Atrophy responses to muscle inactivity. II. Molecular markers of protein deficits

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Atrophy responses to muscle inactivity. II. Molecular markers of protein deficits. J Appl Physiol 95: 791–802, 2003. First published April 25, 2003; 10.1152/japplphysiol.01113.2002.—We examined the expression of several molecular markers of protein balance in response to skeletal muscle atrophy induced by spinal cord isolation (SI; i.e., a complete transection of the spinal cord at both a midthoracic and a high sacral level plus complete deafferentation between the two transection sites). This treatment nearly eliminates neuromuscular activity (activation and loading) of the hindlimb muscles while maintaining neuromuscular connectivity. SI was associated with a reduced transcriptional activity (via pre-mRNA analyses) of myosin heavy chain (MHC) and actin. In addition, there was an increased gene expression of enzyme systems impacting protein degradation (calpain-I; plus enzymes associated with polyubiquitination processes) that could further contribute to the protein deficits in the SI muscles via degradative pathways. IGF-I receptor and binding protein-5 mRNA expression was induced throughout the 15-day period of SI, whereas IGF-I mRNA was induced at 8 and 15 days. These responses occurred in the absence of an upregulation of translational regulatory proteins (p70 S6 kinase; eukaryotic 4E binding protein 1) to compensate for the decreased protein translational capacity. These data collectively demonstrate that 1) the molecular changes accompanying SI-induced muscle atrophy are not necessarily the reverse of those occurring during muscle hypertrophy, and 2) the rapid and marked atrophy that defines this model of muscle inactivity is likely the result of multifactorial processes affecting transcription, translation, and protein degradation.

Premessenger ribonucleic acid; protein translation markers; protein degradation enzymes; insulin-like growth factor I; myogenic regulatory factors

IN A COMPANION STUDY, WE DEMONSTRATED that the model of spinal cord isolation (SI), which results in near complete inactivity of the hindlimb muscles while maintaining an intact neuromuscular connectivity, induces rapid muscle atrophy and a parallel deficit in protein balance (25). Also, we observed a significant reduction in total RNA (i.e., ribosomal RNA) concentration and content in the soleus muscle, as well as significant reductions in mRNAs encoding key contractile and structural proteins, such as myosin heavy chain (MHC) and actin.

In the present study, we addressed five working hypotheses to gain a better understanding of the factors contributing to the deficits in protein homeostasis in the hindlimb muscles of SI rats. 1) The reductions in MHC and actin mRNA are due to decreased transcriptional activity of specific MHC and actin genes. 2) The decreases in the translational capacity are linked to upstream alterations in growth factor expression, e.g., reductions in IGF-I expression, including expression of IGF-I-associated receptor and binding proteins (BPs). 3) The phosphorylation state, and hence functional activity of marker signaling molecules such as ERK1–2 and proteins regulating the initiation steps of protein translation, e.g., p70 S6 kinase (p70S6K) and eukaryotic BP factor [eukaryotic 4E BP-1 (4EBP1)], are reduced in response to SI. 4) Gene expression of enzymes linked to protein degradation is upregulated in response to SI. 5) Myogenic regulatory factor gene expression, i.e., cyclin D1, MyoD, myogenin, and p21, associated with cell proliferation and differentiation processes, is repressed relative to the normal control (NC) state in response to SI. In testing these hypotheses, we examined a variety of marker molecules at different time points during the atrophy process. Based on these analyses, we conclude that the atrophy process in response to SI is heavily impacted by a reduction in the transcriptional activity of genes encoding key sarcomeric proteins (actin and myosin) of the muscle. This response lowers the mRNA substrate available for translation and is not offset by significant increases in the functional activity of enzymes regulating protein translation processes. Furthermore, the protein deficits appear to be exacerbated by increased gene expression of enzymes postulated to be associated with 1) disassembly of the cytoskeletal and myofibril framework, and 2) ubiquitination of proteins targeting them for degradation by the proteasomal machinery. Collectively, these data suggest that the rapid and marked atrophy associated with the SI model is likely the result of multifactorial processes affecting gene transcription and of protein translation and degradation.

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METHODS

Experimental design. Approximately 70 young adult female rats weighing ~230 ± 5 g were assigned randomly to either a NC (n = 35) or a SI (n = 35) group. Animals in each experimental group subsequently were assigned randomly into subgroups and studied on the day of (time = 0) and 2, 4, 8, and 15 days after SI surgery. The rats in the SI group were anesthetized by using an intraperitoneal injection of ketamine hydrochloride (70 mg/kg body wt) and acepromazine maleate (5 mg/kg body wt) and were subjected to complete spinal cord transections at both a midthoracic and an upper sacral level plus bilateral deafferentation between the two transection sites, as described previously (23, 40). These procedures were approved by the Institutional Animal Care and Use Committee at the University of California Los Angeles, and they conformed to the animal care procedures and standards recommended by the American Physiological Society.

Tissue processing. At each time point, animals from the appropriate subgroups were weighed, anesthetized with pentobarbital sodium (100 mg/kg), and then decapitated. The soleus muscles (as well as other limb muscles not reported in this study) were removed, quickly dissected free of connective tissue, quick frozen on dry ice, and subsequently stored at -80°C until used for biochemical and molecular analyses. Analyses for all subgroups involved an n of 6 or 7 per time point, as presented in the data profiles.

Total RNA isolation and RT. Total RNA extraction and RT procedures were as described in the companion paper (25). Pre-mRNA analyses for assessing transcriptional activity of sarcomeric genes. Gene expression can be regulated at several levels in the cells, i.e., at the transcriptional, posttranscriptional/pretranslational, translational, and posttranslational levels. One may gain understanding as to the site of regulation by examining the level of expression of protein, mRNA, and pre-mRNA along with their relationship to one another. To assess transcriptional activity of endogenous genes, nuclear run-on assays have traditionally been the standard procedure. Because of difficulties in obtaining skeletal muscle nuclei, and perhaps the inconvenience of the run-on assay (using large amounts of radioisotopes), very few studies have reported nuclear run-on assay results on muscle-specific gene transcription (22, 26). As an alternative to the nuclear run-on assay, analysis of pre-mRNA expression can be used to assess a gene’s transcriptional activity (19, 35, 37). To our knowledge, this approach has not been applied previously to study MHC or actin gene transcription. In this study, we have explored this approach by investigating transcriptional regulation of the type I MHC and α-skeletal actin genes in the soleus muscle of SI rats.

Type I MHC and actin mRNA and pre-mRNA analyses. All of the RNA samples used for these analyses were treated with DNase to remove any trace of genomic DNA contamination. DNase treatment was performed according to the supplier’s recommendation (Promega). After DNase treatment, the RNA samples were reextracted with TRI Reagent LS, and the RNA pellet was suspended in nuclease-free water. RNA concentration was determined by UV absorption at 260 nm, and the samples were stored at -80°C for subsequent analysis with the RT-PCR.

Semi-quantitative RT-PCR was used to analyze the expression of type I MHC and α-skeletal actin gene expression at the pre-mRNA and mRNA levels in NC and 8-day SI soleus RNA samples. One microgram of total RNA was reverse transcribed by using Superscript II RT, according to the supplier’s recommendations (Invitrogen, Carlsbad, CA), but with substitution of oligo(dT) primers with a gene-specific antisense primer (primer 2 for the β-MHC and primer 6 for the actin, see Table 1). The PCR for amplification of type I MHC and actin mRNA was carried out in 25-μl reactions containing 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.75 unit Biolase DNA polymerase (Bioline, Randolph, MA), 1 μl cDNA template diluted 40-fold, and 15 pmol each of primers 1 and 2 for MHC or primers 5 and 6 for actin (Table 1). Annealing was set at 56°C, and amplification was carried out for 24 cycles by using a 1-min extension period for each cycle, which resulted in a 546-bp PCR product for type I MHC and a 214-bp product for α-skeletal actin. PCR to amplify type I MHC pre-mRNA or actin pre-mRNA was similar to the above, except that the cDNA dilutions were only fivefold. The primers used were primers 3 and 4 for MHC and primers 7 and 8 for actin, and the number of cycles was raised to 30. This resulted in a 186-bp product for MHC and 210-bp product for actin. Primer 3 is derived from the first intron of the β-MHC gene (F. Haddad and K. M. Baldwin, unpublished observations), whereas primer 7 is derived from the first intron of the actin gene. Thus these primers can target only pre-mRNAs. The number of cycles and the PCR conditions for each target mRNA were optimized so that the amplified signal was still on the linear portion of a semilog plot of the yield expressed as a function of the number of cycles. As a check for genomic DNA contamination, PCR reactions were carried out by using an equal amount of nonreverse transcribed RNA. These reactions turned out negative, thus validating the effectiveness of DNase treatment. PCR products were separated on a 2% agarose gel by electrophoresis, and they were stained with ethidium bromide. The signal quantification was conducted by laser scanning densitometry, as reported previously (52).

Table 1. Nucleotide sequence of the PCR primers that were used for amplification of type I MHC and α-skeletal actin pre-mRNA and mRNA

<table>
<thead>
<tr>
<th>Sequence 5’ to 3’</th>
<th>Reference</th>
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<td>Primer 1 βMHC E37 s</td>
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<td>Primer 2* β-MHC E40 as</td>
<td>GTCTCCAGGTTCCTAAGCCAGG</td>
</tr>
<tr>
<td>Primer 3 βMHC I39 s</td>
<td>GAAAGCCCTCAAGGTCGAC</td>
</tr>
<tr>
<td>Primer 4 β-MHC E40 as</td>
<td>AGGCTCCCTAAGGTCGAC</td>
</tr>
<tr>
<td>Primer 5 α-skel actin E6 s</td>
<td>GGGATCCCATGAGACCACCTA</td>
</tr>
<tr>
<td>Primer 6* α-sk actin E7 as</td>
<td>GCCGCCGATCCACACTG</td>
</tr>
<tr>
<td>Primer 7 α-sk actin 11 s</td>
<td>GGGTCTCAGGGCTTCACAGG</td>
</tr>
<tr>
<td>Primer 8 α-sk actin E2 as</td>
<td>CCCCCCTGCGAACGAGGAGT</td>
</tr>
</tbody>
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*Primer was used for the RT reaction to synthesize mRNA specific cDNA. Gb, GenBank accession number. Sequences are from the 5’ to the 3’ direction. MHC, myosin heavy chain; sk, skeletal.
PCR. A relative RT-PCR method, using 18S as an internal standard (Ambion, Austin, TX), was applied to study the expression of specific mRNAs for IGF-I, IGF-I receptor, IGF-BPs (BP-4, and BP-5), myogenin, MyoD, cyclin D1, and p21. Also, additional analyses were made on calpain-1 and -2, ubiquitination enzymes, the E2 14-kDa carrier protein, the E3, ubiquitin (Ub) ligase, and the newly discovered nuclear E3 ligase atrogin-1 (10, 21). The sequences for the various primers used for the specific target mRNAs are shown in Table 2, and these primers were purchased from Life Technology (Invitrogen). In each PCR reaction, the 18S ribosomal RNA was coamplified with the target cDNA (mRNA) to serve as an internal standard and to allow correction for any differences in starting amounts of total RNA. PCR procedure and quantification methods were conducted as described previously (2, 24).

Phosphorylation state of intracellular signaling proteins.

The phosphorylation state of the p70S6 kinases, the 4EBP1, the eukaryotic initiating factor 2 (eIF2) and the ERK1/2 were examined by immunoblotting using phospho-specific antibodies (Cell Signaling Technology, Beverly, MA). These antibodies react with phospho-specific sites of phosphorylation that has been shown to strongly correlate with the kinase biological activity in vivo (Cell Signaling, Beverly, MA). Muscle samples were extracted by homogenization in seven volumes of ice-cold buffer A [50 mM Tris-HCl, pH 7.8, 2 mM potassium phosphate, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM DTT, 3 mM benzamidine, 1 mM sodium orthovanadate, 10 μM leupeptin, 5 μg/ml aprotinin, 200 μg/ml soybean trypsin inhibitor, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride] by using a motor-driven glass pestle. The homogenate was immediately centrifuged at 12,000 g for 30 min at 4°C. The supernatant was immediately saved in aliquots at −80°C for subsequent use in immunoblotting. The supernatant protein concentration was determined by using the Bio-Rad protein assay with BSA as the standard. Approximately 50 μg of supernatant proteins were subjected to SDS-PAGE (12.5% total monomer concentration in the acrylamide gel), according to standard protocol (31), and then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P) by using 10% methanol, 1 mM orthovanadate, 25 mM Tris, and 193 mM glycine, pH 8.3. The enhanced chemiluminescence method was used for detection (Amersham, Piscataway, NJ). Signal intensity was determined by laser scanning densitometry (Molecular Dynamics/Image Quant). For each specific antibody, all of the samples were run under identical (previously optimized) conditions, including the transfer on the membrane, the reaction with the first and secondary antibodies, washing conditions, enhanced chemiluminescence detection, and film exposure. To ensure the consistency of this analysis, at least one representative sample from each group was included in each gel run and Western analysis. In addition, a positive control, provided by the antibody supplier, was run on each gel to allow for normalization. For each set of Western blotting and detection conditions, the detected signal was directly proportional to the amount of protein loaded on the gel over a range of 20–150 μg (data not shown).

Statistical analysis. All values are reported as means ± SE. For each time point, treatment effects were determined by ANOVA with Newman-Keuls post hoc testing by using the Prism software package (Graphpad). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

RESULTS

Transcriptional activity of type I MHC and actin. In the companion paper (25), we reported that mRNA levels for both actin and the type I MHC were mark-

Table 2. Nucleotide sequence of the primers used for the RT-PCR mRNA analyses

<table>
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<tr>
<th>Target mRNA</th>
<th>PCR Primer sequence 5'-3'</th>
<th>Product Size, bp</th>
<th>GenBank Accession No.</th>
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<td>IGF-I</td>
<td>Fwd: GCATTCTGGATGATGCTATGC</td>
<td>202</td>
<td>X06043</td>
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<tr>
<td></td>
<td>Rev: GGCTCTTCTGATATTCTGTA</td>
<td></td>
<td></td>
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<tr>
<td>IGF-BP-5</td>
<td>Fwd: CAAGCTTTGGCAAGGTAAC</td>
<td>214</td>
<td>NM_012817</td>
</tr>
<tr>
<td></td>
<td>Rev: GTGGGGAATTGGGATGGTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-I receptor</td>
<td>Rev: ACTGGGAGACCGGAGAAGAGA</td>
<td>245</td>
<td>L29232</td>
</tr>
<tr>
<td>IGF-BP-4</td>
<td>Fwd: CCGTCTTCTGGACTAAAGA</td>
<td>212</td>
<td>BC019836</td>
</tr>
<tr>
<td>Myogenin</td>
<td>Fwd: ACTACGCGACCTGATAGGAC</td>
<td>233</td>
<td>M24393</td>
</tr>
<tr>
<td>MyoD</td>
<td>Rev: TCACAGCGCCGGATCTACAG</td>
<td>563</td>
<td>M84176</td>
</tr>
<tr>
<td>Cyclin D</td>
<td>Rev: TGGGCCGAGATGTAGGAGA</td>
<td>267</td>
<td>D14014</td>
</tr>
<tr>
<td>p21</td>
<td>Fwd: CCGTCTTCTGGACTAAAGA</td>
<td>233</td>
<td>U24174</td>
</tr>
<tr>
<td>Calpain-1</td>
<td>Rev: ACGAGCGCAGAGGATAGTA</td>
<td>212</td>
<td>NM_019152</td>
</tr>
<tr>
<td>Calpain-2</td>
<td>Rev: CATGGGCGGAGATGGTGG</td>
<td>193</td>
<td>NM_017116</td>
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<td>Ub E2 14 kDa</td>
<td>Rev: GCGTGGAATGAGGAAGAAGAG</td>
<td>273</td>
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<td>Ub E3a</td>
<td>Rev: CATGGGCGGAGATGGTGG</td>
<td>211</td>
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<td>223</td>
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IGF1-BP-4 and Ub E3a primer set sequences are from a conserved mouse mRNA sequence based on comparisons with the human mRNA sequence. Fwd, forward primer; Rev, reverse primer. Sequence listed is from the 5' to the 3' direction. BP, binding protein; Ub, ubiquitin.
edly reduced in response to SI. Consistent with this response, we observed that, during the first 7 days of SI, there were 60–70% reductions in pre-mRNA levels of type I MHC and actin in the soleus muscles (Fig. 1). This response correlated well with the reduction in the mRNA for these two genes that were assessed in the same RNA extracts (Fig. 1, C and F). This significant reduction in transcriptional activity was not due to a general reduction of transcriptional activity of the SI muscle, because we have observed a significant increase in the pre-mRNA levels of the fast-type Iib MHC gene (unpublished observation), which is consistent with the redirection of gene expression to a faster phenotype.

Muscle IGF-I system. IGF-I has been associated with muscle growth via binding to its receptor that, in turn, initiates a cascade of intracellular events favoring a positive protein balance and cell growth. Based on the observed increase in IGF-I mRNA and protein in response to muscle overload (1), we expected a decrease in IGF-I mRNA in atrophying SI soleus. Our results show that IGF-I mRNA (Fig. 2A) levels relative to 18S were not different from control levels up to 4 days of SI, and they increased by 30–40% at days 8 and 15, despite the muscle experiencing severe atrophy. The mRNAs encoding the IGF-I receptor and IGF-I BP-5 were elevated during the experimental period (Fig. 2). In contrast, the IGF BP-4 mRNA level did not change significantly throughout the experimental period (Fig. 2C).

Extracellular response factors and protein translation markers. The “extracellular response kinases” (ERK1/2) are part of an intracellular signaling pathway leading to changes in gene transcription that is activated by a number of growth factors, including IGF-I (20, 51). At the sites detected by the antibodies used in this study, phosphorylation of ERK1 and ERK2 indicates that this pathway was activated. The phosphorylation of ERK1 was increased at day 8 of SI (Fig. 3B), whereas ERK2 was elevated at days 4 and 8 of SI (Fig. 3C). These changes in the ERK phosphorylation levels occurred without altered total ERK expression, as assessed by immunoblotting with an ERK antibody (cell signaling) (Fig. 3).

The phosphorylation of p70S6k and of 4EBP1 has been reported to be associated with an increase in translation of mRNAs encoded for specific components of the translation machinery and for proteins in general, respectively (17). Increases in the phosphorylation of these proteins are known to occur in response to 1) increased muscle loading (8, 24) and/or 2) IGF-I receptor binding (20, 38). Analysis of these markers by Western blotting demonstrates that, in the SI samples, the phosphorylation level of p70S6k tended to be higher at days 4 and 8, although this did not reach statistical significance (Fig. 4A). The total amount of p70S6k was
not altered (Fig. 4B). Levels of 4EBP1 expression and phosphorylation state were not different between NC and SI soleus muscles (Fig. 5, A and B). To gain further insight on additional key marker proteins impacting protein translation, we examined the phosphorylation state of another regulatory protein in the initiation process, i.e., regulatory protein eukaryotic initiation factor (eIF2α). This protein can modulate the activity of eIF2B, the enzyme that controls one of the rate-limiting steps in protein initiation (33, 48). When eIF2α is phosphorylated, it inhibits eIF2B activity, thereby reducing protein initiation. However, in SI, we observed a consistent, although not statistically significant, pattern of dephosphorylation of eIF2α, suggesting that this factor was not functioning in a mode to inhibit eIF2B activity (Fig. 6). Taken together, these findings indicate that there was no overall clear pattern in the soleus muscle of SI rats to suggest marked downregulation in either the functional properties (phosphorylation state) or protein levels of the protein translation system to explain the severe atrophy response.

Markers of protein degradation. In view of the rapid loss in both muscle mass and protein in response to SI, it was of interest to ascertain whether there was altered gene expression of enzyme systems associated with protein degradation. One enzyme system that we focused on involved the calcium-activated calpains. The calpains have been proposed to play a role in dissociating certain proteins such as titin and α-actinin and releasing them from the myofibrillar complex (27). As presented in Fig. 7, there was a clear pattern of increased expression of calpain-1 mRNA that persisted for up to 15 days of SI. Calpain-2 mRNA expression, on the other hand, did not change significantly throughout the SI period.

Because the Ub-proteasome pathway has been proposed to be the primary pathway for degrading the bulk of proteins expressed in skeletal muscle, including those disassociated from the sarcomere infrastructure (15, 44), we examined three key enzymes in the enzymatic cascade of protein ubiquitination, i.e., E2 14-kDa Ub carrier protein, the E3α, Ub ligase, and atrogin-1, an E3 Ub ligase of SCF (Skp/cullin/Fbox) family (21), also called muscle atrophy F-box (10). Our data show no significant difference between NC and SI mRNA levels for E2 14-kDa Ub carrier protein (Fig. 8A). However, there was an elevation in the Ub E3α ligase mRNA levels in the SI soleus muscles throughout the experimental period (Fig. 8B). E2 14-kDa and E3α ligases are critical in degrading myofibrillar and muscle protein (32, 45). Interestingly, although the functional properties of the atrogin-1 gene have not been elucidated (10, 21), it is obvious that this gene was also highly responsive to SI (Fig. 8C). In fact, the response of this gene was relatively greater and occurred at an earlier time point than that of E3α ligase. These findings indicate that there are increases in mRNA expression of enzymes involved in critical steps of protein degradation. Also, the responses seem to be temporally consistent with the rapid stages of protein loss in the soleus muscle of SI animals. Furthermore, it is not clear whether these components of the protein degradation system have any preferential effect on MHC relative to actin in the soleus of SI rats to cause a larger deficit in MHC protein relative to actin, as reported in the companion paper (25).
Gene expression of marker proteins of the cell cycle. Significant increases in the mRNA expression of molecular markers of the cell cycle, such as cyclin D1 and p21, as well as myogenic regulatory factors, such as myogenin and MyoD, were previously reported in skeletal muscle undergoing compensatory hypertrophy (4). Cyclin D1 and p21 are associated with proliferation (entering the cell cycle) and differentiation (leaving the cell cycle) processes involving progenitor cells, e.g., satellite cells, respectively (43, 49). Although myogenin and MyoD also are thought to be associated with the proliferation and differentiation processes of such progenitor cells (13, 49), they also may serve as transcription factors in adult skeletal muscle fibers and may be involved in regulating phenotype properties (29, 34, 36, 41). Thus it was of considerable interest to ascertain how these markers would respond to SI, given that other models of atrophy, such as hindlimb unloading, spaceflight, and spinal cord transection, have been associated with a loss of myonuclei (6). We hypothesized that these markers would be downregulated if the function of satellite cell proliferation and differentiation were inhibited during the atrophy response associated with SI. Surprisingly, with the exception of cyclin D1, which decreased initially and then returned to control levels, the response of MyoD, myogenin, and p21 reflected marked upregulation in response to SI (Fig. 9).

DISCUSSION

The primary goal of this study was to identify the molecular markers of inactivity-induced atrophy of a
slow muscle by using the model of SI that near completely inactivates the muscle, while maintaining intact nerve-muscle connections. Several cellular (25) and molecular (present paper) responses were examined in the soleus muscle when subjected to SI intervention. The SI soleus experiences a severe protein imbalance that results in a rapid and significant loss in protein and muscle mass. To better understand the mechanism of protein loss in the SI muscle, we have assessed markers known to be involved with protein synthesis (transcription and translation) and protein

Fig. 5. Analysis of eukaryotic 4E BP-1 (4EBP1) activity by immunoblotting. A: representative immunoblot for NC and SI samples. 4EBP1 is phosphorylated at different sites. A different amount of phosphorylation (P) results in altered migration on the gel: the δ form, the most phosphorylated, is the slowest migrating; and the α form, the least phosphorylated, is the fastest migrating. The relative expression of each form is obtained via scanning and image analysis by using peak area integration utility (Image Quant Software, Molecular Dynamics). The utilized antibody reacts with 4EBP1, regardless of its phosphorylation state. B: phosphorylation state of 4EBP1 in the different groups at the indicated time points. This was calculated as the ratio between the sum of δ + γ/α + β. C: total 4EBP1 expression was calculated based on the volume density integration of a rectangle containing all of the bands with local average background correction. Values are means ± SE of the average of n = 6 or 7/group at each time point.

Fig. 6. Bottom: bar graph representing the mean values (±SE) of the phospho-eukaryotic initiation factor (eIF2α) for NC and SI muscles at the indicated time points and as determined by Western blotting analysis using a phospho-specific eIF2α antibody. There were no significant differences between NC and SI at any time point. n = 6–7/Group. Top: representative immunoblots for NC and SI muscle samples at 2, 4, and 8 days.

Fig. 7. Calpain-1 (A) and calpain-2 (B) mRNA analysis by RT-PCR. Calpain-1 and -2 mRNA levels are relative to 18S ribosomal RNA in NC and SI muscles. Both panels include a representative gel of the PCR products. Values are means ± SE of the average of n = 6 or 7/group at each time point. *P < 0.05 vs. NC.
degradation, as well as factors involved with cell proliferation and myogenesis. Of the five hypotheses outlined in the Introduction, our findings supported only two of these hypotheses, i.e., the hypotheses that were centered on 1) transcriptional regulation (number 1) and 2) protein degradation control (number 4). Those hypotheses that focused on the role of 1) the IGF-I system (number 2), 2) cell signaling and markers of protein translational efficiency (number 3), and 3) myogenic regulatory factor expression (number 5) in the events of muscle atrophy in the SI model were not supported by our findings. In the following sections, we address the significance of each of these observations.

**Transcriptional regulation of sarcomeric genes.** We have reported that the SI model induces marked reductions in the transcriptional regulation of the type I MHC promoter (28). In the present study, we focused on the endogenous regulation of both slow type I MHC and actin pre-mRNA in view of the marked reductions in actin, total MHC, and especially type I MHC mRNA reported in the companion paper (25). Thus the findings reported herein demonstrate that decreased gene transcription likely plays a role in reducing the amount of mRNA of key sarcomeric proteins in the SI model. The smaller mRNA pools for actin and type I MHC in the slow soleus muscle may limit the ability of the muscle to synthesize these key proteins, especially taking into consideration that the ribosomal RNA pool is also reduced under these conditions (25). Therefore, decreased transcriptional regulation of key skeletal muscle genes plays a significant role in regulating the atrophy responses to muscle inactivity as induced by SI.

**IGF-I and associated BPs.** Considerable evidence has accumulated to suggest that IGF-I is an autocrine and paracrine growth factor that, alone, has skeletal muscle anabolic properties (1). Also, it has been implicated to be strategically involved in compensatory muscle growth in response to increased loading states imposed on the muscle (1, 3). Therefore, we reasoned that the IGF-I system might undergo downregulation and serve as a negative regulatory factor in response to muscle inactivity. The results reported herein negate this hypothesis and suggest that the IGF-I system components either were not changed or were upregulated in the SI muscles.

The role of IGF-I in muscle atrophy is not clear. IGF-I mRNA expression and protein decrease after denervation (11), but, after hindlimb unloading and in human muscle disuse, these levels have been reported to decrease (7), remain unchanged (14), or increase (39). These apparently contradictory findings on the increased expression of IGF-I in atrophied muscle may be attributed to a counterregulatory response to conserve muscle mass. Although we have not determined the protein level of IGF-I in this study, generally there is a good agreement between the protein and mRNA for muscles undergoing atrophy (11) or hypertrophy (3). If IGF-I protein levels parallel the mRNA levels, our results suggest that IGF-I does not play a role in the atrophy process, at least as induced by SI. This is further supported by the fact that IGF-I overexpression did not prevent unloading-induced atrophy (14). The IGF-I cellular action occurs via binding to its receptor that, in turn, can be modulated by various BPs. For example, IGF BP-5 is proposed to have a dual role: it either potentiates or inhibits the action of IGF-I, whereas IGF BP-4 is postulated to be a negative modulator (12, 16). Our observation of an upregulation of BP-5 mRNA in the inactive soleus (Fig. 2) is consistent
with an upregulation in the muscles of hindlimb-unloaded rats (7). Collectively, these results suggest that muscle atrophy in response to inactivity occurs without an obligatory decrease in IGF-I mRNA expression and thus support the conclusion that the molecular responses involving the IGF-I system are not the reverse of what is observed in hypertrophying muscles.

**Markers of cell signaling and protein initiation and translation processes.** In overload-induced hypertrophy, as well as in response to increased muscle activation associated with high-resistance exercise, several markers involved in intracellular signaling that can lead to increased transcriptional activity or increased protein translation and thought to greatly contribute to the anabolic response are activated by phosphorylation (2, 8, 9, 20, 24). These signaling molecules include ERK1/2, p70S6k, 4EBP1, and eIF2α. Because muscle atrophy in unloading models has been attributed partly to a decrease in protein synthesis (47), we anticipated that the activity of these factors would be downregulated in the atrophied SI muscles. Our findings (Figs. 4–6) would argue that these translation markers do not play a significant role in limiting the protein translation processes associated with a loss in protein in the soleus muscles of SI rats. On the other hand, it would appear that the significant decreases in ribosomal RNA observed in the companion paper (25), as well as the inability of the protein initiation apparatus to significantly augment its functional activity, act in combination with the reductions in sarcomeric gene mRNA substrate to impair the SI muscle from carrying out a sufficient level of protein translation to maintain the muscle in a state of normal protein homeostasis.

**Protein degradation markers.** Protein degradation processes play a significant role in the turnover of muscle proteins, and any alteration in the functional level of these pathways can significantly affect the balance of protein maintained in skeletal muscle. Evidence is rapidly accumulating that the Ub-proteasome pathway is a key system in protein degradation and that this may be the primary pathway in degrading the majority of muscle proteins, including the myofibril fraction, which accounts for ~50% of the total muscle protein pool. Evidence also suggests that individual myofibril proteins, e.g., myosin and actin, are not readily degraded by the Ub-proteasome when they are conjugated within the sarcomeric machinery (27, 44–46). Thus it has been proposed that other proteolytic systems must operate in synergy with the Ub-proteasome axis to bring about degradation of complex structures of the muscle, such as the myofibrils. Therefore, we focused on examining 1) enzyme systems thought to be involved in causing dissociation of myofibrils, such as the calpains (27); and 2) the pivotal steps in polyubiquitinating proteins before their subsequent degradation in the proteasome machinery, i.e., marker E2 and E3 Ub enzymes. We found that gene expression for calpain-1 and enzymes representative of the E3 enzyme system were upregulated in the inactive muscles. The marked upregulation of the atrogin-1 gene is most intriguing in that this E3 enzyme contains specific sequences to suggest that it may be preferentially located in the nucleus (10). This raises the possibility...
that atrogin-1 may target the degradation of transcription factors, thereby negatively impacting the transcription of key sarcomeric genes, as discussed above. If these patterns of response at the mRNA level translate to increased expression at the protein level, they could provide a mechanism for increased protein degradation. In combination with an inadequate capacity for protein translation, as discussed above, upregulation of these proteolytic factors could play an important role in the rapid protein loss that occurred in SI muscles.

Gene expression of markers of the cell cycle. During experimental perturbations that lead to an anabolic state followed by subsequent muscle hypertrophy, cell cycle markers thought to regulate proliferation and/or differentiation of myogenic precursor cells and satellite cells, i.e., gene expression of myogenic factors such as cyclin D1, MyoD, myogenin, and p21, are markedly upregulated. Factors such as cyclin D1 and MyoD have been associated with cell proliferative processes, whereas factors such as myogenin and p21 have been implicated to have a role in modulating cell cycling and terminal differentiation (43, 49). These events are thought to be obligatory responses for muscle fibers to hypertrophy and concomitantly increase the number of nuclei within the myofibers to maintain a normal myonuclear-cytoplasmic domain (6). In the context of this well-defined adaptive process during hypertrophy, we hypothesized that these proliferative and differentiation events would be either blunted or nonoperative in the catabolic state of atrophying SI muscles. Surprisingly, with the exception of the cyclin D1 response pattern (which showed little change), the MyoD, myogenin, and p21 upregulation responses were equally as robust as that seen under the conditions of functional overload (2, 4). Assuming that the upregulation of MyoD, myogenin, and p21 has some functional role in the atrophic process, it is apparent that these proteins can mediate responses beyond simply modulating cell proliferation and differentiation. Interestingly, p21 is thought to have antiproliferative activity (53) and thus may be playing an inhibitory role on satellite cell proliferation. In addition, it is important to note the recent observation by Adams et al. (2) that overloaded-irradiated skeletal muscles, which are blunted in their ability to hypertrophy, even when the stimulus is applied for several months, show an increased expression of the same markers noted above for SI muscles. In the atrophying condition, the muscles lose myonuclei via apoptosis (5, 42), whereas, in the “overloaded-irradiated condition,” the hypertrophy process is blunted by irradiation by causing defects in the DNA structure and its ability to repair.

A limitation of our approach is that the markers were analyzed at the whole muscle level. Based on the heterogeneous nature of skeletal muscles, this approach cannot be used to identify which components of the muscle were responsible for the expression of the myogenic-cell cycle markers. The fact that the atrophied muscles did not respond directly opposite to that observed in hypertrophied muscles is intriguing. However, these findings highlight the importance to better define the response in terms of which cell types (muscle fiber, connective tissue, fibroblast, endothelial, etc.) are responsible for the observed responses. These issues could be addressed by using immunohistochemistry in future studies. In view of the MyoD and myogenin responses in muscle atrophy models using immunocytochemical methods, Weis (50) reported that the observed increase in MyoD and myogenin expression in denervated muscle is localized in myonuclei rather than in satellite cells, whereas others report that, after spinal cord transection, myogenin immunostaining increases in both myonuclei and satellite cells whereas myoD increases only in satellite cells of soleus muscle (18). Recently, Hyatt et al. (30; unpublished observations) reported increased expression of MyoD and myogenin protein in the myonuclei in both SI and denervated soleus muscle fibers and that this response markedly exceeded that observed for nuclei outside the boundaries of the muscle sarcolemma, e.g., satellite cells and other nonmuscle cells. Furthermore, it was noted using 5′-bromo-2′-deoxyuridine labeling that the proliferation of satellite cells into new myonuclei incorporated into the fibers could not account for the increased myonuclear expression of MyoD and myogenin. These latter findings suggest that the increased expression of MyoD and myogenin reflects a response in which these factors may be elevated to affect target genes other than those associated with proliferation and differentiation. The exact role of myoD and myogenin in atrophied SI soleus is not clear; however, they may, in part, be involved with the marked phenotype change associated with this model.

Summary. In the present papers (24) and previous studies (25, 40), our laboratory has demonstrated that inactivity results in rapid skeletal muscle atrophy and protein deficits. These responses appear to be occurring by the coordinated events governing processes that impact 1) the reduced transcriptional activity and translational capacity (mRNA substrate and ribosomal RNA) involving key proteins comprising the sarcomeric fraction of the muscle (and other key fractions as well); and 2) the apparent upregulation of genes impacting the degradation processes governing muscle protein breakdown. These processes can occur independently of the IGF-I axis and without necessarily inhibiting protein translation at critical control steps. In addition, there is a reduction in the ability of the muscle to adequately control the DNA content in the muscle. These collective alterations bring about a rapid and marked wasting of the inactive muscle.

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DISCLOSURES

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