-40, 0.25% (wt/vol) gelatin, and 3% (wt/vol) BSA]. Detection of slow and fast sTnI was done using anti-TnI monoclonal antibody (MAb) clones C5 (Research Diagnostics, Flanders, NJ), 8I-7 and 3I-35 (Spectral Diagnostics, Toronto, ON), and 10F2 (Sanofi, Montpellier, France). (All MAb cross-react, but to different degrees, with the three isoforms of TnI; the cardiac isoform of TnI is absent in skeletal muscle.) The various anti-TnI MAb s were epitope mapped according to Van Eyk et al. (31). Detection of TnT was done using three anti-TnT MAb clones: JLT-12 (Sigma, St Louis, MO), and 4TnT conjugated to horseradish peroxidase (Spectral Diagnostics, Toronto, ON). All displayed cross-reactivity to the skeletal and cardiac isoforms. The only MAb to cross-react with another muscle protein was the anti-TnI MAb 3I-35 (~10% with sTnT).

Blots were incubated with anti-mouse IgG antibody-alkaline phosphatase conjugate (Jackson Laboratories, West Grove, PA) and detected by CDP-Star chemiluminescence (NEN-Mandel, Boston, MA) except for 4TnT conjugated to
RESULTS

Inhalation of the hypoxic gas mixture resulted in significant decreases in mean PaO2 (from ~65 to 25 Torr), arterial O2 content, and mixed venous PaO2 (Table 1). Increases in Qt did not reach statistical significance nor did they, in combination with a significant increase in hematocrit, prevent a drop in whole body O2 delivery (D\dot{O}_2). Peak amplitudes of integrated EMG activity of the diaphragm and IO as well as respiratory frequency increased. Death occurred after an average 197 min (range 131–285 min) of breathing the hypoxic gas mixture; in all animals, death was preceded by a cessation of EMG activity in all respiratory muscles. In four dogs, all muscles stopped firing at the same time, but, in the other two, EO stopped firing first by 2 min. Plasma CK levels, a marker of cell necrosis, increased significantly, but its source was not identified.

Myofibrillar Proteins in Respiratory Muscles

TnI. All respiratory muscles contained bands (Fig. 1) corresponding to the fast and slow isoforms of sTnI (~25 and ~26 kDa, respectively, on 12.5% SDS-PAGE, compared with their actual molecular masses of 21.2 and 21.6 kDa), which were identified using samples from exclusively fast (caudofemoralis) and slow (soleus) muscles of cat (Fig. 1A). (The faint band representing a degradation product in soleus is due to ischemia of this particular muscle.) However, only the costal and crural diaphragms showed evidence of hypoxemia-induced degradation of TnI, evident as a band at a position corresponding to a molecular mass of 17.5 kDa. Samples of nonhypoxic diaphragms from two dogs used in other experiments showed no evidence of TnI degradation (Table 2); other nondiaphragmatic respiratory muscles (e.g., IO; Fig. 1B) also showed no evidence of this. The hypoxicemic crural diaphragm of dog 6 also yielded the 17.5-kDa degradation product despite the virtual absence of the band corresponding to the fast

Table 1. Cardiorespiratory parameters before and midway and for last set of complete measurements before death during hypoxemia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time 0</th>
<th>Time 0.5</th>
<th>Time 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>f, min⁻¹</td>
<td>9.0 ± 4.2</td>
<td>49.3 ± 17.1*</td>
<td>44.5 ± 10.4*</td>
</tr>
<tr>
<td>peak f/Dia, AU</td>
<td>6.1 ± 2.5</td>
<td>14.1 ± 8.5*</td>
<td>10.5 ± 6.1*</td>
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<tr>
<td>peak f/EO, AU</td>
<td>1.6 ± 1.2</td>
<td>5.8 ± 2.2</td>
<td>5.4 ± 3.4</td>
</tr>
<tr>
<td>peak f/OE, AU</td>
<td>1.7 ± 1.8</td>
<td>10.2 ± 2.0*</td>
<td>10.4 ± 3.2*</td>
</tr>
<tr>
<td>Q₁, l/min⁻¹ · kg⁻¹</td>
<td>3.3 ± 0.4</td>
<td>5.8 ± 1.9</td>
<td>4.0 ± 2.1</td>
</tr>
<tr>
<td>PaO₂, Torr</td>
<td>82.4 ± 4.9</td>
<td>24.7 ± 2.8</td>
<td>19.8 ± 3.3*</td>
</tr>
<tr>
<td>PaCO₂, Torr</td>
<td>41.5 ± 2.2</td>
<td>53.5 ± 6.7</td>
<td>42.1 ± 9.6</td>
</tr>
<tr>
<td>pHa</td>
<td>7.310 ± 0.060</td>
<td>7.280 ± 0.070</td>
<td>7.241 ± 0.094</td>
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<tr>
<td>PaO₂, Torr</td>
<td>43.9 ± 6.5</td>
<td>15.4 ± 1.3*</td>
<td>10.9 ± 2.4*</td>
</tr>
<tr>
<td>PaCO₂, Torr</td>
<td>43.4 ± 9.2</td>
<td>49.7 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.282 ± 0.027</td>
<td>7.270 ± 0.074</td>
<td>7.220 ± 0.100</td>
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<td>Cao₂, ml O₂/dl</td>
<td>15.8 ± 0.110</td>
<td>6.8 ± 1.2*</td>
<td>4.8 ± 2.3*</td>
</tr>
<tr>
<td>Cvo₂, ml O₂/dl</td>
<td>11.8 ± 0.19</td>
<td>3.6 ± 0.9*</td>
<td>1.9 ± 1.2*</td>
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<tr>
<td>D\dot{O}_2, ml O₂·kg⁻¹·min⁻¹</td>
<td>20.9 ± 4.3</td>
<td>10.8 ± 3.1*</td>
<td>8.5 ± 2.7*</td>
</tr>
<tr>
<td>V\dot{O}_2, ml O₂·kg⁻¹·min⁻¹</td>
<td>5.15 ± 1.9</td>
<td>5.09 ± 1.98</td>
<td>5.95 ± 1.11</td>
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<tr>
<td>Hct</td>
<td>37.9 ± 1.9</td>
<td>43.6 ± 5.3*</td>
<td>45.7 ± 5.6*</td>
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<tr>
<td>Creatine kinase, U/l</td>
<td>170 ± 32</td>
<td>360 ± 163*</td>
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Values are means ± SD. Time 0, time 0.5, and time 1, measurements before, midway, and immediately before death; f, respiratory frequency; peak f/Dia, peak integrated diaphragmatic activity; peak f/EO, peak integrated external oblique activity; peak f/OE, peak integrated internal oblique activity; AU, arbitrary units; Qt, cardiac output; D\dot{O}_2, oxygen delivery; PaO₂, arterial partial pressure of O₂; PaCO₂, arterial partial pressure of CO₂; PaO₂, and PaCO₂, mixed venous PaO₂ and PaCO₂; Cao₂ and Cvo₂, arterial and venous O₂ content; Hct, hematocrit. *Significantly different from control, P < 0.05; †significantly different from time 0.5, P < 0.05. One-way repeated-measures ANOVA with Bonferroni post hoc analysis or, for nonparametric data, Friedmann’s repeated measures ANOVA with Student-Newman-Keuls post hoc analysis was performed.
Table 2. Hypoxemia-induced degradation of TnI

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Control</th>
<th>Hypoxemia</th>
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<tbody>
<tr>
<td>Diaphragm (costal)</td>
<td>&lt;2</td>
<td>20.0 ± 8.9</td>
</tr>
<tr>
<td>Diaphragm (crural)</td>
<td>&lt;2</td>
<td>26.1 ± 8.4</td>
</tr>
<tr>
<td>Internal oblique</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td>External oblique</td>
<td>5.8 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>Transverse abdominis</td>
<td>2.9 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Internal intercostal 4</td>
<td>2.1 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>External intercostal 4</td>
<td>3.4 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Intercostal 10</td>
<td>2.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Quadriceps</td>
<td>2.6 ± 4.2</td>
<td>2.0 ± 1.7</td>
</tr>
</tbody>
</table>

Values are means ± SD. Percent degradation expressed as densitometric "volume" of 17-kDa fragment divided by the sum of the volumes of the fast and slow isoforms of intact troponin I (TnI). *Based on samples from 2 dogs not used in these experiments.

isoform of intact sTnI (Fig. 1A), suggesting that the degradation product originated from, but not necessarily exclusively, slow TnI. This same dog, however, had almost equal amounts of the fast and slow isoforms of TnT (data not shown).

Western blots using anti-TnI MAbs C5 and 3I-35 of representative hypoxic diaphragms and an accessory respiratory muscle, the IO, are shown in Fig. 2. They consistently and unambiguously revealed, particularly with prolonged exposures, a higher molecular mass covalent complex only in the diaphragm (compare lanes c and d in Fig. 2). The failure of the MAb 3I-35, which binds to the extreme COOH terminus (20), to reveal the 17.5-kDa fragment, even with prolonged exposure, indicates that formation of the 17.5-kDa fragment involves proteolysis at the COOH terminus. In contrast, the binding of this MAb to the 42-kDa covalent complex indicates that this complex contains TnI with an intact COOH terminus. The higher molecular mass complexes did not dissociate in the presence of 6 M urea or 1 mM DTT (data not shown), indicating that they are covalent (nondisulfide) in nature.

We believe that the two bands beneath the 42-kDa complex, most visible in lane e (Fig. 2), result from cross-reactivity of 3I-35 to the intact slow and fast isoforms of TnT for three reasons: 1) only this anti-TnI antibody detected the bands, 2) this MAb also detected TnT in purified troponin, albeit with reduced affinity (~10%, data not shown), and 3) the two bands aligned perfectly with the slow and fast isoforms of intact TnT revealed with the anti-TnT MAbs JLT-12 (Fig. 3) or 4D-11.

The extent of TnI degradation in biopsies from all respiratory muscles, expressed as a percentage of the sum of the intact fast and slow TnI isoforms, is presented in Table 2. Hypoxemia-induced degradation was present in the diaphragms of all dogs, averaging 20% and 26% in the costal and crural diaphragms, respectively, but did not exceed 6% in any other respiratory muscle or the quadriceps. There was no significant difference (P > 0.05, paired t-test) between the amount of TnI degradation product in the costal and crural diaphragms (in the five dogs in which samples were available from both parts), suggesting no regional differences in susceptibility to damage. Moreover, because the crural biopsy was always done after the costal one, its later removal did not result in more degradation.

TnT. Different patterns of TnT modification products were detected using three different anti-TnT MAbs (Fig. 3). 4TnT (data not shown) and JLT-12 detected only a single TnT degradation product with a molecular mass of 28 kDa. Although J LT-12 revealed the degradation fragment in three of five crural and in two of six costal diaphragms, another MAb, 4D-11, detected formation of two high molecular mass (42 and 66 kDa) complexes in all hypoxic diaphragms, costal and crural, but not in any other respiratory muscle. However, confirmation that TnT is part of these complexes requires its detection by at least one other MAb with a different epitope.

DISCUSSION

This study is the first, to our knowledge, to report hypoxemia-induced posttranslational modification of
identified myofibrillar proteins (TnI and TnT) in a skeletal muscle. The acute changes observed in our dogs would precede any transcriptional changes such as isoform switches. These posttranslational modifications may, however, account for the impaired performance of respiratory muscles in a variety of acute, and possibly chronic, clinical conditions (for reviews, see Refs. 24 and 32).

Hypoxemia was induced by lowering $F_{O2}$. Cessation of respiratory activity and death occurred after an interval similar to that in dogs in which diaphragmatic fatigue was induced by reducing whole body $D\dot{O}_2$ with cardiac tamponade (2). However, without measurements of regional perfusion, we cannot be certain that diaphragmatic $D\dot{O}_2$ delivery was, indeed, reduced. Although whole body $D\dot{O}_2$, averaged 10.8 and 8.5 ml $O_2$·kg$^{-1}$·min$^{-1}$ at the midpoint and end, respectively, of hypoxemic stress, values similar to the critical value ($\approx 10$ ml $O_2$·kg$^{-1}$·min$^{-1}$) for $D\dot{O}_2$ during hypoxia (6) but only a fifth of that ($\approx 50$ ml $O_2$·kg$^{-1}$·min$^{-1}$) for the contracting canine diaphragm (34), cessation of respiratory activity was probably not due to failure (fatigue) of the respiratory muscles for four reasons. 1) It was preceded by a sudden slowing of respiratory frequency, indicating a change in output of the central respiratory controller. 2) Activity of all muscles ceased simultaneously in four of the six dogs. 3) Protein modification occurred only in the diaphragm; had the diaphragm failed, other muscles would have compensated, possibly leading to hypoxemia-induced protein modification in them. 4) Institution of mechanical ventilation elicited a transient recovery of activity in all muscles. Respiratory arrest and death therefore appear to have been due to central (brain stem) damage resulting from severe prolonged hypoxemia, a conclusion consistent with that of a previous report (35).

TnI and TnT Modifications and Contractile Function

Hypoxemia-induced modifications of TnI and TnT in tissue consisted of both lower molecular mass (17.5 and 28 kDa, respectively) fragments and higher molecular mass complexes (42 and 66 kDa). Modification products other than these and detectable using MABs with different epitopes may, however, also be present. Although misidentification is possible due to cross-reactivity of MABs with other muscle proteins, all products except covalent complexes possibly containing TnT were recognized by MABs with different epitopes to the same protein, a finding that greatly reduces the probability of misidentification. Nevertheless, the observation that TnI and TnT were modified only in the diaphragm indicates both that the MABs we used are specific to acute muscle injury and that, under our experimental conditions (severe hypoxemia), the diaphragm was the sole muscle susceptible to such modification.

Differential binding of the anti-TnI MABs C5, 3I-35, and 10F2 to intact TnI and the 17.5-kDa degradation product suggests initial proteolysis of sTnI at the COOH terminus. We mapped the TnI fragments using different MABs; whereas C5 bonded equally well to all sTnI modification products, 3I-35 and 10F2 bonded only to intact sTnI, indicating that the 17.5-kDa sTnI fragment resulted from proteolysis at or near amino acid residue 162. Although the calculated molecular mass of fragments 1–162 of the slow and fast isoforms are 18.98 and 19.01 kDa, respectively (greater than the measured 17.5 kDa), some proteins, including the troponins, do not migrate according to their molecular masses. The fast and slow isoforms of sTnI migrated "higher" (25 and 26 kDa) than predicted by their actual molecular masses (21.2 and 21.6 kDa, respectively), suggesting that the 1–162 fragment also migrates higher than calculated. Interestingly, Farah et al. (9) observed that the recombinant fast sTnI 1–156 mutant, only six amino acids less, migrated to 22 kDa instead of the expected 19 kDa. These findings indicate that the degradation of sTnI to a 17.5-kDa fragment is too great to be accounted for by anything other than cleavage. The failure of MABs 3I-35 and 10F2 to bind to the fragment could be due to COOH-terminal clipping or block of the epitope at the COOH terminus by posttranslational modification (e.g., phosphorylation, glycosylation). Alternatively, clipping could have occurred first at the NH$_2$ terminus, with binding of the MABs 3I-35 and 10F2 being blocked by posttranslational modification(s) at the extreme COOH terminus.

Covalent Complexes

Two complexes of 42 and 66 kDa formed in the diaphragm during hypoxemia (Figs. 2 and 3). These involve covalent bonds between the proteins because they were stable in the presence of high concentrations (0.1%) of SDS and in 8 M urea, conditions that disrupt noncovalent interactions, and of the reducing agent DTT (1 mM), which also disrupts disulfide bonds. The 42-kDa product is comprised of TnI and possibly TnT. Importantly, the anti-TnI MABs 8I-7, C5, and 3I-35 identified this complex, indicating that the complex is comprised of TnI with an intact COOH terminus. On the basis of the molecular mass of the complex, further
degradation of TnI (from the NH\textsubscript{2} terminus) or TnT
(from either the NH\textsubscript{2} or COOH terminus) probably
occurred, reducing the molecular mass of the smaller
complex from the calculated 49.5 kDa.

The 66-kDa complex may be composed of fragments of
one or more of the three troponin subunits (TnI, TnT,
and TnC); the calculated molecular mass of the 17.5-
kDa TnI fragment and intact TnT and TnC is \( \sim 67 \) kDa.
However, because none of the anti-cTnI MABs (with
different epitopes) bound to this 66-kDa complex, it
either does not contain TnI or the epitopes for the
various TnI MABs antibodies are inaccessible. The
66-kDa complex is also unlikely to be a dimer of TnT
due to the physical separation between "adjacent" TnTs
on the thin filament. Instead, this protein is likely a
covalent complex of TnT and TnC or other unidentified
proteins. Interestingly, McDonough et al. (20) have
identified cTnC in covalent complexes in ischemic/
reperfused rat myocardium.

Skeletal Muscle Proteolysis

To our knowledge, there have been no previous
studies of hypoxemia-induced modifications of myofil-
lar proteins of respiratory muscles. Indirect evidence,
however, suggests that loads modify myofilibrillar pro-
teins of respiratory muscles. Reid et al. (23) recovered
\(~7\)% less myofilibrillar protein (including any TnI and
TnT associated with the actin filament) from the dia-
phragms of hamsters with tracheal banding (compared
with \( \sim 23\% \) modification of TnI in the diaphragms of
our hypoxic dogs); tropomyosin and \( \alpha \)-actinin of
loaded diaphragms also appear to have been degraded
more quickly by calpain. The latter observation sug-
gests that several myofilament proteins were modified,
making them more susceptible to degradation by cal-
pain. Recently, the same group (15) demonstrated
increased calpain-like activity in the costal diaphragms
of rabbits subjected to a moderate inspiratory resistive
load.

There are few descriptions of posttranslational modi-
fications of myofilament proteins in skeletal muscle.
Belcastro et al. (4), based on SDS-PAGE of myofibrils
from fatigued plantaris of rats, described loss of a
58-kDa band (possibly desmin, a cytoskeletal protein)
and the appearance below actin (\(~42\) kDa) of an
unidentified protein in the region of tropinin and
tropomyosin. Belcastro (3) later described faster degra-
dation of tropomyosin and \( \alpha \)-actinin from purified
myofibrils of hindlimb muscles from exercised rats.
Eccentric contraction-induced injury of mouse soleus in
vivo increases the rate of proteolysis (affected proteins
not identified) by \(~60\% \) at 48 h postinjury (19), whereas
eccentric contractions of rabbit extensor digitorum
longus cause rapid (\(<15\) min) myofilament damage in
fast glycolytic fibers, characterized by loss of desmin
(18). Together, these results suggest a spectrum of
exercise-induced damage to myofilaments and that,
with time, there is increasing degradation or loss of
specific myofilament proteins.

Plasma levels of sTnI, analyzed using immunoenzy-
mometric assays, along with "traditional" markers like
CK, have recently been used to assess exercise-induced
skeletal muscle damage in humans. Rama et al. (22), on
the basis of the increases and time course of changes in
both sTnI and CK levels after a triathlon, concluded that
sTnI is superior to CK as a marker because of its
greater sensitivity and faster return to control values.

At the end of a marathon, plasma levels of sTnI,
measured using the anti-TnI MAb 3I-35, were elevated
in only 9 of 46 runners (28); however, since this MAB
does not detect the 17.5-kDa TnI degradation product
(Fig. 2), the incidence of muscle damage may have been
underestimated, especially because, at least in is-
chemic cardiac muscle, cTnI modification products, not
intact cTnI, are preferentially released (20). Although
the appearance of sTnI in the blood is a promising
marker of muscle injury, these studies demonstrate
only that exercise causes membrane damage sufficient
to allow the release of muscle proteins. Moreover,
because we do not yet know what form of sTnI was
measured, the assay may not be quantitative. Until
more is known about the changes in sTnI and other
skeletal muscle proteins during muscle injury and
what forms are released, the utility of sTnI as a marker
of damage is limited.

The precise nature of these modifications and their
similarity to the changes observed in our canine model
of hypoxemia remain to be elucidated. The latter differ,
however, from the transformation to the slow isoforms
observed in the diaphragms of patients with congestive
heart failure (30) and chronic obstructive pulmonary
disease (17), both a form of diaphragmatic "exercise"
(5). Transformation provides no information about the
mechanisms underlying injury and fatigue nor can it
serve as a marker of rapid changes in clinical status.
This could reflect, in part, differences between acute
and chronic injury.

These posttranslational modifications of sTnI and
sTnT are similar in some respects to those recently
described for cTnI in the ischemic-reperfused isolated
rat heart (12, 20). In that model, as the severity of
ischemic-reperfusion injury increases from reversible
to irreversible, contractile function deteriorates in asso-
ciation with progressive degradation of cTnI (31) and
the formation of covalent complexes between its frag-
ments and cTnT or cTnC; little if any intact cTnI is
released (20). In the present study, we have shown for
the first time that severe hypoxemia initiates similar
processes in canine diaphragm. The functional conse-
quences of protein modification in the heart and respi-
atory muscles, however, likely differ. Protein modifi-
cation in the heart impairs contractile function and,
therefore, myocardial performance because the heart
contracts as a syncytium. In contrast, protein modifica-
tion in some diaphragmatic (or any other respiratory
muscle) fibers need not impair ventilation if other
motor units within the diaphragm and/or other respira-
atory muscles compensate. Thus protein modification
may occur well before any ventilatory impairment.

The molecular mechanisms and the enzymes respon-
sible for TnT and TnI modifications in the diaphragm
are unknown. However, in cardiac muscle during ische-