Development of a Diagnostic Method for Detecting Increased Muscle Protein Degradation in Patients with Catabolic Conditions

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Muscle atrophy in catabolic illnesses is due largely to accelerated protein degradation. Unfortunately, methods for detecting accelerated muscle proteolysis are cumbersome. The goal of this study was to develop a method for detecting muscle protein breakdown and assess the effectiveness of anticatabolic therapy. In rodent models of catabolic conditions, it was found that accelerated muscle protein degradation is triggered by activation of caspase-3. Caspase-3 cleaves actomyosin/myofibrils to form substrates for the ubiquitin-proteasome system and leaves a characteristic 14-kD actin fragment in the insoluble fraction of a muscle lysate. Muscle biopsies were obtained from normal adults and three groups of patients: 14 who were undergoing hip arthroplasty, 28 hemodialysis patients who were participating in exercise programs, and seven severely burned patients. In muscle of patients who were undergoing hip arthroplasty, the 14-kD actin fragment level was correlated \( r = 0.787, P < 0.01 \) with the fractional rate of protein degradation. In muscle of hemodialysis patients who were undergoing endurance exercise training, the 14-kD actin fragment decreased to values similar to levels in normal adults; strength training did not significantly decrease the actin fragment. Severely burned patients had increased muscle protein degradation and actin fragment levels, but the two measures were not significantly correlated. The experimental results suggest that the 14-kD actin fragment in muscle biopsies is increased in catabolic states and could be used in conjunction with other methods to detect and monitor changes in muscle proteolysis that occur in patients with mild or sustained increases in muscle proteolysis.


The loss of lean body mass in aging, diabetes, uremia, trauma, burns, and immobilization causes increased morbidity and mortality (1). Unfortunately, methods that are used for detecting muscle atrophy are unwieldy, and the problem usually is unrecognized until there is weight loss or visible muscle wasting. Available methods for detecting loss of lean body mass or judging the effectiveness of therapeutic interventions to correct the problem include estimating muscle size by nuclear magnetic resonance imaging (MRI), calculating amino acid/protein turnover during infusion of isotopically labeled amino acids, or measuring nitrogen balance. The MRI method of measuring protein/amino acid turnover requires at least two measurements, special expertise, and expensive equipment. Nitrogen balance does not assess changes in muscle protein directly. Instead, it measures the net metabolism of all proteins and requires several days of collections. Our goal is to devise a method that directly evaluates changes in muscle protein degradation. It should satisfy the following criteria: It detects muscle protein breakdown in several conditions that cause muscle wasting, it is minimally invasive, it is correlated with measured rates of protein catabolism in muscle, and it identifies an effective intervention that improves muscle protein metabolism and function.

The mechanisms that cause loss of muscle mass in most catabolic conditions involve activation of the ubiquitin-proteasome system (UPP) (2,3). In addition, an initial, rate-limiting step in muscle protein loss is activation of caspase-3 (4). Using animal models of catabolic conditions, we found that activated caspase-3 is capable of cleaving actomyosin to create substrates that are degraded rapidly by the UPP. Caspase-3–mediated protein cleavage leaves a characteristic “footprint” in the myofibril fraction of muscle, a 14-kD actin fragment. In muscles of rodents with catabolic conditions, the level of the 14-kD actin fragment is closely associated with measured rates of protein degradation (4–7). We evaluated the actin fragment level in muscle biopsies from patients with different catabolic condi-
tions to determine whether it yields a method for diagnosing the presence of accelerated muscle proteolysis and monitoring the effectiveness of therapy that is designed to reduce muscle protein losses.

**Materials and Methods**

The studies were approved by the Institutional Review Boards of the University of Texas Medical Branch (UTMB) and Harbor-UCLA Medical Center. Biopsies of chronic hemodialysis patients and normal adults were performed at the Harbor-UCLA Medical Center. Other biopsies and protein kinetic studies were performed in the UTMB General Clinical Research Center and Blocker Burn Unit. Informed consent was obtained from all patients or their families.

**Patient Groups**

We studied normal individuals and three groups of patients with muscle wasting. Fourteen patients who were aged 56 ± 12 yr (±SE) and had severe degenerative hip disease had their leg muscle mass measured by dual energy x-ray absorptiometry (DEXA; Hologic, Natick, MA). During hip replacement surgery, the rate of muscle protein breakdown was calculated from the turnover of amino acids that were labeled with stable isotopes on the basis of dilution of intracellular amino acid enrichment from unlabeled amino acids that arose from protein breakdown. Muscle biopsies were obtained approximately 1 to 1.5 h into the arthroplasty procedure to calculate fractional protein breakdown (8).

There were four groups of maintenance hemodialysis patients (4 h of hemodialysis three times a week) plus normal adults of similar age, gender, and racial/ethnic backgrounds (Table 1). The hemodialysis patients were stable enough to participate in an exercise program that was always supervised by an experienced trainer. They had evidence of abnormalities in factors that regulate muscle protein metabolism (9). The patients were randomly assigned to one of three types of exercise or to no training. Seven group 1 patients had a muscle biopsy before and after approximately 18 wk of endurance training using a calibrated, electrically braked, cycle ergometer (Ergoline 800; Sensor-Medics Corp., Yorba Linda, CA) (10). Seven group 2 patients had biopsies before and after approximately 18 wk of leg strength training using LifeFitness leg extension/curl and LifeFitness leg press/calf extension apparatus (LifeFitness, Schiller Park, IL). Six group 3 patients had biopsies before and after approximately 18 wk of both endurance and strength training. Endurance training was done during the first 90 min of each hemodialysis treatment, and strength training was immediately before a hemodialysis. Eight group 4 patients had no exercise training but had muscle biopsies before and after approximately 18 wk. Finally, six normal adults with no exercise training had two biopsies separated by an average of 9.5 wk.

In hemodialysis patients, biopsies were obtained from the right vastus lateralis muscle, 10 cm cephalad to the superior border of the patella and 1 to 2 cm anterior to the mid-lateral line. A Bergstrom or U.C.H. muscle biopsy needle (Popper & Sons, New York, NY) was used between 9 and 11 a.m. midweek, 1 d after a hemodialysis treatment; the participants fasted overnight.

Seven patients in the UTMB Blocker Burn Unit were studied after their clinical condition had stabilized. Their age was 44 ± 8 yr, and the average burn was 68 ± 6% of body surface area (average of 28 ± 10% third-degree burns) (11,12). Because of severe injury, protein degradation and muscle biopsies (Bergstrom needle) were obtained at variable times after the injury (average 12 d; range 6 to 30 d). Leg protein metabolism was measured using labeled amino acids with arteriovenous balance and the three-pool model to calculate protein synthesis and degradation (13).

**Measurement of the 14-kD Actin Fragment**

Muscle samples were stored at −80°C. At least 30 mg of muscle was weighed and placed in PBS that contained Complete Protease Inhibitors (Roche, Indianapolis, IN) in a ratio of 1:30 (1 mg of muscle in 30 μl of PBS). After homogenization on ice for 3 min (VWR High Viscosity Mixer at 300 rpm; VWR, West Chester, PA), the samples (approximately 100 μl) were centrifuged at 3300 × g for 10 min at 4°C. Pellets were weighed and resuspended in 2× Laemmli sample buffer (1 mg of insoluble tissue with 10 μl of 2× Laemmli sample buffer). Samples were boiled for 20 min, and 20 μl was separated on a 15% SDS gel. After transfer to a 0.2-μm Protran 83A nitrocellulose membrane (Whatman,

<table>
<thead>
<tr>
<th>Table 1. Characteristics of MHD patients and normal adultsa</th>
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<td>MHD Exercisers</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>No. of participants</td>
</tr>
<tr>
<td>Gender (M/F)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
</tr>
<tr>
<td>black</td>
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<td>Hispanic</td>
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<td>otherb</td>
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<td>Age (yr)c</td>
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<tr>
<td>Duration of MHD (mo)c</td>
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<tr>
<td>range</td>
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<tr>
<td>Duration of exercise training (wk)c</td>
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<td>Interval between initial and end muscle biopsies (wk)c</td>
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aMHD, maintenance hemodialysis patients.
bWhite and Asian.
cMeans ± SEM.
coefficients of variation were 9.5 and 10.9%.

In this reproducibility evaluation, the what we used to assay samples from the various groups of on each blot. This procedure was used because it is the same as normalized for the value from the same normal adult that was tested the reproducibility of the method by assaying muscles routinely began the assay with at least 30 mg of muscle. We also obtained this degree of sensitivity, but for repeated testing, we repeatedly. Therefore, we divided a normal rat muscle into six samples and detected the 14-kD actin fragment in six different assays. The assay coefficient of variation was 11.8%. We also measured the actin fragment density in biopsies from six normal adults; the average value in these normal muscles was 39.5 ± 6.6 (SE), and the muscle level that was obtained before and after an average of 9.5 wk differed by an average of 4.4% (NS). Regarding the assay sensitivity, we assessed different amounts of muscle from a normal adult. The 14-kD actin fragment was readily detected by the Infrared scanner when the pixel value was at least 50% above background values. With a minimum of 500 µg of muscle from a hemodialysis patient, we obtained this degree of sensitivity, but for repeated testing, we routinely began the assay with at least 30 mg of muscle. We also tested the reproducibility of the method by assaying muscles from two hemodialysis patients plus a normal muscle sample on seven different Western blots. Each patient sample was normalized for the value from the same normal adult that was on each blot. This procedure was used because it is the same as what we used to assay samples from the various groups of hemodialysis patients. In this reproducibility evaluation, the coefficients of variation were 9.5 and 10.9%.

**Results**

**Reproducibility of Measurements of the 14-kD Actin Fragment in Muscle**

There was insufficient muscle from individual patients to test the intra-assay reproducibility by assay ing a muscle biopsy repeatedly. Therefore, we divided a normal rat muscle into six samples and detected the 14-kD actin fragment in six different assays. The assay coefficient of variation was 11.8%. We also measured the actin fragment density in biopsies from six normal adults; the average value in these normal muscles was 39.5 ± 6.6 (SE), and the muscle level that was obtained before and after an average of 9.5 wk differed by an average of 4.4% (NS). Regarding the assay sensitivity, we assessed different amounts of muscle from a normal adult. The 14-kD actin fragment was readily detected by the Infrared scanner when the pixel value was at least 50% above background values. With a minimum of 500 µg of muscle from a hemodialysis patient, we obtained this degree of sensitivity, but for repeated testing, we routinely began the assay with at least 30 mg of muscle. We also tested the reproducibility of the method by assaying muscles from two hemodialysis patients plus a normal muscle sample on seven different Western blots. Each patient sample was normalized for the value from the same normal adult that was on each blot. This procedure was used because it is the same as what we used to assay samples from the various groups of hemodialysis patients. In this reproducibility evaluation, the coefficients of variation were 9.5 and 10.9%.

**The 14-kD Actin Fragment Level Is Correlated with Protein Degradation in Muscle**

Muscle size in the unaffected leg of hip replacement patients (determined by DEXA) was greater versus values in the affected leg. The muscle mass of the unaffected leg was significantly greater than the muscle mass in the affected leg (unaffected leg lean mass 8896 ± 688 g; difference in lean mass of the affected leg −966 ± 202 g; P < 0.001). Likewise, the muscle mass in the unaffected leg corrected for body weight was significantly greater than in the affected leg (unaffected leg lean mass/kg body weight 0.108 ± 0.005; difference in lean mass/kg body weight in the affected leg −0.012 ± 0.001; P < 0.001). A representative Western blot of the 14-kD actin fragment in muscle of patients who were undergoing hip arthroplasty was increased in comparison with the intensity of the band in a muscle of a normal adult (Figure 1A). In 14 patients, the rate of muscle protein degradation was highly correlated with the intensity of the 14-kD actin fragment (r = 0.787; Figure 1B).

**Actin Fragment Level Predicts a Benefit of a Therapeutic Intervention**

To compare changes in the actin fragment in muscles of the four groups of hemodialysis patients, we normalized its density to that of an internal control, the value from a muscle biopsy of a normal adult. As shown in Table 2, the amounts of the 14-kD actin fragment in the initial muscle biopsy, corrected by the internal control, were remarkably similar in the four groups of hemodialysis patients. By ANOVA, there were no statistical differences in the initial amounts of the actin fragment among the four groups of hemodialysis patients. The overall average of the actin fragment level in muscles of hemodialysis patients was 37.7% higher than the average value in muscles of normal...
adults of similar ages, gender, and racial/ethnic distribution as the hemodialysis patients. To determine whether the amount of the 14-kD actin fragment changes in response to various types of exercise training, we examined muscle biopsies that were obtained before and after 18 wk of thrice-weekly regimens of endurance training, strength training, a combination of endurance and strength training, or no training (10). After 18 wk of endurance training, the level of the 14-kD actin fragment in all seven participants was reduced; the average decrease was 40% (P < 0.01; Table 1). Notably, this level of the 14-kD actin fragment that was obtained after endurance training was comparable to values that were found in muscle of normal adults (Figure 2A). In the hemodialysis patients who were assigned to strength training, there was no change in the 14-kD actin fragment (three values decreased, three values increased, and one did not change) (Figure 2B). In the muscle of patients who were assigned to strength plus endurance training, there was a 9.8%, statistically significant decrease in the amount of the 14-kD actin fragment in muscle. Finally, there was no significant change in the actin fragment in muscles of hemodialysis patients who did not undergo exercise training or in muscles that were obtained from normal adults. These results are consistent with earlier reports. Storer and colleagues (14,15) reported that hemodialysis patients who underwent 8 wk of endurance training had significant improvement in leg muscle endurance capacity, strength, and performance and decreased fatigability. Endurance training also was associated with an increase in the IGF-1 receptor mRNA and a decrease in myostatin mRNA. This pattern of changes in mRNA would be consistent with an increase in the insulin/IGF-1 signaling pathway, and, experimentally, this signaling pathway will decrease actin cleavage and protein degradation in muscle (4–7).

Actin Fragment Level Is Increased in Muscle of Severely Burned Patients

Western blots of the 14-kD actin fragment in muscle of a burned patient and in muscle of a normal adult are shown in Figure 3. Earlier, Biolo et al. (12) and Ferrando et al. (13) reported that such patients have a marked increase in protein degradation. The intensity of this band in each of the seven patients with an average of 68% total body burns was significantly greater than that in muscle biopsies of normal adults. However, we did not find a significant correlation between the intensity of the actin band and the rate of protein degradation.

Discussion

Our goal is to devise a method for detecting the presence of accelerated muscle protein degradation in humans. In rodent models of catabolic conditions, we found a characteristic, 14-kD actin fragment in the insoluble, myofibril fraction of homogenized muscle; it was closely related to the rate of protein degradation (4). In this study of patients who were undergoing hip replacement surgery, we found that the amount of the 14-kD actin fragment in muscle was significantly correlated (r = 0.787, P < 0.01) with the measured rate of protein degradation. Hemodialysis therapy, another catabolic condition (16–19), also yielded information that supports this thesis; the amount of the actin fragment in muscle of hemodialysis patients changed in concert with established physiologic and biologic (e.g., mRNA of myostatin/IGF-1) functions, thereby predicting the effectiveness of therapeutic interventions that are used to improve muscle performance (10,14,15). Therefore, detecting the 14-kD actin fragment in a muscle biopsy might satisfy two requirements for a diagnostic test: It is correlated with the measured rate of muscle protein degradation, and it could be used to monitor a therapeutic intervention that changes muscle proteolysis.

Methods for diagnosing and monitoring accelerated muscle protein degradation in patients include MRI assessment of changes in muscle mass or measurement of nitrogen balance or protein turnover by an isotope method. Only the latter directly evaluates muscle protein metabolism, but it is expensive and clinically cumbersome and requires extensive analysis. A practical method is needed to detect muscle wasting and to monitor therapy that is directed at minimizing this problem (1,16,17,20). Our earlier studies showed that the 14-kD actin fragments that are released during accelerated muscle atrophy are degraded rapidly by the ATP-dependent UPP (4). Consequently, the amount of the 14-kD actin fragment in the soluble fraction of a muscle cell lysate is very low and highly variable unless a

### Table 2. The influence of various types of exercise on the level of the 14-kD actin fragment in muscle of hemodialysis patients

<table>
<thead>
<tr>
<th>Patient Groups</th>
<th>Initial Value (Mean % ± SE)</th>
<th>Posttreatment Value (% Change ± SE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysis + endurance training (n = 7)</td>
<td>148.9 ± 7.2</td>
<td>−40.0 ± 2.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dialysis + strength training (n = 7)</td>
<td>126.6 ± 21.2</td>
<td>0.3 ± 9.9</td>
<td>NS</td>
</tr>
<tr>
<td>Dialysis + endurance + strength training (n = 6)</td>
<td>134.7 ± 13.3</td>
<td>−9.8 ± 2.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dialysis + no training (n = 8)</td>
<td>140.8 ± 8.8</td>
<td>−2.5 ± 11.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Patient samples were assayed along with a sample of muscle from the same normal adult. The initial value for the actin fragment level in muscle from each patient was divided by the level in the muscle of the normal adult and multiplied by 100 (density patient/density control × 100). The posttreatment value was calculated similarly and then subtracted from the initial value. The endurance training significantly reduced the level of the actin fragment, bringing it to levels similar to that in normal adult muscle.
proteasome inhibitor is present. Therefore, we explored events that occur before the degradation of the 14-kD actin fragment. We found the 14-kD actin fragment in the insoluble fraction of muscle, presumably because it is protected by the myofibril complex from additional proteolysis.

Experimentally, caspase-3 generates the 14-kD actin fragment (4). However, measuring caspase-3 activity in muscle samples is not useful as a diagnostic method because we and others have found that caspase-3 activity in muscle samples is low and the “signal to noise” ratio is high even in muscles of rodents with demonstrably high rates of protein degradation. Wei et al. (21) reported difficulty in detecting changes in caspase-3 activity in muscle of septic rats. Sepsis activates the UPP in muscle, so their results might be related to degradation of caspase-3 by the ATP-dependent UPP (22). In contrast, the 14-kD actin fragment is detectable in the insoluble fraction of muscle of septic rats (data not shown), emphasizing why we assessed this product of caspase-3 rather than measuring caspase-3 activity.

Especially important is our finding that the amount of the 14-kD actin fragment is significantly correlated with the rate of muscle protein degradation in patients who undergo hip replacement. The decrease in leg muscle mass measured by DEXA before surgery and the increase in muscle protein degradation measured during surgery presumably reflect disuse atrophy plus inflammation from osteoarthritis as well as the influence of surgery. We emphasize the detection of protein degradation because the actin fragment reflects muscle proteolysis but recognize that decreased muscle protein synthesis could contribute to the loss of leg muscle mass. In the hip surgery patients, protein degradation, expressed as fractional breakdown, was measured using isotope-labeled amino acids, but this requires arterial blood sampling and measurement of amino acid enrichment in these samples (12,13). The correlation that we found (Figure 1B) indicates that muscle proteolysis can be detected by the presence of the actin fragment in the myofibril complex of muscle.

Another important finding is that changes in the level of the 14-kD fragment can be used to monitor the effectiveness of a treatment for muscle wasting. In hemodialysis patients, we used different types of exercise to improve muscle function and the balance between muscle protein synthesis and breakdown (10,23,24). We found that endurance training reduced the level of the actin fragment in muscle of hemodialysis patients to values that were found in the muscle of normal adults (Table 2). This is consistent with the improvement in muscle strength, power, and fatigability that these patients experienced with endurance training (10). Preliminary analyses of their responses
to endurance exercise also show that these patients had a significant decrease in myostatin mRNA (P = 0.006) (14).

A potential mechanism for the decrease in muscle protein degradation with endurance training is stimulation of the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway. Sakamoto et al. (25) found that treadmill exercise or mechanical stretch stimulates the PI3K/Akt pathway in muscle. This is relevant because we have found that an increase in the PI3K/Akt pathway suppresses caspase-3 and reduces the amount of the 14-kD actin fragment (4–7). These experimental results indicate how endurance exercise could reduce the level of the 14-kD actin fragment.

With strength training, the hemodialysis patients had little or no decrease in the level of the actin fragment (Table 2). The underlying mechanism for this result is not clear because strength training can increase muscle regeneration and hypertrophy. However, the severity of training that is needed to elicit these responses is vigorous (26). The patients whom we studied were relatively deconditioned, limiting the intensity of their resistance training (10). Preliminary analyses of the influence of strength or strength plus endurance training suggest that IGF-1 mRNA in muscle increases by 71% and IGF-1 protein by 41% (15). These growth factor responses should increase muscle mass, but in normal adults, strength/resistance training does not suppress protein degradation in muscle and may even increase it (27,28). If similar responses occur in hemodialysis patients, then strength training would not decrease the muscle level of the actin fragment. Another factor is that both strength and endurance training can cause changes in muscle proteins and accrual of new protein. Strength training reportedly leads to muscle hypertrophy, whereas endurance training without causing hypertrophy induces protein remodeling, altering the proportions of type I and type II myosin heavy chains and increasing the levels of enzymes, including those that are involved with oxidative phosphorylation (29–32). Finally, the combination of strength and endurance training could activate different intracellular signaling processes or stimulate specific cell types (e.g., mature muscle cells versus satellite cells), resulting in different responses in terms of protein expression and exercise capacity (25,26,33,34). Regardless, our results indicate that endurance training can benefit hemodialysis patients who exhibit increased muscle proteinolysis. The complexity of responses to strength training does not suggest an explanation for why it did not reduce the presence of the 14-kD actin fragment in muscle.

To evaluate the assay in a condition with extreme loss of muscle, we studied patients with burn injuries that are severe enough to stimulate protein losses that amount to 20 to 25 g/m² per d (12). The amount of the 14-kD actin fragment in muscle of burned patients was uniformly greater than that of normal adults, but this change was not statistically correlated with the degree of protein degradation in muscle. A potential reason for this finding is that severe loss of myofibrils could limit the protective effect of muscle tissue against degradation of the actin fragment by the UPP (4). For example, we found a significant decrease in the amount of the actin band in cultured muscle cells that were treated with 100 nM staurosporine for 24 h (data not shown). Therefore, when there is a high rate of muscle proteolysis, the assay may not provide an accurate estimate of muscle protein breakdown. Another explanation is that a severe burn injury not only increases proteolysis in muscle but also stimulates protein breakdown in other organs, including skin and bone (12,35). The three-pool method that we used to measure overall protein degradation in muscle of burned patients includes values from muscle, bone, and skin (13) and would overestimate the level of proteinolysis in muscle, whereas the actin fragment evaluates protein degradation only in muscle. Future studies should be based on a more direct measurement of the rate of protein degradation and the amount of the 14-kD actin fragment in muscle of patients who are in the early stage of a serious burn injury.

Conclusion

In our research study of a relatively small group of 49 patients with various catabolic conditions, we found that the assay for this marker of accelerated muscle protein degradation can detect an increase in muscle protein degradation and a positive response to therapy. Because it is relatively simple and cost-effective, this method could be used to detect the presence of muscle protein catabolism and to monitor therapy. Additional testing of the clinical usefulness of the method will require a larger number of patients with other types of catabolic conditions. At this stage, our results indicate that detection of the 14-kD actin fragment should be used to complement results from other measures of muscle protein metabolism.

Acknowledgments

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References


