Analysis of human skeletal muscle after 48 h immobilization reveals alterations in mRNA and protein for extracellular matrix components

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1Department of Exercise Science, University of Massachusetts, Amherst; 2US Army Research Institute of Environmental Medicine, Military Nutrition Division, Natick, Massachusetts; 3Research Center for Genetic Medicine, Children’s National Medical Center, Washington, DC; and 4Division of Cardiology, Henry Low Heart Center, Hartford Hospital, Hartford, Connecticut

Submitted 13 February 2006; accepted in final form 17 May 2006

Urso, Maria L., Angus G. Scrimgeour, Yi-Wen Chen, Paul D. Thompson, and Priscilla M. Clarkson. Analysis of human skeletal muscle after 48 h immobilization reveals alterations in mRNA and protein for extracellular matrix components. J Appl Physiol 101: 1136–1148, 2006.—We examined the effects of 48 h of knee immobilization on alterations in mRNA and protein in human skeletal muscle. We hypothesized that 48 h of immobilization would increase gene expression and respective protein products for ubiquitin-proteasome pathway (UPP) components. Also, we used microarray analysis to identify novel pathways. Biopsies were taken from the vastus muscle of five men (20.4 ± 0.5 yr) before and after 48-h immobilization. Global changes in gene expression were analyzed by use of Affymetrix GeneChips. Candidate genes were confirmed via quantitative RT-PCR. Western blotting (WB) was used to quantify protein products of candidate genes and to assess Akt pathway activation. Immunohistochemistry was used to localize proteins found to be altered when assessed via WB. The greatest percentage of genes showing altered expression with the GeneChip included genes involved in the UPP, metallothionein function, and extracellular matrix (ECM) integrity. Quantitative RT-PCR analysis confirmed increases in mRNA for UPP components [USP-6, small ubiquitin-related modifier (SUMO-1)] and the metallothioneins (MT2A, MT1F, MT1H, MT1X) and decreases in mRNA content for matrix metalloproteinases (MMP-28, TIMP-1) and ECM structural components [collagen III (COLIII) and IV (COLIV)]. Only phosphorylated Akt (Ser473, Thr308), COLIII and COLIV protein levels were significantly different postimmobilization (25, 10, 88, and 28% decrease, respectively). Immunohistochemistry confirmed WB showing decreased staining for collagens postimmobilization. Our results suggest that 48 h of immobilization increases mRNA content for components of the UPP and metallothionein function while decreasing mRNA and protein for ECM components as well as decreased phosphorylation of Akt.

Metallothioneins; ubiquitin-proteasome pathway; immunohistochemistry; Akt

SKELETAL MUSCLE ADAPTS RAPIDLY TO CHANGES IN ACTIVITY (12). In fact, humans who have had a limb immobilized because of injury or illness experience significant losses in muscle mass and function within 1–2 wk (20, 36). Although studies in humans have documented the effects of immobilization on skeletal muscle structure and function (4, 5, 25, 36), the underlying mechanism regulating rapid muscle protein degradation remains uncertain. Investigations of limb immobilization in animals have shown that there is a coordinated upregulation of genes encoding for proteins that function in the initiation of the muscle atrophy process (8, 14, 42, 51).

The majority of genes upregulated by muscle atrophy in animal models can be classified into the ubiquitin-proteasome pathway. This pathway is the intracellular proteolytic pathway responsible for the bulk of protein degradation in skeletal muscle, and it is induced in animal models by unloading, spaceflight, immobilization, denervation, or wasting (7, 8, 19, 22, 33, 37, 48, 51). Ubiquitin-mediated proteolysis is ATP dependent and relies on the selectivity of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) to tag and degrade specific proteins within the cell. An increase in proteolytic activity is initiated by an increase in mRNAs encoding essential components of the ubiquitin-proteasome pathway, and this increase is followed by a respective increase in protein products (24, 30, 34, 38, 44, 54).

Bodine et al. (8) analyzed mRNA expression after immobilization of the rat gastrocnemius muscle. The mRNA content of two genes that encode E3 ubiquitin ligases, muscle RING finger 1 (MurF1) and muscle atrophy F-box (MAFbx/atrogenin-1), were upregulated 2 days after immobilization. Multiple immobilization interventions in animals have reproduced these results (8, 30, 51), whereas gene responses of factors in this pathway in human skeletal muscle in response to similar perturbations remain elusive. In an investigation of gene expression profiles at various time points during hindlimb immobilization in rats, Stevenson and colleagues (51) reported an upregulation of mRNA for genes that encode proteasome subunits, as well as the ubiquitin ligases 24 h after immobilization, and this upregulation was not diminished after 14 days of immobilization. Similarly, a microarray analysis of rat muscle undergoing atrophy from various causes (e.g., sepsis, uremia, wasting) showed that genes encoding the E3 ubiquitin ligases (MurF1 and MAFbx), polyubiquitins, and cathepsin L were upregulated during atrophy (32). Others reported that mRNA for E2-conjugating enzymes and E3 ligases are upregulated greater than twofold within 2 days of hindlimb suspension, denervation, or immobilization (14, 18, 42). Collectively, the muscle atrophy work performed in animals demonstrates similar trends in the expression of genes involved in the ubiquitin-proteasome pathway, suggesting that muscle atrophy in animals is secondary to increased protein breakdown via an

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upregulation of the ATP-ubiquitin-dependent proteolytic pathway (22, 23).

Signaling mechanisms that activate the ubiquitin-proteasome pathway and the subsequent loss of muscle tissue are not well defined. Much research has focused on the role of insulin and insulin-like growth factor (IGF-I) signaling pathways in animal models because of the predominant role insulin and IGF-I play in reducing protein degradation (47). Moreover, previous work has shown that insulin resistance is evident in catabolic conditions that are associated with activation of the ubiquitin-proteasome pathway, and treatment with IGF-I is sufficient to attenuate activity of this pathway (9, 15, 19). Recent work in animals has focused on the role of the phosphatidylinositol-3-kinase (PI3K)/Akt (protein kinase B) signaling pathway as a central regulator between insulin and IGF-I receptor-binding and activation of proteolytic or synthesis machinery in skeletal muscle (9, 15, 21, 47). There is strong evidence in animal models that the activity of the PI3K/Akt pathway is a regulator of the muscle atrophy program (9, 14, 30, 45). However, this relationship in humans, particularly in response to disuse, is not known. Few researchers are confirming and extending results of animal models to humans, and without this intermediate step the translation into effective therapies is slowed down.

To date, we are aware of only one study in humans that has investigated the effects of an atrophy-related stimulus on alterations in gene expression in human skeletal muscle (24). Although this study is an important contribution to atrophy literature, the authors used a targeted approach to identify alterations in gene expression, preventing a comprehensive analysis of all genes affected by the immobilization intervention. Moreover, the analysis was performed after 2 wk of immobilization, and early changes in gene expression responsible for the initiation of the atrophy program remain unknown. Finally, these alterations were only explored at the mRNA level, and alterations in protein products were not investigated.

Therefore, we sought to identify alterations at the mRNA and protein level in human skeletal muscle after 48 h of limb immobilization to identify early regulators of the muscle atrophy program in humans. On the basis of previous work in animals (8, 9, 30), we hypothesized that analysis of human skeletal muscle biopsies taken after 48 h of limb immobilization would show an increase in gene expression and respective protein products for components of the ubiquitin-proteasome pathway and alterations in the phosphorylation of proteins involved in the Akt signaling pathway associated with decreased protein synthesis. Furthermore, we used microarray analysis to identify novel pathways involved in the atrophy program activated in response to 48 h of limb immobilization in humans. We chose to use a multifaceted approach of microarray analysis, quantitative real-time polymerase chain reaction (qRT-PCR), immunoblotting, and immunohistochemistry (IHC) to identify critical regulators of the atrophy program at both the mRNA and protein level in humans after 48 h of limb immobilization.

METHODS

Subjects. Five men completed the study. Mean (+/−SE) age, height, and weight of the subjects were 20.4 +/− 0.5 yr, 173.2 +/− 2.2 cm, and 77.7 +/− 1.5 kg, respectively. All participants provided signed consent as approved by the University of Massachusetts and Hartford Hospital Institutional Review Boards and completed a medical history questionnaire. Subjects were sedentary but ambulatory and healthy, with no orthopedic problems that would be exacerbated by the immobilization intervention. Subjects who were taking muscle building supplements or restricting calorie intake were excluded from the study.

Design. At baseline, subjects reported to the laboratory and were given a standardized meal because meal patterns alter the activity of certain genes that are downstream of Akt (15), a potent regulator of the muscle atrophy program. Approximately 2 h later, a biopsy of the right vastus lateralis muscle was taken to use as a baseline control. Ten days after the preimmobilization baseline biopsy, subjects returned to the laboratory. At this time, the left leg was immobilized (knee angle = 160°) in a cylinder leg brace (Donjoy, Vista, CA) that extended from below the groin to the ankle. The brace was secured with Fiberglas casting tape. Subjects were contacted twice daily to ensure compliance and that the immobilized leg was not causing undue discomfort. Forty-six hours postimmobilization subjects were given a standardized meal, and ~2 h later a biopsy of the left vastus lateralis (the limb that was immobilized) was made. This biopsy was scheduled for the same time of day as the preimmobilization biopsy to reduce influence of circadian rhythms on changes in gene expression. We decided to take the pre- and postimmobilization muscle biopsies from opposite legs to avoid any contamination resulting from scar tissue at the earlier preimmobilization biopsy site. In a previous study, we took muscle biopsies from opposite legs (53), and microarray analysis revealed that there were no differences in gene expression when samples were taken 30 days apart, establishing that levels of gene expression are not different between legs.

The brace was removed immediately before the biopsy while the subject was supine on the examination table. Once the biopsy was completed, subjects were given rehabilitation to ensure rapid recovery of knee range of motion.

Immobilization. Subjects had the left leg placed in a long cylinder leg brace (Donjoy). The brace extended from below the groin to above the ankle joint. The brace was placed on the subject while the subject was standing. Scotchcast (3M, St. Paul, MN) casting tape was used to secure the Velcro closures on the leg brace. Securing the Velcro closures with casting tape ensured subject compliance because the casting tape could only be removed with a cast cutter owned by the investigator. The knee angle was placed in a neutral position (160°), and subjects were able to bear weight on the leg. Subjects were able to ambulate normally during the immobilization. Therefore, casting of the knee joint prevented knee extension and prevented use of the vastus lateralis muscle.

Muscle biopsy. Percutaneous needle muscle biopsies were obtained from the right (baseline) and left (48 h postimmobilization) vastus lateralis muscles by use of a Bergstrom 5-mm biopsy needle (Depuy, Warsaw, IN). Skin was first lightly anesthetized with 4 ml of 2% lidocaine hydrochloride solution, a small (5–6 mm) incision was made in the skin and fascia, the biopsy needle was inserted, and ~200 mg of tissue was removed. Tissue was divided into four 50-mg aliquots, snap frozen in liquid nitrogen, and stored at −80°C until analysis.

Gene expression profiling. Microarray analysis was performed using Affymetrix GeneChip (Affymetrix, Santa Clara, CA) technology (Human Genome U133 Plus 2.0 Array) with standard operating procedures and quality control as recently described (13). Total RNA was extracted from biopsies using TRIZOL reagent (Invitrogen, Carlsbad, CA). Three micrograms of total RNA from each biopsy were converted into double-stranded cDNA by using SuperScript Choice system (Invitrogen) with an oligo-dT primer containing T7 RNA polymerase promoter. The double-stranded cDNA was purified by phenol-chloroform extraction and then used for in vitro transcription using ENZO BioArray RNA transcript labeling kit (Affymetrix). Biotin-labeled cRNA was purified by RNeasy kit (QIAGEN, Valencia, CA) and fragmented randomly to 200 hp. cRNA samples of each
biopsy were hybridized to a Human Genome U133 Plus 2.0 Array for 16 h. Each microarray was washed and stained on the Affymetrix Fluidics Station 400, using Affymetrix reagents.

Absolute analysis of Affymetrix “raw” data was conducted using Affymetrix MAS5.0 and DNA-Chip Analyzer software (dCHIP) (v. 1.2, Affymetrix) according to quality control methods developed previously (46, 52a).

The 18,400 transcripts represented on the Affymetrix Human U133 oligonucleotide array describe 14,500 well-characterized genes and 400 expressed sequence tags. In all, 22,000 probe sets were analyzed by using 22 oligonucleotide probes (11 distributed probe pairs) for each probe set, producing multiple independent measurements for each gene. Comparison of the hybridization signal from the perfect match and mismatch probes allows for a specificity measure of signal intensity and elimination of most nonspecific cross-hybridization signal. Values of intensity differences as well as ratios of each probe pair were used to determine (statistically) whether a gene was called present or absent. Data analysis required >10% of profiles to show a present call to be carried into the next statistical analysis. GeneSpring 7.0 (Silicon Genetics, Redwood City, CA) was then used to filter potential candidate genes for statistical significance using Welch’s t-test, and only genes with P values <0.05 were retained for further analysis. Statistical analysis was performed using a longitudinal design, in which each subject served as his own control. This approach removes interindividual variation due to genetic heterogeneity. Combined, these criteria increase the reliability of our microarray analysis, allowing us to reduce false positives. Although multiple testing corrections were performed on our data set, our small sample size resulted in all genes being eliminated from further analysis. Therefore, we took a systemic approach to confirm the microarray analysis using qRT-PCR, Western blotting, and IHC. Also, candidate genes were selected for confirmational analysis. Sta-

Table 1. Primers for qRT-PCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>FBX09</td>
<td>5’-CTG TGA GCT CAG CAT GAG CAA CAT-3’</td>
<td>5’-TCC TGG GAT TAT AGG CAT GAG CCA-3’</td>
</tr>
<tr>
<td>UBE2E</td>
<td>5’-AAG GAG CAG TCG GGC GAC ATC AAT TTA-3’</td>
<td>5’-TCC ACT GTC TGG CCA TTT CTT CAT-3’</td>
</tr>
<tr>
<td>USP-6</td>
<td>5’-ACT TGG GCA TCG TTA CTA TG-3’</td>
<td>5’-GGC GAG TTA CTC CTT TC-3’</td>
</tr>
<tr>
<td>SUMO-1</td>
<td>5’-AAA AGC CAT ATT CCT CAG CA-3’</td>
<td>5’-GAG CAT CAG TTT CCA AGG TC-3’</td>
</tr>
<tr>
<td>MuRF</td>
<td>5’-TGA AGC ACA AGT ATG TTA AGA CG-3’</td>
<td>5’-TGA TGA GTC GCT TGG CAG TC-3’</td>
</tr>
<tr>
<td>Atrogin-1</td>
<td>5’-TGA CAG CTC AGA TGC CTC AGG AG-3’</td>
<td>5’-AGA TTG GCC TGC TGG TTA GC-3’</td>
</tr>
<tr>
<td>MT2A</td>
<td>5’-TCA GTC GTG TTA CGG GGA AAG TAA-3’</td>
<td>5’-CGG TCA CGG TGA GGG TAC TAA AA-3’</td>
</tr>
<tr>
<td>MT1F</td>
<td>5’-TGG TTC TCG CAA CGG CAA AGA GCT-3’</td>
<td>5’-GTA AGC TAG CAA AGC GGT CAA GGT GC-3’</td>
</tr>
<tr>
<td>MT1H</td>
<td>5’-GCT CCT GCA ACT GCA AAA AG-3’</td>
<td>5’-GAG CAT CCA GAT CCA TCT GCC -3’</td>
</tr>
<tr>
<td>MT1X</td>
<td>5’-TCC TCC TCG TCG TCG CCA AAT-3’</td>
<td>5’-AGG ATG TAG CAA AGC GGT CAG GGT-3’</td>
</tr>
<tr>
<td>MMP-28</td>
<td>5’-TGT TCT TGT CCT AGA CTT CTA-3’</td>
<td>5’-AGA CCA AGA CAG ATG TTA TCC CT-3’</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>5’-ACC AGA AGC CAA CGA GAC CAC TCT-3’</td>
<td>5’-AAA ACC CTA GCA AGC CCT TGT CTA-3’</td>
</tr>
<tr>
<td>COLIII</td>
<td>5’-CTG CAG GAC CAG CAG CCA CAG GCT-3’</td>
<td>5’-ATG ACC GAT CCA AGC GAT CCA CAG-3’</td>
</tr>
<tr>
<td>COLIV</td>
<td>5’-TTC GTG TAG TCC AAC CAG GGT TAT-3’</td>
<td>5’-TCT CTC TGG TG TGG GTT CTT CG-3’</td>
</tr>
</tbody>
</table>

and reverse RT-PCR primers (Integrated DNA Technologies, Coralville, IA) were designed by using Affymetrix and NCBI gene sequences with the Primer Express program v. 2.0 (Applied Biosys-

qRT-PCR reactions were run in triplicate, in 96-well plates. On each plate, cDNA samples from preimmobilization (control) and postimmobilization (48 h) were run in triplicate for each gene of interest and for GAPDH, which served as a reference standard. The average threshold cycle value for triplicate samples was used for data analysis. Threshold cycle values were the number of cycles necessary to reach a certain threshold fluorescence level using a Stratagene MX3000P QPCR system (Stratagene, La Jolla, CA). Thermal cycling conditions were as follows: one cycle at 50°C for 2 min for uracil-

GAPDH (Integrated DNA Technologies) was used to normalize the expression level of genes of interest. GAPDH is a constitutively expressed housekeeping gene and was not found to be differentially expressed in our microarray dataset. Relative quantitation of amplified mRNA was normalized to GAPDH, compensating for variations in quantity.

Western blotting. Muscle samples (~50 mg) used for Western blot analysis were homogenized in buffer [100 mM Tris, pH 7.4; 250 mM sucrose/protease inhibitor mixture (1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 µg of leupeptin per ml, 10 µg of aprotinin per ml, 1 mM microcytin-LR, 1 mM PMSF, 10 mM sodium fluoride) and analyzed for total protein concentration (Lowry method) by using commercially available reagents (Sigma, St. Louis, MO). Samples were heated for 5 minutes at 95°C and then subjected to SDS-PAGE using 7–15% gradient gels (Bio-Rad Laboratories, Hercules, CA) before proteins were electrophoretically transferred to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ), which were then incubated in a solution of TBS containing 5% nonfat dry milk (Carnation, Nestle, Solon, OH) and 0.1% Tween-20. Precision Plus Kaleidoscope Protein Standards (Bio-Rad Laboratories) were used as molecular weight markers on each gel (31).

Membranes were incubated overnight at 4°C in a hybridization buffer (1:1,000, rabbit polyclonal, Cell Signaling Technology, Beverly, MA); Phospho-Akt (Ser473), Phospho-Akt (Thr380), PTEN (Ser380), PKD1 (Ser419), GSK3-ß (Ser9), Raf-1 (Ser259) (1:1,000, rabbit polyclonal, Cell Signaling); ubiquitin-specific protease 6 (1:1,000, goat polyclonal, Abcam, Cambridge, MA); SUMO-1 (1:500, rabbit polyclonal, Bio-
HIC. Serial cross-sectional slices (10 μm) of muscle biopsy samples immersed in optimal cutting temperature embedding medium (Fisher Scientific, Pittsburgh, PA) were generated on a MICROM HM 505E cryostat at -25°C (Richard Allan Scientific, Kalamazoo, MI) and placed on glass slides coated with Vectorbond reagent (Vector Laboratories, Burlingame, CA). All baseline and 48-h samples were processed at the same time. Sections were washed in PBS, pH 7.4 (Sigma), blocked with goat normal serum (Jackson ImmunoResearch Laboratories, West Grove, PA) or rabbit normal serum (Zymed Laboratories, San Francisco, CA), and stained overnight at 4°C with primary antibodies used for Western blotting, diluted 1:250–1:500 in 5% BSA (Sigma) in PBS. Sections were washed in PBS and were incubated for 30 min at 37°C with secondary fluorochrome-labeled antibodies against primary antibodies (Alexa green, Alexa red, goat anti-mouse; goat anti-rabbit, rabbit anti-goat) (Molecular Probes, Eugene, OR). Fluorochrome-labeled secondary antibodies, without primary antibodies used for Western blotting, were used as controls. After being washed with PBS, -10 μl of 3,3′-diaminobenzidine (Sigma) and a coverslip were added to each section.

Samples were visualized and photographed with a Nikon Eclipse E600 phase contrast microscope (Nikon, Tokyo, Japan) equipped with a Spot insight QE camera and EclipseNET software, version 1.16. Scion IMAGE for Windows (Scion, Frederick, MD) was used for image analysis. Quantification of antibody staining was performed using the image-analysis system in addition to manual microscopic scoring. Exposure time and brightness were kept constant during image analysis.

Statistical analysis. Values for qRT-PCR, Western blotting, and IHC are given as means ± SE. Statistical comparisons were done using a paired Student’s t-test, with P < 0.05 indicating significance and P < 0.10 indicating a trend.

RESULTS

Microarray analysis. Forty-eight hours of limb immobilization resulted in the differential expression of 737 transcripts according to inclusion criteria (P < 0.05, fold change >1.5) or <-1.5. Of these, 242 had known biological functions according to the Affymetrix NetAffx Gene Ontology database (http://www.affymetrix.com/analysis/netaffx/go_analysis_netaffx4.affx). Overall, 120 were downregulated, and 122 were upregulated. Figure 1 presents a schematic of gene categories that were differentially expressed according to these criteria. In agreement with our hypothesis, among the top 25 upregulated genes were genes associated with the ubiquitin-proteasome pathway. Additionally, several genes involved in metallothionein function and the extracellular matrix (ECM) were among the top 25 up- and downregulated genes, respectively. This analysis provided a snapshot of the molecular changes that occur in response to 48 h of immobilization. We therefore chose to perform additional work at the mRNA (qRT-PCR) and protein (Western blotting and IHC) level on a subset of genes involved in the ubiquitin-proteasome pathway, metallothionein function, and the ECM (Table 2). The specific genes were chosen because they were in the top 25 when ranked according to fold change and P value, have previously been associated with muscle atrophy (1, 2, 8, 23, 24, 32, 42, 48), and had antibodies to the gene product available commercially.

qRT-PCR. UBE2E, an E2 ubiquitin-conjugating enzyme, and FBX09, a ubiquitin-specific ligase (E3), were found by using the microarray to be upregulated 1.6-fold and 1.9-fold. However, the more sensitive qRT-PCR technique revealed that mRNA content was only increased 1.2-fold and 1.1-fold, respectively, and this increase was not considered significant (P > 0.05, NS). We therefore did not perform additional analysis at the protein level for these genes.

In contrast, USP-6, a ubiquitin-specific enzyme that provides a deubiquitinating role in the cell, and SUMO-1, which functions to prevent proteasome-mediated degradation of proteins...
were found to be upregulated 1.8-fold and 1.9-fold, respectively (P ≤ 0.05) via qRT-PCR in agreement with the microarray analysis (Table 2).

Several genes involved in metallothionein function were upregulated in the microarray dataset. We chose to confirm each of these using qRT-PCR, and data showed that there was a 2.6-fold, 2.1-fold, 1.7-fold and 2.0-fold increase in mRNA content for metallothioneins 2A, 1F, 1H, and 1X, respectively (P ≤ 0.05), in agreement with the increase observed via microarray analysis (Table 2).

qRT-PCR analysis of matrix metalloproteinase domain 28 (MMP-28) and tissue inhibitor of metalloproteinase 1 (TIMP-1), enzymes involved in ECM turnover, confirmed the decrease seen with the microarray analysis, revealing a 1.9-fold and 1.8-fold decrease, respectively (P < 0.05). Collagen III (COLIII) and collagen IV (COLIV), components of the ECM, were found to be downregulated 2.3-fold and 1.8-fold, respectively (P < 0.05). 

Western blotting. To identify whether the Akt signaling pathway was affected by the 48-h limb immobilization intervention, we performed Western blotting on total Akt, Akt_{ser473}, Akt_{thr308}, PTEN_{ser380}, PDK1_{ser241}, GSK3-β_{ser9}, and Raf-1_{ser259}. Results of the Western blotting indicated that, after 48 h immobilization, total Akt was unchanged, but there was a significant (P < 0.05), 25 and 10% decrease in phosphorylation of Akt_{ser473} and Akt_{thr308}, respectively (Fig. 2). Western blotting for components upstream of Akt indicates that phosphorylation of PTEN_{ser380} showed a trend for an increase of ~9% (P = 0.05), and phosphorylation of PDK1_{ser241} showed a trend for a decrease of ~22% (P = 0.06) in response to 48 h of immobilization (Fig. 3). Western blotting for downstream components, GSK3-β_{ser9} and Raf-1_{ser25}, showed a trend that

Table 2. Effects of 48-h immobilization on gene expression: candidate genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>GeneChip P Value</th>
<th>Fold Change</th>
<th>qRT-PCR P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin proteasome pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBX09</td>
<td>1.9</td>
<td>0.002</td>
<td>1.1 ± 0.23</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>UBE2E</td>
<td>1.6</td>
<td>0.002</td>
<td>1.2 ± 0.18</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>USP-6</td>
<td>1.7</td>
<td>0.021</td>
<td>1.8 ± 0.26</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>SUMO-1</td>
<td>2.3</td>
<td>0.013</td>
<td>1.9 ± 0.26</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>MuRF</td>
<td>1.1</td>
<td>0.015</td>
<td>1.1 ± 0.15</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>MAFbx</td>
<td>NS</td>
<td>NS</td>
<td>1.1 ± 0.10</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Metallothioneins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MT2A</td>
<td>2.0</td>
<td>0.024</td>
<td>2.6 ± 0.68</td>
<td>&lt;0.01</td>
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<tr>
<td>MT1F</td>
<td>2.3</td>
<td>0.018</td>
<td>2.1 ± 0.36</td>
<td>&lt;0.05</td>
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<tr>
<td>MT1H</td>
<td>1.9</td>
<td>0.041</td>
<td>1.7 ± 0.28</td>
<td>&lt;0.05</td>
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<tr>
<td>MT1X</td>
<td>1.8</td>
<td>0.048</td>
<td>2.0 ± 0.42</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Extracellular matrix</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MMP-28</td>
<td>−2.6</td>
<td>0.046</td>
<td>−1.9 ± 0.07</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>−1.8</td>
<td>0.029</td>
<td>−1.8 ± 0.10</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>COLIII</td>
<td>−1.8</td>
<td>0.028</td>
<td>−2.3 ± 0.11</td>
<td>&lt;0.05</td>
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<tr>
<td>COLIV</td>
<td>−1.7</td>
<td>0.031</td>
<td>−1.8 ± 0.12</td>
<td>&lt;0.05</td>
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qRT-PCR, quantitative RT-PCR; NS, not significant.
phosphorylation of GSK3\textsuperscript{\text{ser9}} was decreased \textasciitilde 21\% (\(P = 0.05\)), although phosphorylation of Raf-1\textsuperscript{ser259} showed only a modest, nonsignificant increase (\(-6\%, \ P = 0.34\)) (Fig. 4).

Western blotting was then performed on protein products of USP-6 and SUMO-1. Despite significant increases in mRNA content, protein levels for USP-6 and SUMO-1 were unchanged (\(P > 0.05\)) (Fig. 5). Also, we used a monoclonal antibody against metallothionein-I to assess protein levels of the metallothioneins after 48 h of immobilization. Despite the increases observed in mRNA content, protein levels of metallothionein did not change as a result of 48 h of immobilization (\(P > 0.05\)) (Fig. 6).

Although mRNA content for MMP-28 and TIMP-1 was increased, Western blotting for protein products of MMP-28 and TIMP-1 showed that protein levels were unchanged as a result of 48 h of immobilization (\(P > 0.05\)) (Fig. 7). In contrast, protein levels of structural proteins COLIII and COLIV were decreased 88 and 28\%, respectively (\(P < 0.05\)) after 48 h of immobilization (Fig. 8).

In addition to Western blotting for components of the Akt pathway and each protein of interest, membranes were also incubated in a polyclonal antibody against GAPDH. Results of the Western blotting analysis revealed that there was no change in protein levels for GAPDH after 48 h of immobilization (\(P > 0.8\), NS). Figure 6 provides a representative blot for GAPDH.

IHC. Immunohistochemical analysis was first performed on COLIII and COLIV because these were the only proteins whose levels were found to be altered as a result of the immobilization intervention. Figure 9 depicts sections from muscle biopsies taken at baseline and 48 h after immobilization by using monoclonal antibodies targeted against COLIII and
COLIV. Both instances show decreased staining for these proteins along the ECM.

To ensure that the protein isolation procedure did not affect our Western blotting results (e.g., elimination of protein products not in the supernatant), we performed IHC on SUMO-1, USP-6, and metallothionein. In accordance with Western blotting results, we did not document any changes in protein levels or location of protein products with IHC analysis (data not shown).

DISCUSSION

The overall aim of the present investigation was to use microarray technology to examine transcriptional alterations in human skeletal muscle after 48 h of limb immobilization and, then, to identify whether candidate genes selected from this analysis showed altered mRNA content (via qRT-PCR) and/or showed altered protein content. We hypothesized that 48 h of limb immobilization in humans would result in an increase in...
Fig. 7. Western blotting for matrix metalloproteinase 28 (MMP-28) and tissue inhibitor of metalloproteinase 1 (TIMP-1). No changes were observed in protein levels after 48 h of immobilization ($P > 0.05$). Representative blots are below each graph, and the control value has been assigned the value 100%.

Fig. 8. Western blotting for COLIII and COLIV. In agreement with microarray and quantitative RT-PCR results showing decreased gene and mRNA content for COLIII and COLIV, Western blotting for protein levels of COLIII and COLIV revealed a 88 and 28% decrease, respectively ($*P < 0.05$) after 48 h of immobilization. Representative blots are below each graph, and the control value has been assigned the value 100%.
gene expression and respective protein products for components of the ubiquitin-proteasome pathway and alterations in the phosphorylation state of proteins involved in the Akt signaling pathway in muscle biopsy samples. Through the combined use of microarray technology, qRT-PCR, Western blotting, and IHC, we were able to identify fundamental changes in mRNA content and protein content for ECM components that are thought to be involved with the initiation of muscle atrophy after 48 h of limb immobilization in humans.

Akt pathway. The Akt signaling pathway plays a critical role in the balance between muscle protein synthesis and degradation (9). Under normal circumstances, activation of PI3K by IGF-I results in the suppression of PTEN, a negative regulator of muscle growth. Subsequently, PDK1 is phosphorylated, which in turn results in the phosphorylation of Akt at two sites (Ser473 and Thr308), resulting in the suppression of proteolytic transcription factors (9). However, in response to reduced upstream signaling from IGF-I, particularly after periods of inactivity, phosphorylation of Akt is inhibited and downstream components involved in proteolytic cascades are activated (45). We used the present study as an opportunity to explore phosphorylation state of critical proteins involved in the Akt signaling pathway (Figs. 2–4). We hypothesized that we would observe alterations in this pathway indicative of reduced protein synthesis. Figure 10 is a schematic of the Akt signaling pathway in response to immobilization indicative of reduced protein synthesis. Specifically, the observed 25 and 10% decrease in the phosphorylation of Akt at both the Ser473 and Thr308 sites, respectively, indicates that 48 h of limb immobilization is sufficient to suppress protein synthesis pathways that are activated by Akt. Furthermore, using Western blotting, we were able to document trends for alterations in other components of the pathway such as an increase in phosphorylation of PTENSer380, which acts as a negative regulator of the Akt pathway, and a decrease in phosphorylation of PDK1Ser241, which functions to phosphorylate Akt (Fig. 3).

Ubiquitin-proteasome pathway. In part, the results from this study support our hypothesis that 48 h of limb immobilization results in an increase in gene expression for components of the ubiquitin-proteasome pathway in humans. Because degradation of proteins via the ubiquitin-proteasome pathway is a multistep process, activation of several components of this pathway is necessary for the accelerated protein breakdown, which occurs in response to catabolic conditions (33, 39). Increases in mRNA content for various components of the ubiquitin-proteasome pathway have been documented in previous muscle atrophy models in animals (7, 8, 14, 19, 22, 24, 32, 37, 48, 51) and humans (24).

We report here that microarray analysis identified increased expression of the E2 conjugating enzyme UBE2E, which functions in the proteasome pathway to attach ubiquitin-tagged proteins to E3 ligases (35, 37). E3 ligases act in the ubiquitin-

Fig. 9. Immunohistochemical analysis for COLIII (A) and COLIV (B). Cross sections of human vastus lateralis muscle (10 μm) stained for COLIII, COLIV, and nuclei. In agreement with Western blotting analysis, immunohistochemistry confirms decreased staining for COLIII and COLIV along the extracellular matrix. A: COLIII, green; nuclei, blue. B: COLIV, red; nuclei, blue. Magnification: ×40.
proteasome pathway to alter the structure of the ubiquitin-protein complex so that it can be inserted into the proteolytic core for degradation (35, 37). The microarray analysis showed that expression of the E3 ligase (FBX09) was also increased after 48 h of immobilization. Despite this increase, we were unable to confirm alterations in UBE2E and FBX09 mRNA content using the more sensitive qRT-PCR technique, suggesting that the microarray technique did not provide a valid assessment of the expression of these genes. Furthermore, these results reinforce the critical need to perform confirmational work using qRT-PCR when performing microarray analysis.

Both the microarray analysis and qRT-PCR documented an increase in expression for USP-6 and SUMO-1, which also function in the ubiquitin-proteasome pathway. USP-6 is a deubiquitinating enzyme that cleaves ubiquitin from the ubiquitin-tagged proteins so that ubiquitin molecules can be recycled and reused, maintaining increased activity of the pathway during catabolic states (3). This is the first paper to show an increase in mRNA content for USP-6 in human skeletal muscle, because previous work has focused on the role of USP-6 in bone as a tumor suppressor gene (41). This gene is also increased in response to muscle atrophy, suggesting that it serves a growth suppression function, perhaps by maintaining increased ubiquitination and subsequent degradation of proteins. Future work is warranted to explore the expression of this gene at various time points during the course of muscle atrophy, because the significance of this alteration at a single time point, with no concomitant alteration in protein levels, is unclear.

SUMO-1, a small ubiquitin-related modifier, was also increased after 48 h of immobilization according to the microarray and qRT-PCR analysis. This finding is unique because previous experiments have shown that SUMO-1 functions to prevent proteasome-mediated degradation of proteins (16). The ability of SUMO-1 to stabilize proteins that are misfolded or tagged for degradation, particularly during conditions that precede muscle atrophy, makes it an ideal target for intervention. Thus we explored protein levels of SUMO-1 at baseline and after 48 h of immobilization. Western blot analysis failed to confirm that protein levels of SUMO-1 were altered at this time point. Immunohistochemical analysis also failed to show any alterations in the location or abundance of SUMO-1 protein as a result of 48-h immobilization. This finding does not rule out the possibility that SUMO-1 protein levels are altered as a result of disuse because additional work is necessary to examine the time course of alterations in gene expression and respective protein products.

Although our findings indicate that increased content of mRNA for two components of the ubiquitin-proteasome pathway, USP-6 and SUMO-1, occurs in response to 48 h of limb immobilization, they do not coincide with previous atrophy models that show an increase in mRNA content for E2 conjugating enzymes and the E3 ligases, atrogin-1, and MuRF (8, 32, 34, 51). In contrast to the present study, Bodine et al. (8) documented a nearly 10-fold increase in the expression of atrogin-1 and MuRF in response to 3 days of hindlimb immobilization in rats. Similarly, Stevenson et al. (51) reported an increase in mRNA content for several E3 ligases and this increase was maintained until 7 days of hindlimb immobilization in rats. In the present study, atrogin-1 expression was not altered after 48 h of immobilization according to the microarray analysis. MuRF is not included on the Human U133 GeneChip, so we performed targeted analysis of gene expression using custom designed primers (Table 1) and the more sensitive qRT-PCR technique. Despite this approach, mRNA content for atrogin-1 and MuRF was unchanged after 48 h of immobilization (Table 2).

The reason for this discrepancy between data from animal models and from our study regarding atrogin-1 and MuRF is unclear. A previous study in humans used targeted gene expression analysis (qRT-PCR) toward genes involved in the ubiquitin-proteasome pathway at 2 wk after limb immobilization (24); although that study showed a 62% increase in atrogin-1 expression ($P < 0.01$), the authors failed to document changes in MuRF and another E3 ligase previously reported to be upregulated in animal models of muscle atrophy. Additionally, a recent study examining the expression of ubiquitin-proteasome pathway components in response to age-associated sarcopenia (54) has also failed to identify alterations in atrogin-1 and MuRF mRNA content. Thus far, work in humans (24, 26, 54) suggests that there is a distinctive pattern of gene expression alterations that regulate the atrophy process compared with animal models. To this end, future work performed to explore the activity of ubiquitin-proteasome pathway components at multiple time points during the atrophy process will
be valuable in facilitating our understanding of the ubiquitin-proteasome pathway and its course of action in humans during the atrophy process.

**Metallothioneins.** One purpose of this study was to use microarray technology to acquire information about novel genes that may play a role in the initiation of the atrophy program in human skeletal muscle. We were successful in this approach because a group of genes, not previously associated with muscle atrophy in humans, showed increased mRNA content after 48 h of immobilization. Marked induction of metallothionein mRNA has been documented in skeletal muscle of animals in various catabolic states (23, 29, 32) and in human skeletal muscle 24 h after an intense exercise bout (43). This increase is thought to be beneficial to skeletal muscle, but the exact mechanism by which metallothioneins are induced and how they exert their protective effect is still not clear. Increased metallothionein content in muscle undergoing atrophy may be necessary to detoxify metal-containing compounds. For example, myoglobin is an iron-containing compound released during muscle protein degradation (23).

Increased metallothionein content has also been associated with exposure to physiological stress situations (e.g., immobilization, sepsis, aging) that result in elevated levels of glucocorticoids and reactive oxygen species (ROS) (23, 28, 29, 43). Metallothioneins preferentially bind zinc, and it has been proposed that increased metallothionein content is induced via glucocorticoids to assist in the accumulation of intracellular zinc (49). Because zinc is a cofactor involved in the synthesis of proteins and antioxidants, increased intracellular zinc accumulation may prevent ROS-mediated DNA or cellular damage (28).

Our results show, in a human model, that gene expression for several metallothionein isoforms is increased in response to 48 h of immobilization. However, we were unable to document an increase in metallothionein protein levels after 48 h of immobilization. These findings indicate that the atrophy program in humans is marked by an early transcriptional response in metallothionein, possibly as a consequence of elevated levels of heavy metals, ROS, or glucocorticoids in response to immobilization. It is possible that protein levels of metallothionein are elevated at later time points in the atrophy program when the rate of protein degradation increases and heavy metals and ROS begin to accumulate in the cell.

**ECM.** An unexpected finding in the present investigation was the number of genes involved in the ECM showing decreased mRNA content after the 48-h immobilization intervention (Table 2). Moreover, Western blot and immunohistochemical analysis showed that protein products of genes involved in ECM structure and integrity, COLIII and COLIV, were decreased.

Our data agree with earlier observations in animal muscle showing a decrease in COLIII and COLIV mRNA in various atrophy conditions (1, 2, 14). Additionally, previous work in animals has shown that immobilization, hindlimb suspension, and microgravity lead to a decrease of total muscle collagen synthesis rate, particularly COLI, COLII, and COLIV (1, 2), and this decrease is evident within 1 day of the intervention. It is well established that skeletal muscle responds rapidly to altered levels of activity (11, 12), and the number of studies that have shown decreased mRNA content for collagen components support the hypothesis that collagen degradation is an initial step in the muscle atrophy process.

We report here that in addition to decreases in mRNA for COLIII and COLIV, protein levels of COLIII and COLIV were reduced 88 and 28%, respectively, within 48 h of immobilization. Collagen is the most abundant protein in the ECM (52), and types III and IV are found in the epi/perimysium and basement membranes, respectively (27). Our results are in agreement with a recent 14-day hindlimb suspension intervention in rats by Giannelli and colleagues (17) reporting decreased levels of COL and COLIV protein according to Western blot and immunohistochemical analysis. The authors suggest that decreased COLIV alters the permeability of the membrane, possibly resulting in a proteolytic imbalance and subsequent atrophy. Others have suggested that decreased protein levels of collagen, such as the documented decrease in COLIII and COLIV after 48 h in the present study, indicate that when a limb is immobilized, the muscle begins to undergo remodeling within a relatively short time period, possibly to accommodate the reduced mechanical load as well as the fixed length of the muscle and loss of contractile activity (10, 52). Thus it is reasonable to hypothesize that reduced protein levels of COLIII and COLIV compromise the integrity of the ECM. The ECM is a dynamic structure that regulates cell behavior through the interaction of ECM molecules with each other, interaction with growth factors, and through ECM signal transduction pathways. Therefore, when the integrity of the matrix is compromised, normal muscle function is affected (52).

Previous work in animals has suggested that decreased collagen within the ECM increases the activity of intracellular signaling cascades involved in protein breakdown (2, 17). ECM proteins also function to interact with cell surface receptors, including growth factor receptors, to initiate signal transduction pathways responsible for muscle growth and maintenance. Thus a reduction in COLIII and COLIV protein levels will have a detrimental effect on downstream pathways responsible for protein synthesis and turnover. Additionally, chronic instability of the ECM due to a loss of collagen content is likely to induce various signaling networks responsible for protein degradation, including the ubiquitin-proteasome pathway.

Breakdown of the ECM components COLIII and COLIV is initiated by matrix metalloproteinases (MMPs) (27). The MMP family of enzymes contributes to tissue remodeling acting as regulatory molecules, both by functioning in enzyme cascades and by processing matrix proteins, cytokines, and growth factors. When induced, MMPs directly affect the stability of the ECM (10, 17). Remodeling of the ECM occurs through tightly regulated protease and protease inhibitor activities. Protease inhibitor molecules include the tissue inhibitor of metalloproteinase (TIMP) family and serine protease inhibitors. The main role of TIMP-1 is to inhibit inflammatory cell invasion, primarily through inhibition of metalloproteinase activity (50).

Although our microarray analysis and qRT-PCR confirmational work showed a decrease in both MMP-28 and TIMP-1 mRNA content, we were unable to document this alteration at the protein level. The observed decrease in MMP-28 mRNA is of interest because we are unaware of previous investigations in atrophy models in humans or animals that have documented a similar decrease. In contrast, MMP-2 and MMP-9 are the most abundant MMPs in skeletal muscle, and their content
tends to increase in response to various atrophy paradigms in which the muscle is in a state of proteolytic imbalance (40). However, our observed increase in TIMP-1 mRNA content after 48 h of immobilization is consistent with previous work in animals showing increases in TIMP-1 at both the mRNA and protein levels, with the latter occurring upward of 7 days (17). Unfortunately, the literature regarding these changes is limited, making it difficult to draw conclusions as to why these alterations occur, and more importantly, how MMPs and TIMPs can be manipulated to attenuate losses to ECM integrity in response to disuse. In fact, Giannelli et al. (17) are the first to investigate MMPs and TIMPs in an “in vivo” model in response to disuse, and this was done in an animal model. Additional studies are necessary to expand our knowledge of the action of MMPs and TIMPs in response to disuse.

In summary, the present study used four different approaches to identify novel candidate genes and proteins affected by 48 h of leg immobilization in humans: gene microarray, qRT-PCR, Western blotting, and IHC. Through this approach we were able to show that the early stages of the muscle atrophy program in humans are marked by several distinct alterations in mRNA content for components of the ubiquitin-proteasome pathway, metallothionein function, and ECM integrity. However, alterations at the protein level in humans after 48 h are limited to those proteins involved in ECM integrity and the phosphorylation state of proteins involved in the Akt signaling pathway. Although we did not find a respective increase in protein products for components of the ubiquitin-proteasome pathway, metallothionein function, and matrix metalloproteinases, it is possible that later time points would show an increase in these proteins in humans. The results of this study serve as a key first step in identifying critical regulators of the muscle atrophy program in humans.

ACKNOWLEDGMENTS

We acknowledge Dr. Rongye Shi for expert assistance in performing expression profiling; Dr. Milena Penkowa who generously supplied the metallothionein antibody; Dr. Larry Schwartz who assisted with protocol development and data interpretation; Dr. Jiguo Yu for IHC advice; Callie Comtois for qRT-PCR, Western blotting, and IHC. Adaptation of muscle mRNA expression to changes in contractile activity. Adv Myochim 1: 205–216, 1987.


