ABSTRACT: Muscle denervation and concomitant high-dose dexamethasone treatment in rodents produces characteristic pathologic features of severe muscle atrophy and selective myosin heavy filament (MyHC) depletion, identical to those seen in acute quadriplegic myopathy (AQM), also known as critical illness myopathy. We tested the hypothesis that defective pre-translational processes contribute to the atrophy and selective MyHC depletion in this model. We examined the effects of combined glucocorticoid–denervation treatment on MyHC and actin mRNA populations; we also studied mRNA expression of the myogenic regulatory factors (MRFs), primary transcription factors for MyHC. Adult female rats were subjected to proximal sciatic denervation followed by high-dose dexamethasone (DD) treatment (5 mg/kg body weight daily) for 7 days. Disease controls included rats treated with denervation alone (DN) or dexamethasone alone (DX). At 1 week the plantaris atrophied by ~42% in DD muscles. DD treatment resulted in selective MyHC protein depletion; actin protein concentration was not significantly changed. Despite an increase in total RNA concentration in DN and DD muscles, MyHC and actin mRNA concentrations were significantly decreased in these muscles. MyHC mRNA showed a significantly more extensive depletion relative to actin mRNA in DD muscles. Glucocorticoid treatment did not influence a denervation-induced increase in the mRNA expression of the MRFs. We conclude that a deleterious interaction between glucocorticoid and denervation treatments in skeletal muscle is responsible for pre-translational defects that reduce actin and MyHC mRNA substrates in a disproportionate fashion. The resultant selective MyHC depletion contributes to the severe muscle atrophy.


MOLECULAR AND CELLULAR DEFECTS OF SKELETAL MUSCLE IN AN ANIMAL MODEL OF ACUTE QUADRIPLEGIC MYOPATHY

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Glucocorticoids and denervation independently are potent inducers of skeletal muscle atrophy, starting as early as 3 days following treatment.3,22,38 In both treatments, muscle atrophy is due to protein imbalance and is the result of the combination of decreased protein synthesis and increased protein degradation. Each of these processes is influenced by cellular and molecular events that could differ slightly in these two models. Glucocorticoids and denervation have diverse effects on insulin-like growth factor-1 (IGF-1) signaling, an important mediator of skeletal muscle atrophy. For example, glucocorticoid treatment is associated with increased insulin resistance in skeletal muscle and inhibition of IGF-1 signaling39; by contrast, denervated plantaris muscle shows an increase in mRNA levels for IGF-124 and increased phosphorylation of p70s6k, a potent inducer of protein translation.17 Although eventually both treatments lead to decreased expression of myofibrillar proteins, it is not clear whether...
the chain of events leading to the protein loss is similar at the mRNA level.

Because dexamethasone and denervation treatments are each potent inducers of muscle atrophy, a catastrophic effect on skeletal muscles can be predicted if these two treatments are combined. Surprisingly few investigators have studied the combined effects of dexamethasone–denervation treatment. Rouleau et al. found that muscles from animals receiving such combined treatment exhibited severe muscle atrophy and selective MyHC depletion. Similar changes were confirmed by the work of Rich and colleagues. The atrophy and selective MyHC depletion reversed once reinnervation was allowed to occur. Rich et al. also showed that muscle membrane inexcitability occurred in this model. The pathologic and neurophysiologic changes in the rodent muscles were recognized to be identical to those seen in acute quadriplegic myopathy (AQM) in hospitalized critically ill patients, a condition characterized by severe muscle weakness, respiratory failure, and significant morbidity and mortality. AQM is also referred to as "critical illness myopathy." The rodent model thus provides a unique opportunity to examine the molecular changes underlying this disorder, and so far represents the only reproducible animal model for this disease. The molecular basis for the severe muscle atrophy or selective MyHC depletion in AQM has not been studied.

In order to better understand the molecular mechanisms underlying the severe skeletal muscle atrophy and selective MyHC depletion in AQM, we chose to examine the effects of this combined treatment on the RNA populations as well as specific mRNA levels of MyHC and actin genes. We hypothesized that, in the combination treatment model, pre-translational events, such as a selective loss of MyHC mRNA, cause selective MyHC depletion. We further examined the effects of combined glucocorticoid–denervation treatment on myogenic regulatory factors (MRFs). MRFs, such as myogenin, MyoD and Myf5, are all members of the basic helix-loop–helix (bHLH) family of muscle-specific transcription factors and represent the primary transcription factors for the MyHC gene family. MyoD is metabolized within the nucleus through one of the muscle-specific ubiquitin ligases (atrogenes), atrogin-1. Atrogin-1 is a highly sensitive marker of skeletal muscle atrophy. One of the presumed mechanisms of atrogin-1–induced skeletal muscle atrophy is increased degradation of MRFs, resulting in reduced transcription of the MyHC gene, and this may underlie the skeletal muscle atrophy seen in denervation as well as with glucocorticoid treatment.

Recovery from atrophy or muscle injury is critically dependent upon effective repair. MRFs are established markers of satellite cell/myogenic progenitor cell activity, an important first step in muscle fiber regeneration. Denervation upregulates the expression of myogenin and MyoD in skeletal muscle. MyoD specifically is also closely linked to gene expression of the nicotinic acetylcholine receptor, another gene that is upregulated immediately after denervation. Protein levels of myogenin and MyoD closely follow those of the respective mRNAs.

There is considerable cross-talk between proteins crucial to the maintenance of the cell cycle and these MRFs. Levels of p21, a cyclin kinase inhibitor and a marker of cell cycle arrest, parallel a rise in MRFs such as MyoD and myogenin. Myogenin and p21 regulate processes of myogenic progenitor cells leaving the cell cycle and undergoing differentiation. From transgenic experiments involving p21 gene homozygous knockouts, it has been determined that p21 is essential for normal functioning of the myogenic progenitor cells in regenerating skeletal muscles. GADD45 is a p53-regulated gene and, as such, behaves as a growth-arrest gene; its expression is also enhanced during apoptosis. Expression of Myf5 alone has been suggested to define an intermediate developmental stage that provides a mechanism for satellite-cell self-renewal and further proliferation and Myf5-positive cells may not participate in muscle regeneration.

Thus, we sought to ascertain how markers of myogenic precursor cell cycle arrest (p21 and GADD45) and MRFs (MyoD, Myf5, and myogenin) responded to combined glucocorticoid–denervation treatment. Similar to the MRFs, levels of p21 and GADD45 mRNA are reportedly upregulated in denervated skeletal muscles. We hypothesized that there is glucocorticoid-induced suppression of elevated mRNA levels of cell cycle arrest genes in denervated muscle. Similar to a suppression of MRFs, such suppression in cell cycle arrest proteins should render muscle incapable of mounting a regenerative response and would have deleterious effects on denervated skeletal muscles, exaggerating muscle atrophy.

**MATERIALS AND METHODS**

**Animal Selection.** Adult female Sprague-Dawley rats (weight 250–310 g) were assigned randomly to one of four groups, so that there were 6 animals in each group. The groups were as follows: normal controls (NC); unilateral denervation (DN); dexamethasone treatment (DX); and unilateral denervation–dexamethasone treatment (DX+).
ethasone treatment (DD). Contralateral limb muscles in DN (untreated) and DD (treated with dexamethasone only) were also collected and analyzed; the findings in these muscles were not statistically different from NC and DX muscles, respectively, and are not included in the data presented herein. The denervation procedure was performed on anesthetized rats as described previously. Dexamethasone was administered as a subcutaneous injection to the paraspinal region at a dose of 5 mg/kg body weight for 7 consecutive days, similar to the previously used dose of dexamethasone in this model. All animals were housed in standard vivarium cages. At the end of 7 days the experiment was terminated and the animals were euthanized with an overdose of pentobarbital sodium (60 mg/kg intraperitoneally). These procedures were approved by our institutional review committee and conformed to national standards.

**Tissue Selection.** At the termination of the experiments, plantaris muscles were rapidly excised, cleaned of connective tissue, weighed, quickly frozen in liquid nitrogen, and stored at −80°C until subsequent analyses. We chose to study plantaris, a predominantly fast-twitch muscle in rats, because in the early stages of the human disease fast-twitch fibers (type IIb) seem to be involved preferentially.

**Muscle Protein and Myofibril Extraction.** A pre-weighed portion of each muscle sample was homogenized in 20 volumes of a homogenization buffer consisting of 250 mM sucrose, 100 mM potassium chloride (KCl), 5 mM ethylene-diamine tetraacetic acid (EDTA), and 10 mM Tris-HCl at pH 6.8. Myofibrillar proteins were quantitatively extracted from a known volume of the total homogenate by a modification of the original procedure described by Solaro et al., and were suspended in 100 mM KCl, 10 mM Tris-HCl, and 1 mM EDTA at pH 7.4. Muscle total protein and myofibrillar protein concentration was determined by the Biuret protein assay method. The whole homogenate was diluted to a final protein concentration of 1 mg/ml in a storage buffer containing 50% glycerol, 100 mM sodium pyrophosphate (Na₂P₂O₇), 5 mM EDTA, and 2 mM 2-mercaptoethanol (pH 8.8), and stored at −20°C until subsequent analyses for MyHC and actin protein content.

**Muscle MyHC and Actin Protein Determination.** Skeletal muscle MyHC and actin proteins were separated on acrylamide gels (10% T,2.5% C) using a standard sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) technique as described previously. Briefly, protein samples were denatured and an equivalent of ~2.5 µg of total protein was loaded per lane. The gels were run at constant current (30 mA) for ~2.5 h at 22°C. At the completion of electrophoresis, the gels were stained with Coomassie brilliant blue G250 (Sigma Chemical, St. Louis, Missouri), destained, and then scanned using a laser-scanning personal densitometer (Molecular Dynamics, Sunnyvale, California). The MyHC and actin bands were identified based on their molecular weight and comparisons with purified protein. The intensity of the bands of interest (MyHC, actin) was calculated via integration of pixel density within a rectangle containing the entire band with local background correction (ImageQuant; Molecular Dynamics). Using this method, MyHC and actin proteins were expressed as arbitrary units per microgram of total protein, and the MyHC and actin protein concentration were calculated from the product of intensity of the bands of interest (arbitrary units per microgram of total protein of each band) multiplied by the corresponding muscle total protein concentration.

**MyHC and Actin mRNA Analyses.**

**RNA Extraction.** Total RNA was extracted from pre-weighed frozen muscle samples using TriReagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer’s protocol. Total RNA was precipitated from the aqueous phase with isopropanol and then washed with ethanol, dried, and suspended in a known volume of nuclease-free water. The RNA concentration was determined by optical density at 260 nm (using an equivalent of 40 µg/ml per unit, optical density = 260). The muscle total RNA concentration was calculated based on total RNA yield and the weight of the extracted sample. The RNA samples were stored frozen at −80°C until subsequent analysis for actin and total MyHC mRNA content by slot blotting, and for specific mRNA expression by reverse transcription–polymerase chain reaction (RT-PCR) methods.

**RNA Slot Blotting.** Total RNA (1 µg) was placed in 20 µl of denaturing buffer consisting of 10% formaldehyde, 67% formamide, and 0.5 × 3-[N-morpholino]propanesulfonic acid (MOPS) at pH 7, at 60°C for 15 min. Samples were brought up to a 100-µl volume with 6 × sodium chloride/sodium citrate (SSC) and were applied onto a positively charged nylon membrane (GeneScreen Plus; NEN, Boston, Massachusetts) by using a slot-blot apparatus (Schleicher & Schuell, Keene, New Hampshire). After ultraviolet fixation, these membranes were hy-
brided with three different probes consecutively as follows: (1) An antisense α-actin mRNA probe was used to determine α-actin mRNA expression, or a common antisense MyHC mRNA probe was used to determine the total MyHC mRNA expression. The MyHC probe is complementary to the coding region 500 nucleotides upstream from the stop codon of type I MyHC mRNA. This region is 100% identical in all of the MyHC isoforms, and the obtained signal corresponds to the total population of MyHC mRNA expressed in the muscle. (2) An oligodeoxynucleoside triphosphate (deoxythymidylic acid, or dT) probe (12–18-mer; Life Technology, Carlsbad, California) was used to detect poly(A) RNA (total mRNA population). (3) An antisense 18S ribosomal RNA probe was used to normalize for possible variability in the amount of loaded RNA per slot. Probes were 5′ end-labeled with 32P by using gamma-adenosine triphosphate (γATP) and T4 polynucleotide kinase. Hybridization and washing procedures were carried out as described previously. Hybridization signals were detected and analyzed by using a PhosphorImager and ImageQuant analysis software (Molecular Dynamics). For each sample, the MyHC mRNA, actin mRNA, and dT poly(A) signals were normalized to the corresponding 18S signal. Both the 18S ribosomal RNA (rRNA) along with 28S rRNA constitute the major components of total RNA (>85%), whereas the remainder is made of transfer RNA, 5S rRNA, and mRNA. The signal of 18S is directly proportional to the amount of loaded total RNA, ranging from 0.25 to 2 μg per slot, and therefore it is valid to use it as control for the amount of RNA loaded to normalize across different samples. The sequence of oligonucleotide probes used for hybridization was as reported by Adams et al. Total MyHC mRNA, total α-skeletal actin mRNA, and total mRNA were expressed as concentration; values were obtained based on total RNA concentration and the specific signal generated relative to corresponding 18S per 1 μg of total RNA.

Reverse Transcription. Total RNA (1 μg) was reverse transcribed for each muscle sample by using SuperScript II RT (Invitrogen Life Technology) and a mix of oligo-dT and random primers (100 ng/reaction) according to the provided protocol. At the end of the RT reaction, the tubes were heated at 85°C for 5 min to stop the reaction and stored frozen at −80°C until they were used in the PCRs.

Polymerase Chain Reaction. A relative RT-PCR method, using 18S as an internal standard (Ambion, Austin, Texas), was applied to study the expression of specific mRNAs for atrogin-1, MyoD, Myf5, myogenin, p21, and GADD45. The primer sequence used for Myf5 was as follows: forward, 5′-CGTACGAGGATGGAACAGGT 3′; reverse, 5′-GAGTGGAGGTCGCGGTG 3′. PCR product size was 211 bp. Those primers were based on an identical sequence between the mouse (Genbank accession no. X56182) and the human (accession no. NM_005593) mRNAs. The primer sequence used for GADD45 was as follows: forward, 5′-ATCGAAAGGTGGGACGCTGT 3′; reverse, 5′-TGAATGGTG GTTCGTCACCA 3′. Primers were based on rat sequences (Genbank accession no. L32591); PCR product size was 348 bp. Primer sequences for other target mRNAs were described in an earlier study from our group.

In each PCR reaction, the 18S ribosomal RNA was co-amplified with the target cDNA (mRNA) to serve as an internal standard and to allow correction for any differences in starting amounts of total RNA. For the 18S amplifications we used the alternate 18S internal standards (Ambion), yielding a 324-bp product. For GADD45 PCR, we used the classic 18S standard, which yields a product of 488 bp. The 18S primers were mixed with competitors at an optimized ratio that could range from 1:4 to 1:10, depending on the abundance of the target mRNA. Inclusion of 18S competitors was necessary to reduce the 18S signal, which allows its linear amplification to be in the same range as the co-amplified target mRNA (relative RT-PCR kit protocol; Ambion).

For each specific target mRNA, the RT and PCR reactions were carried out under identical conditions using the same reagent premix for all samples being compared in the study. To validate the consistency of the analysis procedures, at least one representative sample from each group was included in each RT-PCR run, and each sample was run in duplicate for the PCR reactions.

A 0.1–1-μl RT reaction was used for the 25-μl PCR reactions. The PCR reaction was carried out in the presence of an optimized MgCl2 concentration (1.5–2 mM) by using standard PCR buffer (Bioline, Boston, Massachusetts), 0.2 mM deoxynucleoside triphosphate (dNTP), 1 μM primers, and 0.75 unit of Biolase, a thermostable DNA polymerase (Bioline). Amplifications were carried out in a robocycler (Stratagene, La Jolla, California) with an initial denaturing step of 3 min at 95°C, followed by 25 cycles of 1 min at 96°C, 1 min at 55°C (55°C–60°C depending on primers), 1 min at 72°C, and a final step of 3 min at 72°C. The number of cycles was optimized so that the amplified signal was still in the linear range.

58 Myosin Depletion in AQM MUSCLE & NERVE January 2007
of the semilog plot of the yield expressed as a function of the number of cycles. PCR products were separated on a 2%–2.5% agarose gel by electrophoresis and stained with ethidium bromide; signal quantification was done by laser scanning densitometry, as reported previously. In this approach, each specific mRNA signal was normalized to its corresponding 18S. For each primer set, PCR conditions (cDNA dilutions, 18S competitor/primer mix, MgCl₂ concentration, and annealing temperature) were set to optimal conditions, so that both the target mRNA and 18S product yields were in the linear range of the semilog plot when yield was expressed as a function of the number of cycles.

**Statistical Analyses.** All data are reported as mean ± SE. One-way analysis of variance (ANOVA) with the Newman–Keuls multiple comparison post-test was performed using GraphPad Prism, version 4.01 (GraphPad Software, San Diego, California). Statistical significance was set at $P < 0.05$. In order to assess the interaction between DN and DX, data were analyzed by two-way ANOVA. In the analysis of MyHC to actin protein and mRNA ratios, the ratio values were normalized to the mean of the normal control group and logarithmic transformation was performed to achieve symmetry for ratios above 1 (for an increase) vs. ratios between 0 and 1 (for a decrease). In these transformations, a ratio of 1 is equal to 0, a ratio of 2 is equivalent to that of a ratio of 0.5 (i.e., 0.3), an increase equal to 0, a ratio of 2 is equivalent in absolute decrease). In these transformations, a ratio of 1 is associated with a 16% and 27% increase in MyHC and actin, respectively.

**RESULTS**

**Assessment of Muscle Atrophy and Protein Alterations.** Compared to NC, a 42% reduction was observed in absolute muscle weight of DD animals at 7 days; significantly smaller reductions were observed in the DN and DX muscles (20% and 18%, respectively). These values confirm an added atrophic effect of the combined denervation–dexamethasone treatment (Table 1). No significant change in protein concentration was seen in any of these groups, suggesting that the muscle mass decline was due to a proportionate decrease in water and protein. Myofibrillar protein concentration, however, was significantly lower in DD muscles than in other groups, suggesting a disproportionately higher myofibrillar protein loss relative to the total protein, which includes sarcomeric as well as non-sarcomeric proteins. Two-way ANOVA confirmed this loss of myofibrillar protein to be on the basis of an interactive effect of the combination treatment. Myofibrillar protein concentration remained unchanged in DN and DX muscles.

**Selective MyHC Protein Depletion.** Figure IA shows a representative MyHC-actin separating gel. Compared to NC, the DN plantaris showed a 17% decrease in MyHC expression, whereas the DD plantaris showed an exaggerated loss of MyHC, averaging 64% less MyHC expression (Fig. 1B). The actin expression did not change significantly in either the DN or DD group (Fig. 1C). DX treatment was associated with a 16% and 27% increase in MyHC and actin, respectively.

**Total RNA and mRNA Concentration, MyHC, and Actin mRNA Expression.** Muscle total RNA concentration was elevated in DN and DD muscles by 60% and 28%, respectively, relative to NC (Fig. 2A), whereas total RNA concentration was unchanged in DX muscles. Figure 2B shows representative slot-blot images that were used for densitometric analyses. Total [poly(A)] mRNA concentration was significantly elevated relative to NC in DN and DD muscles, reflecting an overall increase in the concentration of messenger RNA in these muscles (Fig. 2C). A marked reduction in MyHC and actin mRNA levels was observed in the denervated muscles, DN and DD (Fig. 2D and E). MyHC mRNA concentration was reduced by 42% and 53%, respectively, in DD and DX muscles relative to NC muscles; values for actin mRNA concentration were reduced similarly by 44% and 40%, respectively. DX muscles, in contrast, showed significantly higher MyHC mRNA levels (up by 49%) and an unchanged actin mRNA concentration relative to NC muscles.

The ratios of MyHC to actin at both protein and mRNA levels showed a significant decrease in DD muscles (Fig. 3). There was no significant difference in the protein ratios in the DN and DX muscles (Fig. 3A),

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**Table 1. Markers of muscle atrophy.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Absolute muscle weight (mg)</th>
<th>Normalized muscle weight (mg/g)</th>
<th>Protein concentration (mg/g muscle)</th>
<th>Myofibrillar concentration (mg/g muscle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>295.3 ± 7.5</td>
<td>1.033 ± 0.023</td>
<td>192.4 ± 3.7</td>
<td>108.8 ± 5.4</td>
</tr>
<tr>
<td>DN</td>
<td>236.0 ± 4.6</td>
<td>0.857 ± 0.014</td>
<td>196.8 ± 2.6</td>
<td>118.0 ± 2.4</td>
</tr>
<tr>
<td>DX</td>
<td>241.8 ± 4.7</td>
<td>0.992 ± 0.009</td>
<td>187.7 ± 3.7</td>
<td>107.7 ± 3.9</td>
</tr>
<tr>
<td>DD</td>
<td>172.8 ± 12.8</td>
<td>0.749 ± 0.047</td>
<td>194.2 ± 3.2</td>
<td>88.13 ± 3.4</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM; n = 6 animals/group.  
*Significant difference compared to normal control.  
†Significant difference compared to all groups.  
‡Inhibitory interaction between denervation and dexamethasone treatments in DD muscles.
whereas there was a preferential increase in the MyHC/actin mRNA ratio for the DX group (Fig. 3B). Two-way ANOVA of both the MyHC/actin protein ratio and MyHC/actin mRNA ratio showed significant interaction between denervation and dexamethasone treatment \( (P < 0.0001 \text{ and } P = 0.0004, \text{ respectively}) \).

![MyHC-Actin Separating Gel](image)

**FIGURE 1.** (A) SDS-PAGE gel for MyHC and actin stained with Coomassie brilliant blue. Lanes labeled NC, DN, DX, and DD represent normal control, denervated, dexamethasone-treated, and denervation–dexamethasone-treated muscles, respectively, at day 7. (B) MyHC protein concentration (arbitrary scan unit per gram of muscle). (C) Actin protein concentration (arbitrary scan unit per gram of muscle). §Inhibitory interaction between denervation and dexamethasone treatments in DD muscles. Values are mean ± SEM; \( n = 6 \)/group. *Significant difference compared to normal control; †significant difference compared to all groups. MyHC was abundant in NC and DX muscles. A slight decrease in MyHC was seen in DN muscles. MyHC was prominently decreased in DD muscles. Actin was abundant in all groups.

![MyHC Concentration](image)

![Actin Concentration](image)

**FIGURE 2.** Effects of various treatments on total RNA (A), poly(A) mRNA (C), MyHC (D), and actin mRNA (E). Total RNA concentration is expressed as milligrams of RNA per unit of muscle (in grams), whereas the total poly(A), MyHC, and actin mRNA concentrations are expressed in arbitrary scanned units per gram of plantaris muscle. (B) Composite slot-blot image showing mRNA signal after hybridization to antisense probes for MyHC, actin, and 18S ribosomal subunit, and an oligomer dT [poly(A) mRNA] probe. Values are mean ± SEM; \( n = 6 \) animals/group. *Significant difference compared to normal control; †significant difference compared to all groups. A denervation-related increased in total RNA concentration was seen but MyHC and actin mRNA concentrations were decreased in muscles receiving denervation treatments (DN and DD).
**Expression of Atrogin-1 mRNA in Atrophic Muscles.**

All three treatment groups showed significant but similar degree of elevation (~2.5-fold) in mRNA levels of atrogin-1 in DN, DX, and DD muscles relative to NC at 1 week (Fig. 4A).

**Gene Expression of Cell Cycle Markers and Muscle Regulatory Factors.** Significant increases in expression of the message for all three MRFs (myogenin, MyoD and Myf5) were seen in DN and DD muscles, relative to NC and DX muscles (Fig. 4B-D). This effect was driven by the denervation treatment, common to both DN and DD muscles. Relative to NC muscles, MyoD mRNA levels were increased 3-fold in DN and DD muscles (Fig. 4B); myogenin mRNA levels were increased 10-fold in DN muscles and 8-fold in DD muscles (Fig. 4C); and Myf5 mRNA was increased 2-fold and 6-fold in these muscles, respectively (Fig. 4D).

**FIGURE 3.** MyHC/actin protein (A) and mRNA (B) ratios. Values are mean ± SEM; n = 6 animals/group. *Significant difference compared to normal control; †significant difference compared to all groups. §Inhibitory interaction between denervation and dexamethasone treatments in DD muscles. MyHC protein and mRNA are preferentially depleted in combined denervation–dexamethasone treatment.

**FIGURE 4.** Messenger RNA expression for atrogin-1 (A), MyoD (B), myogenin (C), and Myf5 (D). Messenger RNA expression is shown as arbitrary scanned units normalized to the 18S subunit. Values are mean ± SEM; n = 6 animals/group. *Significant difference compared to normal control; †significant difference compared to dexamethasone treatment only; ‡significant difference compared to all groups. §Inhibitory interaction between denervation and dexamethasone treatments in DD muscles. A rise in mRNA levels for atrogin-1 was seen in all treatment groups and no additional effect of combined treatment was noted. Expression of MyoD and myogenin mRNA was increased in relation to a denervation effect in DN and DD muscles, whereas Myf5 mRNA expression had an interactive effect from combined treatment.
4D). For Myf5 mRNA levels, an interaction between glucocorticoid and denervation treatment was seen in DD muscles (P < 0.0001).

Denervation treatment also influenced the expression of p21 mRNA (Fig. 5A). Expression of p21 mRNA was increased in DN (~2.5-fold) and DD (~1.5-fold) muscles. The levels of p21 mRNA were, however, lower in DD than predicted based on denervation treatment alone; further analysis showed an interaction between denervation and glucocorticoid treatments in determining the p21 levels in this group (P = 0.04). Expression levels of p21 mRNA in DX muscles were not different from those of NC.

Figure 5B shows mRNA expression for the GADD45 gene. GADD45 mRNA paralleled p21 mRNA levels and showed a similar robust elevation in DN and DD muscles (75- and 50-fold increases relative to NC, respectively). Unlike p21 mRNA, however, this rise in GADD45 mRNA levels was purely a denervation-related effect with no interaction between the two treatment groups. GADD45 mRNA in DX muscles was not significantly changed.

**DISCUSSION**

This rodent model is fundamentally different from the human disease AQM in that only focal pathologic changes are present and, unlike human patients, these rodents are not critically ill. However, there are striking similarities in the pathologic and neurophysiologic changes seen in this model vs. those seen in human AQM disease. We believe that findings from this model are relevant to understanding the mechanisms underlying skeletal muscle atrophy and may be relevant to human AQM.

**Skeletal Muscle Inactivity, Through Denervation, as a Contributory Factor in Development of AQM.** Concurrent treatment with non-depolarizing pharmacological neuromuscular blockers (functional denervation) and glucocorticoids are the primary risk factors predisposing to the development of AQM in humans. The association of glucocorticoids with the development of this myopathy is well established, but the contribution of “denervation” is not as well defined. Based on our findings we believe that a denervative substrate, by virtue of causing skeletal muscle inactivity, is required to cause AQM.

Emerging evidence indicates that most patients who develop this myopathy also have an underlying neuropathy. Furthermore, similar decreases in skeletal muscle levels for MyHC and actin mRNA, as seen in our experiments, were shown previously in both denervation (with disruption of neural influence on skeletal muscle from axotomy) and with inactivity caused by spinal cord isolation, a physiologic paradigm distinct from denervation, because the neural influences on skeletal muscle remain intact even though the spinal cord has undergone complete isolation, resulting in electrical inexcitability of the muscle. In both paradigms the decrease in MyHC and skeletal muscle actin mRNA was related to transcriptional dysregulation of the respective genes. Furthermore, a selective MyHC protein depletion in inactive muscle was also reported in spinal cord–inactivated muscles, suggesting that muscle fiber inactivity may also play a role in development of this selective loss.

Based on the aforementioned observations, we believe that, in human AQM, a similar interactive effect of glucocorticoid treatment in the setting of denervation (either from an underlying neuropathy...
or a functional denervation from prolonged neuromuscular blockade) contributes to the development of the skeletal muscle atrophy and MyHC protein loss.

**Preferential MyHC Depletion in AQM.** Our results suggest that the selective loss of MyHC protein occurs because of an exaggerated depletion of MyHC mRNA relative to actin mRNA. We propose that this selective depletion is due to a deleterious interactive effect of glucocorticoid treatment on denervated muscles, occurring at a pre-translational level. Even though other mechanisms, such as decreased translation of MyHC or increased and targeted degradation of MyHC, should also be considered, we believe this selective loss of MyHC RNA is an important contributor to the protein loss.

Our results are supported by an earlier report by Larsson et al., who, using in situ hybridization on human skeletal muscle samples, found that, in the acute stages of acute quadriplegic myopathy, α-skeletal-actin transcripts were present in most fibers, whereas transcripts for MyHC were few or absent in most fibers from AQM patients. During early recovery from the myopathy, mRNA and protein were normally expressed in all fibers.

Sparing of actin protein indicates that MyHC and actin proteins have different turnover rates. It is possible that, if the experimental period was longer, a reduction in actin protein could have emerged. However, in the original report on this model, actin protein levels were not affected as far out as 28 days. This further suggests that MyHC and actin are lost at different rates during the atrophy process in AQM. The MyHC loss is either faster or greater than that of actin, and this could be the result of the former possessing a higher turnover rate than the latter. Furthermore, the preferential MyHC depletion has been shown to persist for up to 28 days in AQM and spinal cord isolation. This suggests a more selective vulnerability of MyHC to inactivity than actin protein.

The finding of an increase in skeletal muscle MyHC mRNA (and MyHC protein) in muscles treated with dexamethasone alone, although surprising, is in keeping with findings by Muangminsuk et al., who demonstrated dexamethasone-induced cardiac hypertrophy in newborn rats (ranging from 25% at day 1 to 59% at 9 days), accompanied by increased expression of α-MyHC and decreased expression of β-MyHC. The effects on α-MyHC were mediated in part through transcriptional mechanisms.

Myogenic Regulatory Factors and Markers of Cell Cycle Arrest. The findings of persistent elevation of p21 and MRFs in denervated muscles are in keeping with similar changes reported earlier in denervated (axonotomized) muscles, and in spinal cord–isolated muscles. Similar increases in myogenin mRNA levels have been reported earlier in this rodent model of AQM, and an upregulation in the gene expression of the p21 and GADD45 genes was recently also noted in a DNA microarray study of human muscles from patients with AQM.

Although our study is limited by the fact that we only studied mRNA levels of these MRFs, previous studies showed protein level increases parallel to mRNA in DN muscles. The elevation of these markers in a disuse model, such as denervation, is not entirely unexpected, and reflects increased satellite cell proliferative and differentiative activity. It is not completely clear whether this satellite cell activity is an attempt at repair or a response related to the loss of electrical activity in these denervated (inactive) muscles.

It is also possible, however, that increased expression of MRFs may be directed at other compensatory mechanisms in a denervated muscle. Hyatt and colleagues demonstrated increased expression of MyoD and myogenin protein in the myonuclei in both spinal cord–injured and denervated soleus muscle fibers and suggested 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.

In addition to repair, MyoD and myogenin control the transcription of a number of key proteins such as the fast type IIB MyHC isoform, insulin-like growth factor-1, and myostatin. Upregulation of MRFs is closely linked to gene expression of the nicotinic acetylcholine receptor, another gene upregulated immediately after denervation. Myogenin plays an important role in the reconstruction of damaged neuromuscular connections in denervated muscle, through upregulation of acetylcholine receptor α-subunit expression. A decrease in the protein levels of these MRFs, through increased ubiquitination and degradation, such as via atrogin-1 activation, can have extremely deleterious effects on muscle.

Upregulation of p21 in denervated muscles may have negative consequences by inhibiting satellite cell proliferation (impairing repair) and by promoting myonuclear apoptosis (resulting in loss of myonuclei). Both of these proteins (as well as the MRFs) are very sensitive to the innervation state.
and are downregulated upon reestablishment of innervation. Even though the exact role of upregulation in message for MRFs, as well as activation of p21 and GADD45, in atrophied denervated muscles is not clear, these changes may, in part, be involved with the phenotype change associated with muscle atrophy in denervation and disuse and may reflect compensatory changes in muscle preparing for repair or reinnervation.

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REFERENCES