Development of an UPLC-mass spectrometry method for measurement of myofibrillar protein synthesis: application to analysis of murine muscles during cancer cachexia

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Running Head: Myofibrillar protein synthesis in skeletal muscle

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ABSTRACT

Cachexia, characterized by skeletal muscle mass loss, is a major contributory factor to patient morbidity and mortality during cancer. However, there are no reports on the rate of myofibrillar protein synthesis (MPS) in skeletal muscles that vary in primary metabolic phenotype during cachexia, in large part because of the small-size muscles and regional differences in larger muscles in the mouse. Here we describe a sensitive method for measurement of MPS and its application to analysis of MPS in specific muscles of mice with \((Apc^{Min/+})\) and without \((C57BL/6)\) cancer cachexia. Mice were injected with a loading dose of deuterated phenylalanine \((D_5F)\) and myofibrillar proteins extracted from skeletal muscles at 30 min. The relative concentrations of \(D_5F\) and naturally occurring phenylalanine \((F)\) in the myofibrillar proteins and the amino acid pool were quantified by ultra-performance liquid chromatograph (UPLC)-mass spectrometry (MS). The rate of MPS was determined from \(D_5F: F\) ratio in the protein fraction, compared to the amino acid pool. The rate of MPS, measured in 2-5 mg muscle protein, was reduced by up to 65% with cachexia in the soleus, plantaris, diaphragm, and oxidative and glycolytic regions of the gastrocnemius. The rate of MPS was significantly higher in the oxidative vs. glycolytic gastrocnemius muscle. A sufficiently sensitive UPLC-MS method requiring very small amount of muscle has been developed to measure the rate of MPS in various mouse muscles. This method should be useful for studies in other animal models for quantifying effects of cancer and anti-cancer therapies on protein synthesis in cachexia, and particularly for analysis of sequential muscle biopsies in a wide range of animal and human studies.
Skeletal muscle is a highly plastic tissue, which can undergo hypertrophy or atrophy in response to a variety of stimuli, including: disuse, variations in nutritional and hormonal status, aging, exercise, chronic diseases and associated metabolic syndromes (15, 21, 27). Cachexia, a chronic wasting condition associated with inflammatory diseases such as cancer and is characterized by severe loss of both muscle and adipose tissue mass (11, 20). Losses in muscle mass lead to muscle weakness and impaired muscle function, which can negatively impact quality of life, response to therapy, and can increase mortality (22, 24, 26). Cachexia affects up to 80% of patients with advanced cancers and more than 20% of patients die as a result of cachexia-related complications (1, 2, 29). The rapid progressive muscle loss rooted in cachexia is the result of an imbalance between decreased protein synthesis (MPS) and increased protein degradation (28). Because of its importance as a marker of cachexia, measurement of MPS is a valuable tool for evaluating the effectiveness of strategies to combat cachexia.

Although there are several different animal models that are used to study cachexia, the genetically-engineered \( Apc^{Min/+} \) mouse model (Min: multiple intestinal neoplasia) is commonly utilized; the slow-progressive cachexia and associated side effects exhibited by this model closely mimic human cachexia (4, 6). The \( Apc^{Min/+} \) mouse is heterozygous for a point mutation in the adenomatous polyposis coli (\( Apc \)) gene, which leads to development of familial adenomatous polyposis (FAP), a form of colon cancer. The mouse develops intestinal polyps at approximately 4 weeks of age, and loses body weight gradually between 14 and 20 weeks of age. At 20 weeks of age, the mouse is usually severely cachectic, having lost more than 15% of body mass compared to its peak body mass (4, 6, 25). Muscle phenotype related to the capacity for oxidative metabolism can affect myofiber susceptibility to wasting (17), and primarily glycolytic
myofibers in the $Apc^{Min/+}$ mouse demonstrated more atrophy during the development of cachexia (5). The standard procedure for measuring protein synthesis in vivo involves measurement of incorporation of isotopically labeled tracers into myofibrillar protein, using liquid scintillation counting, isotope ratio mass spectrometry (IRMS), and gas chromatography/mass spectrometry (GC-MS) (16, 23, 31). Although these methods have been useful, they require relatively large amounts of tissue (typically 20 - 50 mg wet weight), which in some cases is not readily available, except by pooling samples, and in any case, limits the availability of tissue needed for other analyses (e.g. western blots, RT-PCR, etc) (14, 23). To date, many researchers have been utilizing various mouse models of cachexia to understand the mechanisms responsible for muscle wasting, and the role of muscle phenotype in the process (7, 9, 12, 18).

Tandem Mass spectrometry (MS/MS), coupled with ultra-performance liquid chromatography (UPLC), provides high resolution, specificity and sensitivity for measurement of amino acids in tissues (8). In this paper we describe a method for analysis of MPS in individual mouse muscles using UPLC-MS/MS.

METHODS

Materials

Nonafluoropentanoic acid (NFPA, 97%) and sodium hydroxide (NaOH) were purchased from Sigma (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from ThermoFisher Scientific (Waltham, MA). L-[ring-$^2$H$_5$]phenylalanine (D$_5$F) was from Cambridge Isotope Laboratories (Andover, MA), and HPLC-grade water and methanol from VWR, Inc. (Radnor, PA).
Male $Apc^{Min/+}$ mice on a C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME) and crossed with C57BL/6 female mice at the University of South Carolina’s animal resource facility. Offspring were genotyped as heterozygotes by RT-PCR for the $Apc$ gene as described by Mehl et al. (18, 19) All mice were housed in standard cages. The room was maintained at 24 °C on a 12:12 light:dark cycle with the light period starting at 0700. All mice were provided with standard rodent chow (Harlan Teklad Rodent Diet, #8604, Madison, WI) and water ad libitum. Male C57BL/6 and $Apc^{Min/+}$ mice ($n = 6-7$) were sacrificed at 20 weeks of age.

Thirty minutes prior to sacrifice, all mice received an intraperitoneal injection of 150 mM D$_5$F in a 75 mM NaCl solution at a dose of 0.02 mL/g body weight. Subsequently, mice were given a subcutaneous injection of a ketamine-xylazine-acepromazine cocktail (1.4 mL/kg body weight). Muscles (plantaris, soleus, gastrocnemius, and diaphragm) were excised under anesthesia, rinsed in PBS, weighed, snap frozen in liquid nitrogen, and stored at -80 °C until further analysis. Red and white fibers were isolated from the gastrocnemius as previously described (32). All animal experimentation was conducted in accordance with procedures approved by the University of South Carolina’s Institutional Animal Care and Use Committee.

Protein Extraction and Isolation

Frozen muscle tissues were processed using the method of Welle et al (31) for measurement of myofibrillar protein synthesis by gas chromatography mass spectrometry. Tissue samples (~5 mg) were weighed in a 1.5 mL Eppendorf plastic centrifuge tube, to which 200 µL
of ice cold homogenizing buffer (HB: 50 mM KPO₄, pH 7.0, 0.25 M sucrose, 1% Triton X-100) was added, and kept on ice. Each sample was sonicated twice for 20 seconds on ice. The sonicate was centrifuged at 10,000 g for 10 min at 4 °C, and the supernatant containing the free amino acid pool was transferred to a fresh tube and dried under vacuum (Speed Vac, Thermo Electron Corp., Milford, MA), then reconstituted in 1% aqueous TFA (1 mL) and stored at -20 °C. The pellet fraction was resuspended by sonication in 350 μL of HB, and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was discarded, and this washing procedure was repeated once with HB (350 μL) and twice with ice-cold deionized water (500 μL).

NaOH (0.3 M, 200 μL) was added to the final pellet followed by centrifugation at 10,000 g for 10 min at 4 °C. The supernatant, containing myofibrillar proteins, was diluted with 6 M HCl (1 mL) and the protein hydrolyzed at 110 °C for 24 h in 2 mL screw-closure polypropylene O-ring sealed vials (Sarstedt, Nümbrecht, Germany). HCl was removed under vacuum (Speed Vac, Thermo Electron Cooperation, Milford, MA), and the dried protein hydrolysate was reconstituted in 1% aqueous TFA (1 mL) and stored at -20 °C. The free amino acid pool and myofibrillar protein hydrolysate were applied to a 3 mL Supelco C₁₈ cartridge (Waters, Milford, MA), equilibrated in 1% aqueous TFA. The aromatic amino acid fraction was eluted with 1% TFA in methanol:water (20:80, v/v, 3 mL). The eluate was dried under vacuum and reconstituted in aqueous 5 mM NFPA (45 μL) prior to analysis by UPLC-MS/MS.

**LC-MS Conditions and Instrumentation**

Samples were fractioned on a Waters (Manchester, UK) Acquity UPLC-MS/MS system using a BEH C₁₈ column (2.1 x 50 mm, particle size 1.7 μm), which was maintained at room
temperature. Samples were eluted at a flow rate of 0.2 mL/min, eluting for 2.0 min with 100% solvent A (aqueous 5 mM NFPA), followed linear gradient to 50% solvent B (acetonitrile) over 10 min, then washed with 80% solvent B for 15 min. The injection volume was 5 µL (equivalent to ~3 nmol of F).

Mass spectrometry experiments and optimization of the method were performed utilizing a Micromass Quattro Premier XE Tandem Mass Spectrometer (Waters). Multiple reaction monitoring (MRM) was conducted operating the MS in positive ion electrospray mode. Table 1 details the MRM conditions of analysis. Other MS operating conditions used were: scan duration, 1s; capillary voltage, 3 kV; cone voltage, 25 eV; source temperature, 100 °C; desolvation temperature, 350 °C. Sample peaks corresponding to phenylalanine, naturally occurring 13C-phenylalanine and D5F were integrated. Data were analyzed using MassLynx software (version 4.1), supplied by Waters. Analytes were quantified by comparing the peak area of deuterated compound: unlabelled compound. Percent protein synthesis per day was calculated, using the general formula (31) by dividing the ratio of D5F/F in myofibrillar protein pellet by the ratio of D5F/F in the free amino acid pool and expressing it as a percentage of newly synthesized protein, using the following formula:

\[
\%MPS/\text{day} = \left( \frac{D_5F/F_{\text{MPP}}}{D_5F/F_{\text{FAAP}}} \right) \times 100 \times 24h \div 0.5h
\]

*MPS- myofibrillar protein synthesis, MPP- myofibrillar protein pellet, FAAP- free amino acid pool*

*Statistical Analysis*
All statistical analyses were performed using GraphPad Prism (La Jolla, CA). All comparisons were analyzed using a Student’s two-tailed t-test, with significance set at $p \leq 0.05$.

RESULTS AND DISCUSSION

UPLC-MS conditions were optimized to resolve phenylalanine in a short chromatographic run. Addition of 5 mM NFPA, an ion-pairing agent, to the mobile phases improved signal intensity and resolution. MRM conditions were manually optimized for each compound in positive ESI ionization mode to obtain maximal response. The mass spectrum of phenylalanine is shown in Fig. 1A. The precursor ion ($m/z$ 166) was selected for fragmentation and the fragment corresponding to the immonium ion ($m/z$ 120), which was the most abundant fragment in the spectrum, was recorded by the second mass analyzer. Chromatograms obtained for F, $^{13}$C-F and D$_3$F are shown in Fig. 1B and 1C for myofibrillar protein pellet and the amino acid pool, respectively. The natural abundance peak of $^{13}$C-F, which is 1.1 % the intensity of the $^{12}$C-F peak, was used for internal standardization since the $^{12}$C-F signal saturated the detector for some samples.

All peaks were normalized to the strongest signal in the pellet and amino acid pool fractions, respectively. As expected, the myofibrillar pellet contained an abundant amount of F, followed by naturally occurring $^{13}$C-F, which accounts for ~ 10% of F, and D$_3$F. Although the peak intensity for the D$_3$F in pellet was only ~ 0.14% of F in the myofibrillar protein, this was well within the linear range of the instrument and yielded a strong signal during MRM analysis (Fig. 1B, insert).

Using the optimized MRM conditions and the formula above, myofibrillar protein synthesis was quantified in various skeletal muscles (Fig. 2). The mice chosen for the study, at
20 weeks of age, were chosen from a group of severely cachectic $Apc^{Min/+}$ mice (Table 2). Cachectic $Apc^{Min/+}$ mice experience an average of ~20% weight loss between their 12th and 19th week of age (32). Not all of the weight changes the result of loss of skeletal muscle - there are comparable losses of adipose tissue mass. Even with adjustment for the lifespan of mice, compared to humans, the rate of weight loss in untreated severely cachectic mice is extreme. In humans, pre-cachexia is generally defined as $\leq$5% weight loss during a 6-month period, while weight loss $\geq$6% during this period is described as cachexia (3).

The rate of MPS varied from ~1.25 %/d to ~2.5 %/d in white and red gastrocnemius, respectively, to ~4.3 %/day in the diaphragm muscle of control animals at 20 weeks (5 months) of age (Fig. 2), in reasonable agreement with the 3% estimate of MPS in the gastrocnemius of C57BL/6 mice at 6 months of age, analyzed by the GC-MS/MS procedure (31). MPS was significantly reduced in all muscles in cachectic animals, but varied significantly with the specific muscle, from ~60% in the red gastrocnemius and diaphragm muscles, to ~75% in the plantaris muscle (Fig. 2). In a previous study, using a GC-MS/MS procedure, we observed an ~50% decrease in the rate of gastrocnemius muscle protein synthesis in a group of animals with $\geq$20% weight loss (33), which is reasonably consistent with the results of the UPLC-MS/MS method applied to a more severely cachectic group of mice. There is also an ~150% increase in the rate of muscle protein degradation in these animals (33), which further contributes to the decrease in muscle mass. Percentage reductions in MPS were observed across all muscle types, including the soleus and plantaris muscles, which could not be measured (without pooling samples) by the GC-MS/MS method. We also show for the first time that the absolute rate of MPS is significantly higher in the red portion of the gastrocnemius and the soleus muscle compared to the white portion of the gastrocnemius and the plantaris muscle in the mouse. This
is consistent with the observation that MPS in glycolytic muscle fibers is more susceptible to wasting compared to oxidative fibers in humans and rats (10, 13, 30). However, to the best of our knowledge, no other studies have examined the rate of MPS in different muscle phenotypes of cachectic mice due to limitations regarding tissue mass needed for the analysis.

To further validate the method, MPS was measured in younger $Apc^{Min/+}$ mice group compared to the C57BL/6 controls animals (aged 10 wk) (Fig. 3). These results illustrated that there was no significant difference in MPS across genotypes, which supports the observation that $Apc^{Min/+}$ mice are not cachetic at this age (33), hence MPS is not decreased. Compared to the GC-MS/MS method (33), which requires ~ 50 mg of tissue per analysis, the current method requires only 5 mg of tissue, which permits triplicate injections and applies more straightforward calculations. The intra-assay variance within a group of animals was < 2. Limit of detection is ~ 50 fmol for F standard injected on the column. Compared to GCMS, which requires derivatization and longer analysis time, this method is far more advantageous due to its simplicity of sample preparation and short analysis time. The small sample requirement for the UPLC-MS/MS assay also allows for analysis of MPS in subcellular fractions of mouse muscles, and should also be applicable to the analysis of sequential biopsy samples from animal and human studies.

In summary, a specific, sensitive, and relatively simple method has been developed to measure MPS in mouse tissues and has been applied to analysis of MPS in control and cachetic mice. This method has been applied to analysis of small muscles (soleus and plantaris) muscles and to analysis of MPS in different regions of the gastrocnemius muscle (red vs. white) of $Apc^{Min/+}$ mice. These results show that the rate of MPS is greater in red, oxidative muscles, and that the rate of MPS is comparably decreased in all of these muscles including the
involuntary skeletal muscle diaphragm in \(Apc^{Min/+}\) mice during cachexia. Future studies on the
rates of protein turnover in these tissues will provide a better understanding of the relative roles
of changes in muscle protein synthesis vs. turnover in the loss of muscle mass during cachexia.

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GRANTS

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DISCLOSURES

The authors have declared that they have no competing interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: ML SS RTE JWB JAC. Performed the
experiments: ML SS RTE JAC. Analyzed the data: ML SS RTE JWB JAC. Contributed
reagents/materials/analysis tools: ML JWB JAC. Wrote the paper: ML SS RTE JWB JAC.


Table 1. MRM condition of analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent Mass (Da)</th>
<th>Daughter Mass (Da)</th>
<th>Cone Voltage</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_5$-F</td>
<td>171</td>
<td>125</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>$C_{13}$-F</td>
<td>167</td>
<td>121</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>F</td>
<td>166</td>
<td>120</td>
<td>25</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 2. Body weights and muscle weights of C57BL/6 and $Apc^{Min/+}$ mice

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>$Apc^{Min/+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>29.2 ± 1.3</td>
<td>18.6 ± 0.7 *</td>
</tr>
<tr>
<td>Soleus (mg)</td>
<td>10.0 ± 0.6</td>
<td>6.3 ± 0.5 *</td>
</tr>
<tr>
<td>Plantaris (mg)</td>
<td>18.1 ± 0.6</td>
<td>10.5 ± 0.8 *</td>
</tr>
<tr>
<td>Gastrocnemius (mg)</td>
<td>134.3 ± 3.5</td>
<td>68.5 ± 6.1 *</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>71.3 ± 1.6</td>
<td>30.5 ± 2.7 *</td>
</tr>
</tbody>
</table>

* Significantly different from age-matched C57BL/6 control mice ($p < 0.05$)
Figure 1. Optimization of mass spectrometric conditions for analysis of phenylalanine. Fragmentation pattern of phenylalanine ions (A), extracted ion chromatograms for phenylalanine (F), naturally occurring Carbon 13 F (C13 F), and Deuterated F (D5F) incorporated in the myofibrillar protein pellet (B) (D5F was magnified 100 X, insert shows the well resolved chromatogram for D5F without magnification) and supernatant (C). All peaks are normalized to the largest occurring peak either in the pellet or supernatant fraction.

Figure 2. Skeletal muscle protein synthesis is decreased during cancer cachexia. Myofibrillar protein synthesis was measured at 20 weeks of age in gastrocnemius (white, glycolytic portion) (A) and gastrocnemius (red, oxidative portion) (B) plantaris (C) soleus (D) and diaphragm (E) muscle. Values are mean ± SE, n ≥ 5. *Signifies difference from wild type (p < .05) #Signifies difference from white gastrocnemius (p <0.05)

Figure 3. Skeletal muscle protein synthesis is similar in Min mice at younger age. Wild type and ApcMin/+ mice were sacrificed at 10 weeks of age. Myofibrillar protein synthesis was measured in whole gastrocnemius. Values are ± SE, n = 3.
Figure 1.
Figure 2.
Figure 3.