Mechanisms Underlying Skeletal Muscle Weakness in Human Heart Failure
Alterations in Single Fiber Myosin Protein Content and Function

Mark S. Miller, PhD; Peter VanBuren, MD; Martin M. LeWinter, MD; Stewart H. Lecker, MD; Donald E. Selby, PhD; Bradley M. Palmer, PhD; David W. Maughan, PhD; Philip A. Ades, MD; Michael J. Toth, PhD

Background—Patients with chronic heart failure (HF) frequently experience skeletal muscle weakness that limits physical function. The mechanisms underlying muscle weakness, however, have not been clearly defined.

Methods and Results—This study examined the hypothesis that HF promotes a loss of myosin protein from single skeletal muscle fibers, which in turn reduces contractile performance. Ten patients with chronic HF and 10 controls were studied. Muscle atrophy was not evident in patients, and groups displayed similar physical activity levels, suggesting that observed differences reflect the effects of HF and not muscle atrophy or disuse. In single muscle fibers, patients with HF showed reduced myosin heavy chain protein content (P<0.05) that manifested as a reduction in functional myosin-actin cross-bridges (P<0.05). No evidence was found for a generalized loss of myofilament protein, suggesting a selective loss of myosin. Accordingly, single muscle fiber maximal Ca\textsuperscript{2+}-activated tension was reduced in myosin heavy chain I fibers in patients (P<0.05). However, tension was maintained in myosin heavy chain IIA fibers in patients because a greater proportion of available myosin heads were bound to actin during Ca\textsuperscript{2+} activation (P<0.01).

Conclusions—Collectively, our results show that HF alters the quantity and functionality of the myosin molecule in skeletal muscle, leading to reduced tension in myosin heavy chain I fibers. Loss of single fiber myosin protein content represents a potential molecular mechanism underlying muscle weakness and exercise limitation in patients with HF. (Circ Heart Fail. 2009;2:700-706.)

Key Words: exercise ■ heart failure ■ mechanics ■ myosin ■ skeletal muscle

Patients with chronic heart failure (HF) have a reduced capacity for physical work. Although cardiac dysfunction is the primary pathological insult, the resulting syndrome of HF alters numerous physiological systems to impair functional capacity. Alterations in skeletal muscle are of particular importance, most notably atrophy, weakness, and reduced endurance. These peripheral skeletal muscle adaptations limit physical function independent of cardiac impairment and persist despite correction of cardiac dysfunction.

Clinical Perspective on p 706

Aerobic fitness is commonly assumed to be the primary determinant of physical function in patients with HF because exertional fatigue and dyspnea are predominant symptoms. However, aerobic capacity is a relatively poor predictor of performance in activities of daily living. This is because most activities are limited instead by skeletal muscle strength. Accordingly, functional capacity can be increased in patients with HF in the absence of alterations in aerobic capacity by improving muscle strength. Despite its potential relevance to physical disability in patients with HF, few studies have explored the mechanisms underlying reduced skeletal muscle strength.

Skeletal muscle weakness in HF is not explained by muscle atrophy or reduced motor activation, suggesting defects in the intrinsic contractile properties of individual muscle fibers. Recent studies in rats and humans in chemically skinned single muscle fibers have shown reduced contractile function in HF, implicating impaired myofilament protein function in muscle weakness. Because myosin is the most prevalent myofilament protein in muscle and the primary determinant of single fiber contractile mechanics, alterations in fiber function can be linked to variation in the quantity or function of the myosin molecule. In this context, our previous work demonstrating a reduction in myosin heavy chain (MHC) protein content in skeletal muscles of patients with HF.
suggests that contractile dysfunction may result from a reduced quantity of myosin. Indeed, a recent study in rats with HF has shown reduced MHC protein content and force production in single diaphragm muscle fibers.\textsuperscript{11} In addition, this study found alterations in myosin kinetic properties that could diminish contractile function.\textsuperscript{11} However, whether similar alterations in the quantity and functionality of myosin occur in skeletal muscle of patients with HF has not been examined.

The aim of this study was to examine the effect of HF on single skeletal muscle fiber myofilament protein composition, function, and sarcomeric structure. To accomplish this objective, we evaluated single muscle fibers from the vastus lateralis muscle of patients with chronic HF and sedentary controls. Controls were recruited to match the low physical activity levels typically observed in patients with HF,\textsuperscript{16} which obviates the effects of muscle activity level on single fiber structure and function.\textsuperscript{17}

### Methods

#### Subjects

Ten patients (7 men and 3 women) with physician-diagnosed HF were recruited. The population consisted of patients with both systolic dysfunction (left ventricular ejection fraction <40%; n=6; 26.0±2.7%; range, 17% to 35%) and preserved systolic function (ejection fraction >40%; n=4; 46.8±2.18%; range, 45% to 52%). At the time of study, there were 1 New York Heart Association class I, 5 class II, and 4 class III patients. The etiology of HF was ischemic in 3 volunteers and nonischemic in 7. Three patients had type 2 diabetes mellitus. All patients were clinically stable and had not been hospitalized for at least 6 months before testing. None had evidence of hepatic, renal, or peripheral vascular disease or an active neoplastic process. Patients were receiving angiotensin-converting enzyme inhibitors/receptor blockers (100%), diuretics (70%), and 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (20%), and 1 female patient was receiving levothyroxine. Plasma creatine kinase levels were normal in all patients, and none were smokers or undergoing sex steroid replacement therapy.

Controls (n=10; 6 men and 4 women) recruited were sedentary or minimally active, as defined by self-report of ≤2 sessions of ≥30 minutes of exercise per week and not participating in any exercise training. Controls were nonsmokers; had a stable body weight (±2 kg during previous 6 months and not participating in a weight loss program); had no signs or symptoms of HF, coronary heart disease, or diabetes (fasting blood glucose >112 mg/dL); had normal left ventricular ejection fraction (>55%); had normal complete blood counts and routine biochemical values; and were not undergoing sex steroid replacement therapy. Four controls had a history of hypertension, and 3 were treated with diuretics and 1 with an angiotensin-converting enzyme inhibitor. All were normotensive at testing and showed no evidence of left ventricular hypertrophy or atrial enlargement by echocardiography. Two controls were on stable doses of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, and 1 woman was on levothyroxine. Plasma creatine kinase levels were normal in all controls. Informed consent was obtained from each volunteer, and the protocol was approved by the Committees on Human Research at the University of Vermont. Data showing reduced knee extensor muscle strength in patients with HF from this cohort have been published.\textsuperscript{3}

#### Experimental Protocol

Eligibility was determined during screening visits, at which time medical history, physical examination, blood samples, whole muscle strength testing, a treadmill test, and echocardiography were performed. At least 1 week later, eligible volunteers underwent an inpatient visit. Before admission, medications were maintained per normal dosing regimens, except coumadin (n=3), which was stopped 5 days before this visit. On the following morning, in the fasted state, body composition and mid-thigh muscle cross-sectional area were measured, and muscle tissue was obtained through percutaneous biopsy (Bergstrom needle, 5-mm outer diameter) of the vastus lateralis.

#### Total and Regional Body Composition

Total and regional fat mass and fat-free mass were measured by dual energy x-ray absorptiometry, as described previously.\textsuperscript{14} Bone mass data are not reported. Mid-thigh muscle area was measured by computed tomography, as described previously.\textsuperscript{18}

#### Peak Oxygen Consumption

Peak oxygen consumption (peak VO\textsubscript{2}) was determined using the Naughton protocol.\textsuperscript{19}

#### Accelerometry

Free-living physical activity was measured using a single-plane accelerometer, as described previously.\textsuperscript{20}

#### Muscle Tissue Processing

Approximately two thirds of the biopsy material was placed immediately into cold (4°C) dissecting solution (see online-only Data Supplement for composition of solutions). Remaining tissue was frozen in liquid nitrogen and stored at −80°C. Muscle fibers were dissected into bundles and tied to glass rods at 4°C and then placed in sinning solution for 24 hours at 4°C, storage solution with 50% (vol/vol) glycerol for 16 hours at 4°C, and finally stored at −20°C until study (within 4 weeks).

#### Tissue Homogenate MHC Protein Content and Isoform Distribution

Tissue MHC protein content and isoform distribution were determined on frozen muscle tissue, as described previously,\textsuperscript{21} with minor modifications (see Data Supplement).

#### Single Muscle Fiber Morphology and MHC Protein Content

Segments (~3 mm) of single muscle fibers were measured in relaxing solution (20°C) to estimate fiber volume (see Data Supplement for details). Aliquots (1.5 mm\textsuperscript{3} fiber volume) of sample were analyzed for MHC and actin protein content in triplicate, according to previously published methods,\textsuperscript{7,22} with modifications (see Data Supplement). Thereafter, each fiber was analyzed for fiber type by means of MHC isoform expression (see Data Supplement).

#### Single Fiber Mechanical Measurements

Segments (~2.5 mm) of single fibers were isolated and their ends fixed with glutaraldehyde, as described elsewhere,\textsuperscript{23} with modifications (see Data Supplement). Top and side diameter measurements were made in relaxing solution (pCa 8) at 3 positions to calculate cross-sectional area, and the fiber was incubated in dissecting solution containing 1% Triton X-100 (vol/vol) for 30 minutes. The fibers were attached to a piezoelectric motor and a strain gauge in a solution containing 1% Triton X-100 (vol/vol) for 30 seconds and then to activating solution (pCa 4.5), and tension was recorded at plateau. At plateau, sinusoidal measurements were performed at 15°C. The fiber was transferred to preactivating solution for 30 seconds and then to activating solution (pCa 4.5), and tension was recorded at plateau. At plateau, sinusoidal length oscillations (10 cycles of 0.125% fiber length at 250 Hz) were imposed to measure fiber dynamic stiffness. Duplicate measurements of maximal Ca\textsuperscript{2+}-activated tension and stiffness were obtained for each fiber. Thereafter, the fiber was placed in rigor solution, and at the plateau of tension, dynamic stiffness was measured. The amplitude of dynamic stiffness in the rigor state is proportional to the total number of available myosin heads that can bind actin (ie, total cross-bridge number), assuming all myosin heads bind to actin in rigor. The ratio of pCa 4.5 to rigor dynamic stiffness, therefore, provided an estimate of the fraction of available myosin
heads that bind actin during Ca\(^{2+}\) activation. After mechanical measurements, single fibers were analyzed for MHC isoform composition to identify fiber type.

**Ultrastructural Measurements**
Electron microscopy measurements were conducted on intact skeletal muscle fiber bundles, as described previously\(^{24}\) (see Data Supplement).

**Protein and Gene Expression**
Immunoblotting techniques were used to assess MHC degradation products, according to the method of Ball et al.,\(^{25}\) and the quantity of ubiquitinated MHC (see Data Supplement). MHC isoforms, actin, muscle ring finger-1, and atrogin-1 mRNA levels were determined by real-time polymerase chain reaction (see Data Supplement). MHC isoforms, actin, muscle ring finger-1, and atrogin-1 mRNA levels were determined by real-time polymerase chain reaction (see Data Supplement).

**Statistics**
All data are reported as mean±SE. Unpaired Student \(t\) tests were used to compare groups. Analysis of covariance was used to compare peak \(\text{VO}_2\) data between groups after adjusting for differences in fat-free mass using analysis of covariance.

*The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.*

**Results**
Groups were similar for age, body size, body mass, and total and regional body composition. Peak \(\text{VO}_2\) adjusted for fat-free mass was lower \((P<0.01)\) in patients (Table 1). Daily physical activity level measured by accelerometry over 7.6±0.4 days was similar between groups.

Patients with HF had lower MHC protein content in tissue homogenates compared with controls (Figure 1A; \(P<0.01\)), with no group differences in the relative distribution of MHC isoforms (Figure 1B).

Fibers from patients had greater average cross-sectional area compared with controls \((P<0.01;\) Table 2) when all fibers were pooled, with or without inclusion of those fibers with MHC band densities less than or equal to background.

When partitioned into fiber types, patients had greater cross-sectional area in MHC IIA and IIA/X fibers \((P<0.01\) for both) but not in MHC I fibers.

Patients with HF showed lower MHC protein content in single fibers whether or not fibers with MHC band densities less than or equal to background \((n=39\) fibers from patient group; \(n=25\) fibers from control group) were included \((P<0.01;\) Figure 2A). Actin was detected in all fibers examined (see Data Supplement). In fibers with detectable MHC bands, patients with HF had lower MHC content in MHC I \((P<0.05)\), MHC IIA \((P<0.01)\), and MHC IIA/X \((P<0.01)\) fibers (Figure 2B). There were too few MHC IIX and MHC I/IIA fibers to permit comparisons.

No group differences were evident for myofibrillar volume fraction, A-band length, or thick-to-thin filament ratio (Figure

**Table 1.** Clinical Characteristics and Physical Activity Levels of Controls and Patients With Heart Failure

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Heart Failure</th>
<th>n (Controls/ Heart Failure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>69.3±4.4</td>
<td>72.2±4.4</td>
<td>10/10</td>
</tr>
<tr>
<td>Height, cm</td>
<td>168.1±3.3</td>
<td>170.4±3.0</td>
<td>10/10</td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>81.9±4.0</td>
<td>92.4±9.9</td>
<td>10/10</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>29.9±2.3</td>
<td>32.7±5.3</td>
<td>10/9</td>
</tr>
<tr>
<td>Fat-free mass, kg</td>
<td>49.1±3.3</td>
<td>51.8±5.1</td>
<td>10/9</td>
</tr>
<tr>
<td>Arm fat-free mass, kg</td>
<td>5.3±0.50</td>
<td>5.27±0.61</td>
<td>10/9</td>
</tr>
<tr>
<td>Leg fat-free mass, kg</td>
<td>15.4±1.8</td>
<td>14.8±2.2</td>
<td>10/9</td>
</tr>
<tr>
<td>Midthigh muscle cross-sectional area, cm(^2)</td>
<td>108.8±8.8</td>
<td>109.0±11.2</td>
<td>8/9</td>
</tr>
<tr>
<td>Peak oxygen consumption, L/min*</td>
<td>1.93±0.12</td>
<td>1.24±0.12†</td>
<td>9/9</td>
</tr>
<tr>
<td>Physical activity level, kcal/d</td>
<td>215±32</td>
<td>251±45</td>
<td>9/10</td>
</tr>
</tbody>
</table>

*Data are mean±SE.†Peak oxygen consumption data were adjusted for fat-free mass using analysis of covariance.

\(P<0.01\).

**Figure 1.** Tissue homogenate MHC protein content (A, arbitrary densitometry units [AU] per microgram of protein) and relative isoform distribution (B, % of total) in controls (C; \(n=10\)) and in patients with HF (\(n=9\)) with representative sections of gels. Bar graphs represent mean±SE. \(*P<0.01.\)

**Table 2.** Average Cross-Sectional Area of Single Skeletal Muscle Fibers From Controls and Patients With Heart Failure

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Heart Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>All fibers, (\mu m(^2))</td>
<td>5880±145</td>
<td>6824±160*</td>
</tr>
<tr>
<td>Fibers with detectable MHC content, (\mu m(^2))</td>
<td>5916±159</td>
<td>6748±169*</td>
</tr>
<tr>
<td>MHC I fibers, (\mu m(^2))</td>
<td>6613±242</td>
<td>6538±247</td>
</tr>
<tr>
<td>MHC IIA fibers, (\mu m(^2))</td>
<td>5422±210</td>
<td>7419±305*</td>
</tr>
<tr>
<td>MHC IIA/X fibers, (\mu m(^2))</td>
<td>4450±193</td>
<td>6091±347*</td>
</tr>
</tbody>
</table>

*Data are mean±SE and reflect the average cross-sectional area from top and side diameter measurements every 250 \(\mu m\) along the length of each fiber. Fibers with detectable MHC include only those with band densities greater than background, whereas all fibers include those with band densities equal to or less than background. There were too few MHC IIX and I/IIA fibers to permit comparisons between groups.

\(*P<0.01.\)
tension in MHC I fibers ($P<0.05$), whereas MHC IIA fibers remained similar to controls (Figure 4A). pCa 4.5 stiffness was decreased in MHC I fibers ($P<0.01$) and unchanged in MHC IIA fibers (Figure 4B), whereas rigor stiffness was decreased in both MHC I and MHC IIA fibers (both $P<0.05$; Figure 4C) in patients. The pCa 4.5/rigor stiffness ratio did not differ between groups in MHC I fibers but was increased in MHC IIA fibers ($P<0.01$; Figure 4D) in patients. Finally, there was a subset of fibers in controls (n=7) and patients (n=20) that failed catastrophically (ie, tore) on Ca$^{2+}$ activation.

No differences were found between patients (n=7) and controls (n=5) in mRNA abundance for MHC I (controls: $1.00\pm0.19$ versus patients with HF: $1.10\pm0.33$ relative expression), MHC IIA (controls: $1.00\pm0.14$ versus patients with HF: $0.87\pm0.09$), MHC IIX (controls: $1.00\pm0.37$ versus patients with HF: $1.68\pm0.54$), or actin (controls: $1.00\pm0.12$ versus patients with HF: $0.96\pm0.10$). Similarly, no group differences were noted for mRNA of any of these genes when data were analyzed using 18S RNA as the housekeeping gene (see Data Supplement). In addition, no differences between patients and controls in the amount of MHC I breakdown fragments (Figure 5 A and C), ubiquitinated protein corresponding to MHC (Figure 5 B and D), or expression of E3 ubiquitin ligases (Figure 5 E and F) were found (see Data Supplement for further details).

**Discussion**

In this study, we found reduced single fiber MHC protein content in patients with HF, which was manifested as a decreased number of functional myosin-actin cross-bridges. However, no evidence was found for a generalized loss of myofibrillar proteins, suggesting a selective loss of myosin. In keeping with reduced MHC protein/cross-bridge number, single fiber maximal Ca$^{2+}$-activated tension (force per cross-sectional area) was reduced in MHC I fibers from patients. Interestingly, tension was maintained in MHC IIA fibers from patients because a greater proportion of myosin heads bound...
in single skeletal muscle fibers in humans. The effect of HF on the quantity and kinetic properties of the myofilament protein content and function that are specific to HF. Physical activity patterns is necessary to identify alterations in muscle adaptations to HF—a switch to a fast-twitch phenotype—may simply be a repercussion of the muscle disuse imposed by the disease state. Thus, experimental control for physical activity patterns is necessary to identify alterations in myofilament protein content and function that are specific to HF.

At the single fiber level, MHC loss was evident in all fiber types examined from patients with HF (Figure 2B). These results extend evidence from rat model to demonstrate, for the first time, that myosin is lost from single muscle fibers as a consequence of human HF. Further reinforcing our MHC content data, we found a decrease in single fiber dynamic stiffness in patients at the plateau of rigor tension (Figure 4C), suggesting a loss of functional myosin heads that can bind actin to form cross-bridges. Because myosin comprises 25% of total skeletal muscle protein and 40% of myofilament protein, this reduction may simply reflect a loss of myofilament protein secondary to muscle atrophy. However, the fact that we found no evidence for muscle atrophy in patients and no group differences in single fiber actin protein content (see Data Supplement) or myofibrillar volume fraction argues against this conclusion and implies a selective loss of myosin protein. This type of selective myosin depletion is not unique to HF and has been observed in acute quadriplegic myopathy patients and a rat model of cancer, suggesting that this phenotype may be common to a variety of acute and chronic disease states.

From a structural standpoint, loss of myosin could result from a decreased number of thick filaments, shortening of the thick filaments, and/or loss of myosin at random points along the thick filament. We found no evidence for a loss of thick filaments (thick-to-thin filament ratio) or shortening of the A-band (ie, thick filament) length in patients, implying a loss of myosin along the entire length of the thick filament. This structural phenotype of myosin depletion agrees with the fact that the thick filament is remodeled by replacing myosin at random points along the length of the filament.

Single fiber tension is directly related to the number of functional myosin heads, the proportion of these heads bound to actin during Ca\(^{2+}\) activation, implying altered myosin kinetic properties. Our results represent the first demonstration of an effect of HF on the quantity and kinetic properties of the myosin molecule in single skeletal muscle fibers in humans. Patients with HF are profoundly inactive relative to age-matched, healthy controls, which complicates group comparisons because inactivity modulates muscle structure and function. To mitigate the effect of inactivity, we recruited sedentary controls to match patients for muscle use and confirmed that their activity levels were similar to patients. In addition, patients were tested 6 months after inpatient admissions to eliminate any acute effects of muscle disuse on single fiber structure or function. An interesting observation that highlights the importance of these experimental considerations is the similar MHC isoform distribution (ie, fiber type) in patients and controls (Figure 1B). Our fiber type results agree with studies that have similarly controlled the activity status of controls but differ from those that have not. In these latter studies, a shift in fiber type toward a fast-twitch phenotype was observed. Because muscle disuse increases the proportion of fast-twitch fibers, these results collectively suggest that one of the presumed hallmark muscle adaptations to HF—a switch to a fast-twitch phenotype—may simply be a repercussion of the muscle disuse imposed by the disease state. Thus, experimental control for physical activity patterns is necessary to identify alterations in myofilament protein content and function that are specific to HF.

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Interestingly, despite the reduction in MHC content, no group differences in tension were found in MHC IIA fibers. The reason for this disparity was revealed when fiber stiffness was evaluated under maximal Ca\(^{2+}\)-activated and rigor conditions, which demonstrated a greater proportion of myosin heads bound during Ca\(^{2+}\) activation in MHC IIA fibers in patients (Figure 4D). As detailed above, a greater proportion of myosin heads bound to actin would increase tension. This adaptation could be explained by a change in myosin kinetic parameters, which increases the amount of time myosin is bound to actin or decreases the amount of time myosin is...
detached from actin or both. Thus, our results suggest that HF alters both the quantity and the kinetic properties of the myosin molecule in skeletal muscle, with functional consequences that are fiber-type specific.

Our current results in single fibers differ from our previous findings in isolated myosin and thin filaments evaluated using the in vitro motility assay, which showed no effect of HF on force production or contractile velocity. These differences between studies are likely due to differences in the 2 assays of myofilament function. In the in vitro motility assay, the quantity of myosin and thin filament proteins used is standardized, which negates the functional effect of MHC protein depletion evident in skinned fibers from patients with HF. Moreover, in the mixed fiber preparations used in the motility assay in our previous study, myosin kinetic properties are dominated by MHC I molecules. Hence, alterations in MHC IIA kinetics evident in skinned fibers (Figure 4D) would be masked in the motility assay. In addition, our results differ somewhat from previous studies that showed large reductions (>30%) in single fiber tension in MHC I and IIA fibers from patients with HF. Here again, direct comparisons between studies are difficult because controls in this previous study were 12 years younger than patients and groups were not matched for physical activity level. Thus, the large tension reductions may have been related to aging and/or muscle disuse. In contrast, patients and controls in our study were well matched for age and physical activity, making our findings more reflective of the direct effects of the HF syndrome.

In light of the potential relevance of MHC protein depletion, we sought to identify the mechanisms whereby HF promotes a loss of skeletal muscle myosin by measuring MHC mRNA abundance and indices of MHC protein degradation. No group differences in MHC mRNA abundance were found, suggesting no alteration in MHC gene transcription. These results differ from our previous work in which we observed a trend toward reduced MHC mRNA in patients. This previous finding is likely explained by the fact that we did not match controls and patients for physical activity. Consequently, reduced MHC mRNA was explained entirely by decreased MHC I mRNA, which is likely due to inactivity-induced reductions in MHC I gene expression. This further emphasizes the importance of considering the activity status of controls. In addition, by using multiple techniques, we found no evidence for elevated MHC protein degradation in patients (Figure 5). These results contrast with a recent work in a rat model of HF showing that pharmacological treatment with an inhibitor of protein breakdown can prevent the loss of MHC from single diaphragm fibers. Reasons for differing results are not clear but may relate to the fact that we expect the HF syndrome to be more severe and rapidly progressive in this animal model compared with well-treated, clinically stable patients. These differences in disease status between animal models and patients highlight a potential explanation for the MHC protein content depletion observed in our study; specifically, that reduced MHC gene expression and increased proteolysis occur in patients and precipitate MHC protein loss during periods of acute disease exacerbation and hospitalization. These episodes are characterized by neurohumoral/immune activation and physical inactivity, both of which could contribute to the depletion of MHC protein.

In conclusion, our results suggest selective myosin protein depletion from individual muscle fibers as a potential molecular mechanism contributing to skeletal muscle weakness in patients with HF. Because controls and patients were similar for age and physical activity level and there was no evidence for muscle atrophy in patients, we believe that this phenotype is reflective of the effects of the HF syndrome on skeletal muscle rather than the effects of aging, muscle atrophy, or physical inactivity. Moreover, the fact that these observations were made in well-treated patients with mild to moderate HF suggests that single muscle fiber myosin depletion is not merely a manifestation of end-stage disease but rather a distinct feature of the skeletal muscle myopathy of HF.

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Disclosures

None.

References


Patients with chronic heart failure (HF) frequently experience skeletal muscle weakness, which limits physical function and contributes to their high rates of physical disability. Our study evaluated the mechanisms underlying skeletal muscle weakness in human HF by comparing myofilament protein content, function, and sarcomeric structure in single skeletal muscle fibers isolated from patients and nondiseased controls. Our results show that HF is characterized by a selective loss of the contractile protein myosin from single muscle fibers, which contributed to reduced tension in slow-twitch muscle fibers. This functional impairment may be of clinical significance because slow-twitch fibers are recruited for repetitive movements typically encountered in daily activities and are an important determinant of exercise tolerance. In this context, loss of myosin protein from single muscle fibers represents a potential molecular mechanism underlying physical disability and exercise intolerance in patients with HF. Moreover, the fact that these observations were made in well-treated patients with mild to moderate HF suggests that single muscle fiber myosin depletion is not merely a manifestation of end-stage disease but rather a distinct feature of the skeletal muscle myopathy of HF.
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MECHANISMS UNDERLYING SKELETAL MUSCLE WEAKNESS IN HUMAN HEART FAILURE: ALTERATIONS IN SINGLE FIBER MYOSIN PROTEIN CONTENT AND KINETICS

Mark S. Miller, Peter VanBuren, Martin M. LeWinter, Stewart H. Lecker, Donald E. Selby, Bradley M. Palmer, David W. Maughan, Philip A. Ades, Michael J. Toth

Supplemental Methods:

Solutions. For solutions used for Muscle tissue processing, dissection solution contained (in mM) 20 N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 5 EGTA, 5 MgATP, 1 free Mg$^{2+}$, 1 DTT and 0.25 phosphate (Pi) with an ionic strength of 175 mEq, pH 7.0, and at pCa 8 (pCa = -log$\,_{10}$ [Ca$^{2+}$]), skinning solution contained (in mM): 170 potassium propionate, 10 imidazole, 5 EGTA, 2.5 MgCl$_2$, 2.5 ATP-Na$_2$H$_2$, 0.05 leupeptin and 0.05 antipain at pH 7.0, and storage solution was identical to skinning solution, but without leupeptin and antipain. For solutions used for Single fiber mechanical measurements, calculations were performed using the equations of Fabiato and Fabiato$^1$ and stability constants of Godt and Lindley.$^2$ Relaxing solution was identical to dissecting solution with 15 mM creatine phosphate (CP) and 300 units/ml of creatine phosphokinase (CPK), pre-activating solution was identical to relaxing solution, except at an EGTA concentration of 0.5 mM, activating solution was the same as relaxing solution, except at pCa 4.5 and rigor solution was the same as activating solution, except that it lacked MgATP, CP and CPK. All solutions used for mechanical experiments were adjusted to proper ionic strength (175 mEq) using sodium methane sulfate. Finally,
loading buffer used for various gel electrophoresis applications was 2% SDS, 62.5 mM Tris, 10% glycerol, 0.001% bromophenol blue, 5% β-mercaptoethanol, pH 6.8.

Tissue homogenate MHC protein content and isoform distribution. Muscle tissue (~20 mg) was homogenized (in mM: 250 sucrose, 2 EDTA, 10 Tris, pH 7.4), the homogenate was centrifuged (10,000 g at 4°C) for 10 min and the supernatant, which contains soluble (ie, non-myofibrillar) proteins, was decanted and the pellet resuspended in three volumes of cold (4°C) extraction buffer (in mM: 100 Na₄P₂O₇, 5 EDTA, 1 DTT, pH 8.5), incubated on ice for 30 min and then centrifuged (10,000 g at 4°C for 10 min). The supernatant, which contains myofibrillar proteins, was analyzed for protein content (BioRad; Hercules, CA). An aliquot of the supernatant was added to loading buffer (described above in Solutions section), heated for 2 min at 65°C and analyzed by SDS-PAGE.

For MHC protein content, 2 μg protein was loaded onto gels (stacking: 4% acrylamide-\text{-}N,N'\text{-}methylene-bis-acrylamide (bis) and the resolving 7.5% acrylamide-bis) and run at 70V for 3 hr, which permits visualization of MHC isoforms as a single band. Gels were stained with coomassie blue, scanned and MHC band intensity determined by densitometry (Quantity One; BioRad; Hercules, CA). MHC protein content data are expressed as densitometric units per μg of protein loaded and reflect the average of duplicate measures. MHC content measurements were not performed on one patient because of lack of tissue.

The relative distribution of MHC isoforms (MHC I, IIA, IIX) was determined according to standard methods, as described previously,\textsuperscript{3} with minor modifications. Briefly, 0.2 μg protein was loaded. The stacking gel contained 4% acrylamide-bis/5% glycerol (w/v) and the resolving gel 7% acrylamide-bis/30% glycerol (w/v). Gels were run at 70V for 3.5 hr, followed by 200V for 20 hr at 9°C. This permits separation of the three
isoforms of MHC into three distinct bands. The gel was silver stained (Silver Snap; Pierce, Rockford, IL), scanned, the MHC isoforms quantified by densitometry (Quantity One; BioRad; Hercules, CA) and data expressed as a percentage of total MHC densitometric units.

**Single muscle fiber morphology and MHC protein content.** Fibers had T-clips were placed at either end, were mounted in dissecting solution (20°C) under a compound microscope, pulled taught and clip-to-clip length measured using a manual micrometer. Using a right-angled, mirrored prism, top and side fiber diameters were measured to the nearest tenth of a μm at 250 μm intervals along the length of the fiber at 100X using a digital filar eyepiece micrometer (Lasico, Los Angeles, CA). Following measurements, the fiber was cut adjacent to each T-clip, placed in loading buffer (75 μl), sonicated at 40°C for 1 hr, heated for 5 min at 65°C and stored at -80°C until analysis. Top and side diameters and fiber length were used to calculate the volume of the fiber, assuming an elliptical cross-section. The volume of the fiber per μl of loading buffer was then used to standardize gel loads per unit fiber volume (ie, μl required to obtain 1.5 μm³ of fiber volume). Because of this requirement for the loading volume (ie, 1.5 μm³ of fiber volume), we selected a lower bound for fiber diameters of 60 μm to insure that there would be enough sample to run triplicate analysis on each fiber. Thus, the fibers used in these analyses do not reflect a random sample from each group.

For MHC protein measurements, samples were run on 4% acrylamide stacking/7.5% acrylamide resolving gels at 70V for 3 hr and silver stained (Silver Snap; Pierce, Rockford, IL). For actin protein measurements, samples from a sub-set of patients (n=4/group) were run on 4-15% acrylamide/bis gradient gels at 150 V for 1.5 hr and processed similar to MHC gels. Gels were scanned and the background-adjusted brightness area product (BAP) of each MHC or actin band, run in triplicate, was
quantified (Quantity One; BioRad; Hercules, CA), as described. The BAP for each sample was then adjusted to the running internal standard. Samples in which no MHC band was apparent (density ≤ background) were given a value of zero.

**Single fiber mechanical measurements.** Aluminum t-clips were placed at both ends of the fiber and the fiber segment was mounted on hooks in dissecting solution at 20°C. Fibers were fixed at two points approximately 1 mm apart with glutaraldehyde, as described, with modifications. Briefly, fibers were placed in rigor solution (in mM: 134 potassium propionate, 10 imidazole, 7.5 EDTA and 2.5 EGTA; 20 2,3-butanedione monoxime at pH 6.8) and glutaraldehyde fixative (6% toluidine blue (w/v), 30% glycerol (v/v), 2% glutaraldehyde(v/v)) was applied (15 s/end) using the gravity feed method. The fiber was then placed in dissecting solution with 1% bovine serum albumin to absorb any remaining glutaraldehyde. Fibers were removed from the hooks, t-clipped in the fixed region, and the fiber material beyond the new t-clips removed.

**Ultrastructural measurements.** Shortly after obtaining tissue from the muscle biopsy, a bundle of muscle fibers was tied to a glass rod at slightly stretch length (~20% of initial bundle length) and was fixed in 1.5% glutaraldehyde, post-fixed with osmium tetroxide, stained with uranyl acetate and embedded in epoxy resin. The muscle bundle was cut in cross-section (~100 nm) and contrasted with lead and uranyl acetate prior to transmission electron microscopy. The number of thick and thin filaments per unit fiber cross-sectional area was assessed at 60,000X in 8 fibers per patient (Sterio Investigator v8.0; MBF Bioscience; Chicago, IL). Briefly, a measurement area was manually selected on the muscle fiber that contained clearly discernable thick and thin filaments. Counting frames (1 μm²) were automatically overlaid in a grid pattern and thick and thin filaments counted manually using standard rules. Data were expressed as the ratio of thick to thin
filaments. The muscle bundle was then cut 90° to the cross-sectional cut (~100 nm) and processed as above for electron microscopy (JEOL 1210 Transmission Electron Microscope; JEOL, Inc.; Peabody, MA). A-band (25 measurements/volunteer) and sarcomere length (50 measurements/volunteer) measurements were performed at 5,000X, while the percentage of fiber area occupied by myofibrils (3 images for 275 um²/volunteer) was performed at 8,000X, as described. All cross-sectional area, A-band length and sarcomere length measurements were made using NIH Image (Image J, National Institutes of Health, Bethesda, MD).

Protein and gene expression. Myofibrillar proteins were isolated from muscle tissue (~20 mg), as described above for tissue homogenates. All buffers were ATP free and contained 2 mM N-ethylmaleimide to inhibit proteasomal degradation and deubiquitinating isopeptidases. After protein content determination, samples were diluted in loading buffer and subjected to 4-12% acrylamide-bis gradient SDS-PAGE and routine Western blotting using specific monoclonal antibodies (slow MHC #MAB1628, 1:10,000; Millipore, Temecula, CA; ubiquitin #sc-8017, 1:2000; Santa Cruz Biotech, Santa Cruz, CA). After washing, blots were incubated with sheep anti-mouse IgG (#NA931, 1:2000, Amersham, Piscataway, NJ) conjugated to horseradish peroxidase for chemiluminescent detection (Pierce, Carlsbad, CA). We chose to assess MHC breakdown fragments using an antibody directed at the MHC I isoform because this is the most prevalent MHC isoform in human vastus lateralis muscle (Figure 1B). The pattern of MHC I breakdown fragments (Figure 5A) is similar to what has been previously noted in chymotrypsin digests of rat soleus muscle using this antibody, which likely reflects high molecular weight degradation fragments of myosin. Bands/portions of lanes were quantified by densitometry (Quantity One; BioRad; Hercules, CA). For MHC degradation fragments, the primary MHC band and the MHC
fragments were quantified separately. The densitometric signal for MHC degradation fragments was expressed relative to the primary immunoreactive MHC band to control for any variation in MHC protein content and isoform distribution among groups. To quantify the amount of ubiquitinated MHC, the ubiquitin signal corresponding to the MHC band was quantified and expressed relative to the total MHC band density determined from Simple Blue (Invitrogen, Carlsbad, CA) stained gels run concurrently.

For MHC and actin mRNA measurements, RNA was extracted from muscle tissue (≤8 mg) using the MELT Total Nucleic Acid Isolation System (Ambion, Austin TX). Multiplexed amplification reactions were performed using GAPDH as an endogenous control (Applied Biosystems, Assay ID: Hs99999905_m1) using the Quanta Perfecta QPCR Super Master Mix (Quanta Biosciences, Gaithersburg, MD). The following settings were used: Stage 1 (reverse transcription): 45°C for 5 min; Stage 2 (denaturation): 95°C for 3 min and Stage 3 (PCR): 95°C for 15 s and 60°C for 45 s for 40 cycles. The MHC I, IIA, IIX and actin oligonucleotides were purchased from Applied Biosystems (Assay ID: Hs01110632_m1; Hs00430042_m1; Hs00428600_m1 and Hs00559403_m1, respectively). For atrogene mRNA measurements, total RNA was extracted from muscle tissue (~25 mg) using Triazol reagent. Multiplexed amplification reactions were performed using 18S rRNA as an endogenous control (Applied Biosystems; Foster City, CA) using the TaqMan One step PCR Master Mix reagents kit (Applied Biosystems). The following settings were used: Stage 1 (reverse transcription): 48°C for 30 min; Stage 2 (denaturation): 95°C for 10 min and Stage 3 (PCR): 95°C for 15 s and 60°C for 60 s for 40 cycles. The MuRF-1 oligonucleotides were purchased from Applied Biosystems (Assay ID: Hs00261590). The sequences of the forward, reverse and double-labeled oligonucleotides for atrogin-1 were: forward 5'-CTT TCA ACA GAC TGG ACT TCT CGA -3'; reverse 5'-CAG CTC CAA CAG CCT TAC TAC GT-3'; TaqMan probe: 5'- FAM-TGC CAT CCT GGA TTC CAG AAG ATT CAA C-TAMRA-3'.
MHC and actin samples were run in duplicate and atrogene samples in triplicate. All fluorescence data were analyzed by SDS software (Applied Biosystems) and the threshold cycle (Ct) values for each reaction were used to calculate gene expression relative to controls, according to published algorithms (Applied Biosystems).

**Supplemental results:**

*Missing data.* Several data points are missing because of logistical and technical problems. For total body composition measurements, one heart failure patient was not tested because he exceeded the weight limit of the dual energy x-ray absorptiometry scanner. Because of this, peak VO₂ data for this subject are also excluded because they could not be corrected for fat-free mass. For computed tomography scans, one patient and one control did not have data because the scan files were corrupted upon transfer from the scanner computer to the storage database and in one control because logistical problems prevented the measurement from being completed. Data for peak VO₂ and accelerometry are not available on one subject because logistical problems prevented completion of these tests. For biochemical assessments (ie, protein and gene expression), variable sample sizes was due to limitations in tissue availability.

*Non-parametric statistical analysis.* Comparisons between heart failure patient and control groups using non-parametric statistical procedures (Mann-Whitney U test) did not alter the statistical significance of any of the differences noted within the body of the manuscript, as defined by parametric statistical procedures.

*Single fiber MHC and actin protein content.* In fibers used for MHC protein content measurements, actin bands were evident in all fibers studied, including those with MHC band density less than background. As the actin bands were not quantifiable on gels
used for MHC protein measurements, in a sub-set of patients (n=4/group) for which there were sufficient fibers for triplicate analysis, single fiber actin protein content was evaluated in the same fibers. We did not find differences in actin protein content between controls and patients (157 ± 7 vs. 149 ± 5 BAP X 10^3; n=51 and n=65, respectively).

**MHC degradation and gene expression.** MHC I breakdown fragments, the amount of ubiquitinated protein corresponding to MHC and expression of E3 ubiquitin ligases in a sub-sample of patients (n=4) and controls (n=4) are shown in Figure 5. We found no differences between heart failure patients and controls in the amount of MHC I breakdown fragments (Figure 5A,C) when assessing the densitometric signal of the fragments (C: 1671 ± 426 vs. HF: 1709 ± 362 arbitrary units (AU)) or when this signal was expressed relative to the primary immunoreactive MHC I band to account for MHC protein depletion in heart failure patients (C: 1.00 ± 0.21 vs. HF: 1.05 ± 0.24 AU). We also found no difference in breakdown fragment signal when expressed relative to total MHC protein determined from Simple Blue stained gels (C: 1.17 ± 0.26 vs. HF: 1.29 ± 0.34 AU). Similarly, there was no difference in the ubiquitin signal corresponding to intact MHC (Figure 5B,D; C: 1414 ± 44 vs. HF: 1301 ± 44 AU) or when the ubiquitin signal was expressed relative to total MHC protein determined from Simple Blue stained gels (C: 0.97 ± 0.30 vs. HF: 1.13 ± 0.07 AU). Parenethetically, in the non-myofibrillar protein fraction, there was no evidence for increased protein ubiquitination on an absolute basis (C: 1236 ± 71 vs. HF: 1268 ± 92 AU; representative gel in Supplemental Figure 1) or when expressed relative to the total MHC protein content determined from concurrently run Simple blue stained gels (C: 0.45 ± 0.01 vs. HF: 0.47 ± 0.03 AU), suggesting that there is no evidence for a general up-regulation of protein breakdown in heart failure patients. Finally, in a sub-set of volunteers (n=4 heart failure; n=6 controls; Figure 5E,F),
no differences were observed in the expression of the E3 ubiquitin ligases: MuRF-1 (C: 1.00 ± 0.20 vs. HF: 1.05 ± 0.20) or atrogin-1 (C: 1.00 ± 0.22 vs. HF: 1.00 ± 0.21).

Using 18S as a housekeeping gene, there was no difference in MHC I (C: 1.00 ± 0.12 vs. HF: 1.07 ± 0.20), MHC IIA (C: 1.00 ± 0.11 vs. HF: 1.02 ± 0.21), MHC IIX (C: 1.00 ± 0.43 vs. HF: 2.00 ± 0.75) or actin mRNA abundance (C: 1.00 ± 0.08 vs. HF: 1.03 ± 0.08).
REFERENCES


Supplemental Figure 1. Representative blot of ubiquitinated proteins in the non-myofibrillar fraction of skeletal muscle tissue homogenates in controls (C; n=4) and heart failure patients (HF; n=4).