Selective Diaphragm Muscle Weakness Following Contractile Inactivity During Thoracic Surgery

Willem N. Welvaart¹,², Marinus A. Paul¹, Ger J.M. Stienen², Hieronymus W.H. van Hees⁶, Stephan A. Loer³, R.A. Bouwman³, Hans Niessen⁴, Frances S. de Man⁵, Christian C. Witt⁸, Henk Granzier⁹, Anton Vonk-Noordegraaf⁵ and Coen A.C. Ottenheijm²,⁹

Depts of ¹Surgery, ²Physiology, ³Anesthesiology, ⁴Pathology and Cardiac Surgery, and ⁵Pulmonology, VU University Medical Center/ Institute for Cardiovascular Research, Amsterdam, ⁶Dept of Pulmonary Diseases, Radboud University Nijmegen Medical Center, ⁷Dept of Surgery, Rivierenland Hospital, Tiel, the Netherlands, ⁸Dept of Anesthesiology and Intensive Care, Universitätsklinikum Mannheim, University of Heidelberg, Germany, and ⁹Dept of Physiology, University of Arizona, USA.

Abstract

Rationale
Postoperative pulmonary complications are significant contributors to morbidity in patients who have undergone upper abdominal, thoracic, or cardiac surgery. The pathophysiology of these complications might involve postoperative inspiratory muscle weakness. The nature of postoperative inspiratory muscle weakness is unknown.

Objective
To investigate the effect of surgery on the functioning of the diaphragm, the main muscle of inspiration.

Methods
Serial biopsies from the diaphragm and the latissimus dorsi muscle were obtained from 6 patients during thoracotomy for resection of a tumor in the right lung. Biopsies were taken as soon as the diaphragm had been exposed (t(0)) and again after 2 hours (t(2)). The contractile performance of demembranated muscle fibers, as well as fiber morphology and markers for proteolysis, was determined.

Results
In all patients, the force-generating capacity of diaphragm muscle fibers at t(2) was significantly reduced (~35%) compared with that at t(0), with a more pronounced force loss in type 2 fibers compared with type 1 fibers. Diaphragm weakness was not part of a generalized muscle weakness as contractile performance of latissimus dorsi fibers was preserved at t(2). Diaphragm fiber size and myofibrillar structure were not different at t(2) compared with t0, but myosin heavy chain type 2 was significantly reduced at t(2) and MuRF-1 mRNA and protein levels were elevated at t(2).

Conclusions
Only 2 hours of thoracic surgery causes marked, and selective, diaphragm muscle fiber weakness.
7.1 Introduction

Postoperative pulmonary complications, such as atelectasis, pneumonia and respiratory failure, are significant contributors to morbidity in patients who have undergone thoracic, cardiac, or upper abdominal surgery (1,2). Several reports (3-8) suggest that these pulmonary complications after surgery are related to postoperative inspiratory muscle weakness. For instance, preoperative inspiratory muscle training, which is known to prevent postoperative inspiratory muscle weakness (7), improves postoperative lung function (6) and reduces the duration of postoperative mechanical ventilation (7). Furthermore, recent studies by Hulzebos et al. (4) revealed that preoperative inspiratory muscle training in patients undergoing cardiac surgery reduces the incidence of postoperative pulmonary complications by approximately ~50% and reduces the duration of postoperative hospitalization.

The nature of this postoperative inspiratory muscle weakness is unknown. Normal respiratory functions are profoundly altered during general anesthesia, intrathoracic or abdominal surgery, and mechanical ventilation. Although neural structures of the inspiratory muscles might be involved, postoperative inspiratory muscle dysfunction might also be the result of intrinsic muscle fiber weakness. It is well known that anesthetics, as well as inflammatory mediators, such as cytokines, can directly affect muscle fiber function (9,10). In addition, mechanical ventilation-induced inspiratory muscle unloading can result in rapid diaphragm muscle fiber alterations, as shown recently by Levine and colleagues (11).

The objective of the present study was to investigate for the first time whether a short-term interruption of normal inspiratory muscle activity during surgery causes weakness of the diaphragm, the main muscle of inspiration. To this end, we used a longitudinal approach and determined the contractile performance of single diaphragm muscle fibers at the end of thoracic surgery and compared this with that at the start of surgery - note that we chose to study patients undergoing thoracic surgery, rather than upper-abdominal or cardiac surgery, as during thoracic surgery the diaphragm is readily accessible. This approach, using single diaphragm
muscle fibers, should reveal whether intrinsic diaphragm muscle fiber weakness develops during thoracic surgery, independent of patient motivation or effects on neural input. To test whether changes in diaphragm contractile performance are part of a generalized muscle weakness, we evaluated as a control the functioning of the non-respiratory latissimus dorsi muscle.

7.2 Methods

7.2.1 Patients

Biopsies from the diaphragm and the latissimus dorsi muscle were obtained from 6 patients during thoracotomy for resection of a tumor in the right lung (T1-3NoMx). After induction of general anesthesia (propofol 3 mg/kg, sufentanil 0.20 mg/kg) a double-lumen tube was placed and anesthesia was maintained with propofol and sufentanil. In addition, patients received a thoracic epidural for postoperative pain therapy. During single lung ventilation one lung was allowed to deflate while the other was ventilated. From five patients, biopsies were taken as soon as the diaphragm had been exposed (t0) and again after two hours (t2). The sixth patient (#6 in table 1) had only one hour between the first and second biopsies; this patient is discussed separately. Note that the biopsy locations at t0 and and t2 were approximately ~ 2 cm apart, and that the diaphragm biopsies were obtained from the ‘non-ventilated’ side of the diaphragm. Three of the six patients (number 1, 4, and 6) had mild airway obstruction (table 1) and none of the six patients had a history of chronic heart failure or neuromuscular disease. General patient characteristics are shown in table 1. Informed consent was obtained from each subject, and the study was approved by the local ethical committee.

Table 1
Patients characteristics

<table>
<thead>
<tr>
<th></th>
<th>Sex</th>
<th>Age (y)</th>
<th>BMI</th>
<th>FEV1 (% predicted)</th>
<th>FEV1/VC (%)</th>
<th>Surgery</th>
<th>Time from start anesthesia to 1st biopsies (min)</th>
<th>Time between 1st and 2nd biopsies (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>M</td>
<td>56</td>
<td>23</td>
<td>88</td>
<td>58</td>
<td>Tumor resection</td>
<td>60</td>
<td>2</td>
</tr>
</tbody>
</table>
### 7.2.2 Biopsy handling

The fresh biopsy (~75 mg) was divided in four parts: one part for determination of single fiber contractile performance (stored in cold relaxing solution containing 50% glycerol (v/v); for composition of relaxing solution see (12)); one part for electronmicroscopy (fixed in 2% glutaraldehyde); one part for immunohistochemistry (stored in liquid nitrogen), and one part for real-time polymerase chain reaction (PCR) (stored in liquid nitrogen).

Contractile performance of demembranated single muscle fibers

Composition of relaxing and activating solutions used for contractile measurements, as well as the contractile protocol, were reported previously(12,13), and are presented in the supplement. In brief, single muscle fibers were isolated from the biopsies and demembranated (i.e., skinned). The skinning procedure renders the membranous structures in the muscle fibres permeable, which enables activation of the myofilaments with exogenous Ca$^{2+}$. Preparations were mounted between a displacement generator and a force transducer element. Steady-state force, measured while bathed in the activating solution, was measured.

### 7.2.3 Immunohistochemistry and electronmicroscopy

Immunohistochemistry and electronmicroscopy were performed as described previously (14). For details see supplement.

MAFbx and MuRF-1 expression with real-time quantitative PCR

The methodology for MAFbx and MuRF-1 expression was as reported previously (15) and is presented in the supplement.
7.2.4 Protein analyses
The methodology for analysis of titin and myosin heavy chain (MHC) protein content and of myosin heavy chain isoform composition, as well as for Western blotting of MuRF-1, are presented in the supplement and have been described previously (16-19).

7.2.5 Statistical analysis
Differences in parameters at t2 and t0 were analyzed with repeated measures two-way ANOVA, followed by Bonferroni post-hoc tests (SPSS 16.0 for Windows, SPSS, Chicago IL). All data were verified for normal distribution. Data are presented as mean ± SEM, and a p-value < 0.05 was considered statistically significant.

7.3 Results

7.3.1 Diaphragm muscle fiber function
We determined the contractile performance of 180 muscle fibers (110 from the diaphragm, and 71 from the latissimus dorsi) obtained from five patients (#1-5) at the start of thoracic surgery and again after two hours of surgery. Maximum force generation of single muscle fibers is proportional to the cross sectional area (CSA) of the muscle fiber, and force was normalized to CSA. These experiments revealed significantly reduced maximum force generating capacity of diaphragm muscle fibers at the end of surgery (t2). This marked reduction was significant within all five patients (reduced to 63 ± 6%, 67 ± 8%, 55 ± 7%, 46 ± 3%, and 74 ± 5% of force at t0, patients 1-5 respectively; per patient 10-12 diaphragm fibers were analyzed for each time point). The human diaphragm mainly consists of type slow and type 2A fibers (i.e., muscle fibers expressing the slow myosin heavy chain isoform or the 2A isoform) (13). To study whether the magnitude of force reduction at t2 was different between fiber types, we also analyzed the force reduction per fiber type. As shown in figure 1A, both slow and 2A fibers displayed significant force reduction at t2, but with a more pronounced reduction in 2A fibers (~46% vs. ~28% in slow fibers).

In contrast to the force reduction in diaphragm fibers, no alterations in the contractile performance of single muscle fibers from the latissimus dorsi was observed at t2 (figure 1B; per patient 5-7 muscle fibers were
analyzed for each time point). Note that latissimus dorsi muscle almost exclusively consisted of type slow fibers. Thus, the force generating capacity of diaphragm muscle fibers was reduced by approximately ~35% after two hours of thoracic surgery, with the most pronounced reduction in 2A fibers, and with no effect on latissimus dorsi muscle fibers.

Figure 1
Contractile performance of demembranated diaphragm muscle fibers in five patients (pt) immediately after start of thoracic surgery (t0) and directly before the end of surgery approximately two hours later (t2).
(A) Left panel: all patients show a marked reduction in the maximal force generating capacity of diaphragm muscle fibers after two hours of surgery, with the most pronounced reduction in type 2A fibers. Right panel: typical force response of a type slow diaphragm fiber from patient #2 at t0 and t2 superimposed; note the reduced force generation at t2.
(B) Left panel: the force generating capacity of type slow latissimus dorsi muscle fibers was not different between t0 and t2. Right panel: Typical force response of latissimus dorsi fiber from patient #2 at t0 and at t2. For clarity of presentation, force at t2 is shown as a percentage of force at t0. Data presented as mean ± SEM.

7.3.2 Myofibrillar structure and muscle fiber cross sectional area
To test whether the observed diaphragm muscle fiber weakness at the end of surgery (t2) was related to myofibrillar damage, we performed electronmicroscopy on diaphragm myofibrils. These experiments revealed well-aligned z-disks, and regular myofibrillar structure at t2 (figure 2A), suggesting that the myofibrillar structure was preserved during surgery.

(A) Typical example of myofibrillar ultrastructure, evaluated by electronmicroscopy, in diaphragm muscle fibers at t0 and at t2. Note the well-aligned z-disks and normal myofibrillar structure in diaphragm muscle fibers at t2. Bar = 4 µm.

(B) Immunohistochemical analysis of diaphragm sections (7 µm thick), using antibodies directed against slow (brd-5) and fast (sc-71) myosin heavy chain, as well as an antibody reactive to laminin to outline muscle fibers. No difference in diaphragm fiber cross sectional at t2 compared to t0 is seen.
Fibers reacting with the myosin heavy chain antibody appear green. Bar = 60 µm. Table shows average diaphragm fiber cross sectional areas in patients at t2 and t0 for both fiber types.

Next, we investigated whether diaphragm fiber cross sectional area was altered at t2 vs t0. As shown in figure 2B (and table), diaphragm fiber cross sectional area was comparable between t2 and t0 for both slow and fast fibers in all patients. No significant difference was observed in fiber type proportion at t2.

7.3.3 Myofibrillar protein content and proteolysis

To investigate whether changes occurred in myofibrillar protein content and rate of proteolysis in the diaphragm at t2, we first studied the content of titin, a protein highly sensitive to proteolysis. As shown in figure 3A, titin/MHC ratio’s were not different at t2 compared to t0, and no T2 band was detectable (T2 is a well established titin degradation product (20)).

Next, we studied MHC isoform levels. As shown in figure 3B, the levels of MHC 2A relative to MHC slow were reduced at t2, suggesting elevated degradation of MHC 2A. To test whether proteolytic pathways were activated at t2, we first determined MAFbx and MuRF-1 mRNA levels in the diaphragm by means of real-time quantitative PCR. Compared to t0, diaphragm samples from all patients had higher levels of MuRF-1 at t2, with on average an approximately ~2 fold increase (figure 3C). MAFbx levels were on average higher at t2 compared to t0, but this increase did not reach significance. To verify whether the increase in MuRF-1 mRNA levels resulted in elevated MuRF-1 protein, Western blotting was used. As shown in Figure 3D, there was a significant increase in MuRF-1 protein at t2 compared to t0 in all patients studied. Thus, after two hours of surgery proteolytic pathways were activated.
A) SDS-agarose gel analysis revealed no difference in titin/MHC ratio’s at t2 compared to t0, and no T2 band was detectable (T2 is a well established titin degradation product. As a protein marker a human left ventricle (hh) sample is shown.

B) Specialized SDS-PAGE to separate MHC isoforms revealed reduction of MHC 2A isoforms, relative to MHC slow, at t2 compared to t0.

C) E3-ligase mRNA levels in the diaphragm at t2 vs t0. MuRF-1 levels are higher at t2 compared to t0 in all patients, with an average 2-fold increase.

D) In the line with the mRNA data, Western blotting showed elevated MuRF-1 protein in the diaphragm at t2 compared to t0. A typical MuRF-1 Western blot is shown below. Actin was used to normalize protein loading, and as controls muscle tissue from MuRF-1 wt (+) and knockout (-) mice were run. Data presented as mean ± SEM.

7.3.4 Protein nitration in diaphragm muscle fibers

It is well established that a systemic inflammatory response, which is known to occur during surgery, can induce nitrosative stress and thereby impair muscle fiber function (9,21). Therefore, we determined the levels of nitrotyrosine, a widely used marker for nitrosative stress (22), within diaphragm muscle fibers. As shown in figure 4, nitrotyrosine levels were significantly and homogenously reduced in all patients at t2 compared to t0 (average reduction 28%).
Unlike patients 1-5, who had two hours between biopsies, patient #6 had only one hour between biopsy collections (table 1). Interestingly, this patient displayed no differences in protein nitration and MuRF-1 mRNA expression in diaphragm fibers at t1 compared to t0, and displayed the smallest reduction in maximal force generating capacity of diaphragm fibers when compared to the five patients with two hours between biopsies (reduced to 76 ± 6% of force at t0, n = 10 fibers per time point).

7.4 Discussion
To our knowledge, this is the first longitudinal study to reveal the development of marked diaphragm muscle fiber weakness during thoracic surgery. Development of diaphragm muscle fiber weakness was associated with reduced contractile protein content and activation of proteolytic pathways. Importantly, loss of function was not observed in the non-respiratory latissimus dorsi muscle fibers. We speculate that this
selective diaphragm muscle fiber weakness contributes to the development of postoperative pulmonary complications.

7.4.1 Development of selective diaphragm muscle fiber weakness during thoracic surgery

Reliable determination of in vivo diaphragm muscle fiber function is hampered by methodological constraints, even more so during surgery. An elegant alternative to investigate diaphragm function is the use of demembranated muscle fibers isolated from patient biopsies. In demembranated muscle fibers, the membranous structures, such as the sarcolemma and sarcoplasmic reticulum, are made permeable, while leaving the contractile machinery (i.e. myofibrils) intact. By attaching these demembranated fibers to a force transducer and exposing them to exogenous calcium, their contractile performance was evaluated. These experiments revealed an approximately ~35% loss of the force generating capacity of diaphragm muscle fibers during two hours of thoracic surgery (figure 1A). This loss of muscle fiber function is not part of a generalized muscle weakness as function was preserved in the non-respiratory latissimus dorsi muscle (figure 1B). Considering that in demembranated fibers only the myofibrils are left intact, the loss of function in diaphragm fibers is likely to be caused by damage to, or loss of, myofibrillar structures or by posttranslational modification of myofibrillar proteins. Our findings suggest that, whereas myofibrillar (ultra-) structure is preserved (figure 2), the level of nitrosative modification of proteins is reduced in both type slow and 2A muscle fibers of the diaphragm during thoracic surgery (figure 4). We speculate that this ‘reductant nitrosative stress’ was involved in the impaired force production of diaphragm muscle fibers at t2. This speculation is based on the paradigm of Reid, et al. (21,23), which predicts that an optimal cellular redox state exists whereby conditions are ideal for force production. It follows that a deviation from the optimal redox balance leads to a loss of force production. Thus, considering that the basal redox level in skeletal muscle is slightly reduced (23), a further reduction of the redox status in the diaphragm at t2 might have impaired force generation. Interestingly, the force reduction during surgery was more pronounced in type 2A compared to slow diaphragm fibers. This exaggerated response of type 2A fibers is likely to be caused by an increased loss of myosin heavy chain in these fibers (figure 3B).
During the past decade, several studies have used diaphragm biopsies obtained during thoracotomy to investigate the effect of, for instance, COPD (13,15,24-32) and heart failure (33,34), on the diaphragm. However, the marked effects of only two hours of thoracic surgery on diaphragm muscle fibers clearly warrants caution when performing such studies: to prevent confounding effects of the surgery itself on diaphragm function, future research should carefully control the time of biopsy.

7.4.2 Potential mechanisms underlying the development of diaphragm muscle weakness

The mechanism(s) underlying the development of diaphragm muscle fiber weakness during thoracic surgery need further studies. During thoracic surgery many systemic alterations occur, such as increased levels of inflammatory mediators (35), which can directly affect muscle fiber function (36-39). However, if such a systemic etiology is at play, the diaphragm and latissimus dorsi muscle are expected to be both affected. As our findings indicate that the diaphragm and latissimus dorsi are affected differentially, a major role for systemic alterations is not likely.

Anesthetic agents can impair diaphragm function as well. Previous studies have shown that propofol, the anaesthetic used in the six patients described here, impairs diaphragm function (40). However, propofol affected peripheral skeletal muscles to a similar extent (40), an affect that was not observed in the present study. Furthermore, propofol’s mode of action was mainly directed at phenomena upstream in the process of excitation contraction coupling, such as the neuromuscular transmission, rather than at myofibrillar function (40). Thus, considering that (1) the function of the latissimus dorsi muscle was not affected in the present study and that (2) we studied demembranated muscle fibers, in which neuromuscular transmission does not play a role, it seems unlikely that propofol plays a major role in the development of diaphragm muscle fiber weakness during thoracic surgery.

Alternatively, the sudden mechanical inactivity of the diaphragm during surgery might play a role. The functioning and morphology of the diaphragm, unlike non-respiratory muscles, has been shown to be very sensitive to mechanical ventilation-induced muscle unloading (11,41) (for review see (42,43)). For instance, Levine and colleagues (11) reported that 18-69 hours of mechanical ventilation of brain-dead patients causes severe diaphragm muscle fiber atrophy, whereas fiber size of the non-respiratory pectoralis major muscle was preserved. This selective
diaphragm muscle fiber atrophy was associated with a markedly increased expression of the E3-ligases MAFbx and MuRF-1 (11), which are considered key markers of proteolytic activity in muscle (44). Increased expression of E3-ligases in the diaphragm was also reported by another recent study on mechanically-ventilated brain-dead patients (45). Thus, the selective diaphragm muscle fiber weakness and the increased expression of MuRF-1 in the present study (figures 3C and D), after only two hours of mechanical ventilation, is in line with previous reports on the effects of mechanical ventilation on human diaphragm, and suggests rapid activation of proteolytic machinery in the diaphragm during thoracic surgery. Interestingly, recent studies indicate that MuRF-1 is a muscle fiber type 2 associated factor (18), and that expression of MuRF-1 is required for remodeling of type 2 fibers during pathophysiological stress states (18). Furthermore, myosin heavy chain and myosin binding proteins have been shown to be substrates for MuRF-1-mediated degradation (46,47). Thus, our data suggest that during two hours of thoracic surgery, upregulation of MuRF-1 caused increased degradation of MHC 2A, which contributed to the more pronounced force impairment in type 2A fibers compared to type slow fibers. That the size of both slow and 2A diaphragm fibers was still preserved at t2 (figure 2B), despite activation of proteolytic machinery, is not surprising as the extensive muscle fiber remodeling needed to reduce muscle fiber size is likely to require longer than only two hours.

It is noteworthy that previous work on animal models reported no change in nitrotyrosine levels in the diaphragm after 18 hours of mechanical ventilation (48), whereas we found decreased levels of nitrotyrosine in the diaphragm at t2. Although this discrepancy might suggest that mechanical ventilation alone can not explain the observed changes in protein nitration at t2 compared to t0, it can not be ruled out that nitrotyrosine formation in the diaphragm during mechanical ventilation might be time-dependent with lower formation in the first hours after start of mechanical ventilation followed by increased formation during prolonged ventilation. To our knowledge, so far no studies have investigated the effect of short-term (0-2 hours) mechanical ventilation on diaphragmatic nitrosative stress.

7.5 Clinical implications
Postoperative pulmonary complications, as well as prolonged duration of postoperative mechanical ventilation and hospitalization, have been suggested to be partly caused by postoperative diaphragm weakness. The present study reveals, for the first time, that during the course of only two hours of thoracic surgery marked diaphragm muscle fiber weakness develops. It could be argued that this diaphragm muscle fiber weakness is not clinically relevant, as the patients studied here did not experience postoperative pulmonary complications. However, we would like to emphasize that these patients had no history of severe chronic lung or heart disease and were not at high-risk for postoperative pulmonary complications. In our opinion the development of such profound diaphragm weakness during surgery might seriously affect postoperative outcome in patients who are at high risk for pulmonary complications or in patients who already have a compromised diaphragm function, such as patients with moderate-to-severe COPD (13) or heart failure (33). This is corroborated by previous work showing that preoperative inspiratory muscle training reduced the incidence of postoperative pulmonary complications by 50% in high-risk patients undergoing cardiac surgery (4). Future studies on high-risk patients should evaluate whether the magnitude of diaphragm muscle fiber weakness correlates with the incidence of pulmonary complications. Furthermore, as the diaphragmatic changes were much less pronounced in the patient (#6) with only one hour between biopsies, it could be speculated that the development of diaphragm weakness during surgery is time-dependent. Consequently, the development of diaphragm weakness might be even more exaggerated during longer-term (>2 hours) (cardio-) thoracic surgery. Such time-dependency of diaphragm muscle weakness should be evaluated in future work by studying biopsies obtained at multiple time-points during longer-term surgery.

The diaphragm muscle fiber weakness that develops during thoracic surgery is the result of myofibrillar dysfunction. Importantly, myofibrillar function can be enhanced by pharmaceutical agents, such as calcium sensitizers. These agents target the myofibrillar proteins directly and significantly augment force production, as shown previously in diaphragm fibers from patients with chronic obstructive pulmonary disease (26). Thus, the present findings may open new treatment windows for the prevention/attenuation of postoperative diaphragm weakness.
**7.6 Conclusions**

In conclusion, we found that two hours of diaphragm inactivity during thoracic surgery causes marked, and selective, diaphragm muscle fiber weakness. Future studies should address whether these findings cross specialty disciplines, and also occur during other surgeries that have been associated with inspiratory muscle weakness, such as upper abdominal surgery and cardiac surgery (3,7,8). We speculate that the pathophysiology of pulmonary complications after thoracic, cardiac, and upper abdominal surgery shares the development of diaphragm muscle fiber weakness.

**References**


7.7 METHODS

7.7.1 Contractile performance of demembranated single diaphragm muscle fibers

Single muscle fibers were isolated from the biopsies and demembranated (i.e., skinned) for 30 minutes at ~4°C in relaxing solution (in mM: 20 BES, 10 EGTA, 6.56 MgCl₂, 5.88 NaATP, 1 DTT, 46.35 K-propionate, 15 creatine phosphate, pH 7.0 at 20°C) containing 1% (v/v) Triton X-100. To prevent protein degradation, the solutions contained protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), 0.5mM; Leupeptin, 0.04mM; E64, 0.01mM). The skinning procedure renders the membranous structures in the muscle fibres permeable, which enables activation of the myofilaments with exogenous Ca²⁺. Preparations were washed thoroughly with relaxing solution and mounted between a displacement generator and a force transducer element (AE 801, SensoNor, Norway) using aluminum T-clips. Sarcomere length was set at 2.5 μm using a He-Ne laser diffraction system. Fiber width and diameter were measured at three points along the fiber and the cross-sectional area was determined assuming an elliptical cross-section. Three different bathing solutions were used during the experimental protocols: a relaxing solution, a pre-activating solution with low EGTA concentration, and an activating solution with high calcium concentration. The composition of these solutions was as described previously (1). Steady-state force, measured while bathed in the activating solution, was normalized to fiber cross-
sectional area. At the end of the single-fiber contractile measurement protocol, myosin heavy chain isoform composition of the fiber was identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as described previously (2).

7.7.2 Immunohistochemistry and electronmicroscopy
Cryosections (7 μm thick) were rehydrated for 10 minutes in phosphate buffer and subsequently blocked with phosphate buffer containing 0.3% (w/v) bovine serum albumine. Subsequently, cryosections were incubated with primary brd-5 (slow), sc-71 (fast) or WGA antibody, followed by appropriate fluorescent ALEXA labeled secondary antibodies (Molecular Probes, Eugene, Oregon, USA). Following each incubation, cryosections were washed three times for five minutes with phosphate buffer. Separate sections were incubated with a primary antibody (rabbit) raised against rat nitrotyrosine (A-21285; Molecular Probes) and then incubated with a secondary antibody as described above. Finally, the sections were embedded in Mowiol (10% (w/v) in 0.1M Tris-HCL, pH 8.5 / 25% (v/v) glycerol / 2.5% (w/v) NaN₃). Sections were quantitatively analyzed with use of an inverted digital imaging microscopy workstation (Intelligent Imaging Innovations (3i) equipped with a motorized stage and multiple fluorescent channels. For quantification of nitrotyrosine staining, the mean intensities of the red channel were normalized per fiber area after subtraction of non-specific staining (which was obtained from experiments on serial sections in which the primary antibody was omitted). A cooled charge-coupled device camera (Cooke Sensicam; Cooke Co., Eugene, OR) was used to record images. Exposures, objective, montage, and pixel binning were automatically recorded and stored in memory. Dedicated imaging and analysis software (SlideBook, version 4.2, 3i) was obtained from Intelligent Imaging Innovations (Denver, CO). For electronmicroscopy, samples were fixed in 2% (vol/vol) glutaraldehyde for 30 minutes and 1.5% (wt/vol) osmium tetroxide for 10 minutes, dehydrated with acetone, and embedded in Epon812. Ultrathin sections were collected on 300-mesh Formavar-coated nickel grids. The sections were contrasted with uranyl acetate and lead citrate and were examined in a Jeol-1200EX electron microscope.

7.7.3 MAFbx and MuRF-1 mRNA determination with real-time quantitative PCR
Total RNA was extracted from diaphragm samples using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The extracted RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and the concentration was determined by spectroscopy at 260 nm using the Ultrospec 1000 UV/Visible Spectrophotometer (Pharmacia Biotech, Foster City, CA). Total RNA was then reverse transcribed into cDNA using 50 ng of total RNA in a 20μl reaction volume by using SuperScript™ Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed in a total reaction volume of 25 μl per reaction. The 25 μl reaction mixture contained 12.5 μl of a SYBR green mix (Bio-Rad, Salt Lake City, UT), 10 pmol of each forward and reverse primer, 1μl cDNA and nuclease-free water to make up the reaction volume. Specific primers were selected using express software (Applied Biosystems, Foster City, CA). Forward and reverse oligonucleotides used were as following: MAFbx, 5’-CATCCCTATGTACACTGTCAC-3’ and 5’-ATCCGACCATGATTTGTAAT-3’, MuRF-1, 5’-AATTTGGAAGCAGCTGATCTG-3’ and 5’-TAGGGATTTGCAGCCTGGA-3’; Glyeraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-ATTCCACCACATGGCAAATTC-3’ and 5’-ATTCCACCATGGCAAATTC-3’. These primers were synthesized by Sigma Genosys. PCR runs were performed in triplicate using MyiQ real time PCR detection system (Bio-Rad, Salt Lake City, UT). Levels of MAFbx and MuRF-1 mRNA were normalized to that of GAPDH in arbitrary units, as well as to TBP (TATAbox binding protein) and to B2M (beta-2-microglobuline). Normalization to GAPDH, TBP, and to B2M rendered similar results.

7.7.4 Gel Electrophoresis and Western Blots
For titin analyses 1% SDS-agarose electrophoresis was performed as previously described (3). Wet gels were scanned and analyzed with One-D scan EX (Scanalytics Inc., Rockville, MD, USA) software. The integrated optical density of titin, nebulin, and myosin heavy chain were determined as a function of the volume of solubilized protein sample that was loaded (a range of volumes was loaded on each gel). The slope of the linear range of the relation between integrated optical density and loaded volume was obtained for each protein. For determination of myosin heavy chain isoform composition muscle samples (or single
muscle fiber) were placed in SDS sample buffer containing 62.5 mM Tris_HCL, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.001% (wt/vol) bromophenol blue at a pH of 6.8. The samples were stored at -80°C until assayed. The samples were denatured by boiling for two minutes. The stacking gel contained a 4% acrylamide concentration (pH 6.7), and the separating gel contained 7% acrylamide (pH 8.7) with 30% glycerol (v/v). Sample volumes of 10 µl were loaded per lane. The gels were run for 24h at 15°C and a constant voltage of 275V. Finally, The gels were silver-stained, scanned, and analyzed with One-D scan EX software. For MuRF-1 Western blotting a total of 100 µg of solubilized extracts were loaded on gel, and separated on 4–12% gradient SDS-acrylamide gels (Invitrogen) followed by transfer onto PVDF membranes. The protein levels of MuRF1 were determined with a specific MuRF1 antibody previously described [Labeit et al, 2010; Moriscot et al, 2010]. After incubation with primary antibodies, specific bands were visualized by enzymatic chemiluminescence using horseradish peroxidase-conjugated secondary antibodies, (Super Signal West Pico; Pierce, Bonn, Germany), that were quantified by densitometry using a onedimensional scan software package (Scanalytics, Rockville, USA). Loading variations were monitored by Coomassie-staining.

References