Transcriptional pathways associated with skeletal muscle disuse atrophy in humans

Yi-Wen Chen,1 Chris M. Gregory,2,5 Mark T. Scarborough,3 Rongye Shi,1 Glenn A. Walter,4 and Krista Vandenborne2

1Center for Genetic Medicine Research, Children’s National Medical Center, and George Washington University, Washington, District of Columbia; Departments of 2Physical Therapy, 3Orthopedics, and 4Physiology and Functional Genomics, University of Florida, Gainesville; and 5Malcom Randall Veterans Affairs Medical Center, Brain Rehabilitation Research Center, Gainesville, Florida

Submitted 2 June 2006; accepted in final form 30 August 2007

Chen Y-W, Gregory CM, Scarborough MT, Shi R, Walter GA, Vandenborne K. Transcriptional pathways associated with skeletal muscle disuse atrophy in humans. Physiol Genomics 31: 510–520, 2007. First published September 5, 2007; doi:10.1152/physiolgenomics.00115.2006.—Disuse atrophy is a common clinical phenomenon that significantly impacts muscle function and activities of daily living. The purpose of this study was to implement genome-wide expression profiling to identify transcriptional pathways associated with muscle remodeling in a clinical model of disuse. Skeletal muscle biopsies were acquired from the medial gastrocnemius in patients with an ankle fracture and from healthy volunteers subjected to 4–11 days of cast immobilization. We identified 277 misregulated transcripts in immobilized muscles of patients, of which the majority were downregulated. The most broadly affected pathways were involved in energy metabolism, mitochondrial function, and cell cycle regulation. We also found decreased expression in genes encoding proteolytic proteins, calpain-3 and calpastatin, and members of the myostatin and IGF-I pathway. Only 26 genes showed increased expression in immobilized muscles, including apolipoprotein (APOD) and leptin receptor (LEPR). Upregulation of APOD (5.0-fold, $P < 0.001$) and LEPR (5.7-fold, $P < 0.05$) was confirmed by quantitative RT-PCR and immunohistochemistry. In addition, atrogin-1/MAFbx was found to be 2.4-fold upregulated ($P < 0.005$) by quantitative RT-PCR. Interestingly, 96% of the transcripts differentially regulated in immobilized limbs also showed the same trend of change in the contralateral legs of patients but not the contralateral legs of healthy volunteers. Information obtained in this study complements findings in animal models of disuse and provides important feedback for future clinical studies targeting the restoration of muscle function following limb disuse in humans.

immobilization; gene expression; profiling

THE LOSS OF SKELETAL MUSCLE mass secondary to inactivity or disuse is a common clinical phenomenon and is increasingly becoming recognized as a purposeful biological compensatory mechanism (35). Disuse or inactivity has been shown to result in significant muscle remodeling, manifested by alterations including but not limited to 1) a loss of myofibrillar proteins (66); 2) a shift in phenotypic expression from slow to fast (37); 3) alterations in metabolic pathways (13); and 4) deleterious adaptations within the local neuromuscular junction, cytoskeleton, and microvascular environment (37, 89, 93). Over the past decade, significant effort has been put forth in identifying the molecular pathways involved in mediating muscle remodeling during disuse. In general, the adaptive muscle response has been shown to involve pathways regulating protein synthesis/degradation (49), oxidative stress (21, 44), energy metabolism, and maintenance of cellular structure (20). Specifically, induction of the transforming growth factor-β (TGF-β)/MAPK pathway (MAPK, c-Jun, c-Myc) (20) and IGF pathway (34, 40), upregulation of members of the ubiquitin proteosome pathway (atrogin-1/MAFbx, ubiquitin proteasomes, muscle ring finger proteins) (27, 29, 48, 65, 75), and altered expression of members of the calpain proteolytic system (calpain proteases 1–3 and calpastatin) (34, 39, 47, 87) have all been hypothesized to play an important role in regulating muscle atrophy. However, most of our knowledge is based on information extrapolated from animal models of disuse and studies examining age-related muscle loss (sarcopenia) in healthy individuals. To date, few studies have directly examined the regulatory pathways involved in muscle remodeling in a clinical model of disuse in humans.

Recently, gene expression profiling has created the opportunity for an expanded examination of the complex nature of events involved in regulating biological processes at the transcriptional level. Microarray technology employs a robust evaluative approach that indiscriminately surveys (probes) the response of thousands of potential candidate genes, using only small amounts of tissue, and can easily be employed in a patient population. The purpose of this study was to implement genome-wide expression profiling of skeletal muscle to examine the transcriptional pathways associated with muscle remodeling in a clinical model of disuse in humans. It is our hope that information garnered in this study will complement findings in animal models and provide feedback for future clinical studies investigating the pathophysiology of muscle atrophy in patient populations.

METHODS

Subjects. Fifteen adults (mean age 31.5 ± 2.6 yr) participated in this study. Subjects included eight patients (4 females, 4 males) with medial or lateral malleolar fractures requiring cast immobilization of the lower leg and seven noninjured participants (4 females, 3 males). Patients were immobilized with the ankle in a neutral position, using a short leg cast. The fractures were treated by open reduction-internal fixation followed by cast immobilization of the ankle joint. All subjects gave their signed and informed consent before participating in the study; study protocols were approved by the Institutional
Review Board at the University of Florida. None of the subjects was involved in a resistance or endurance training program, and none presented with a prior history of musculoskeletal injury to the lower extremity. Patients reported taking standard prescription anti-inflammatory and analgesic medications following injury, but the utilization of glucocorticoids and corticosteroids was considered an exclusion criterion.

**Tissue collection.** To identify the genes that were differentially expressed during cast immobilization using genome-wide expression profiling, skeletal muscle biopsies were acquired bilaterally in both ankle fracture patients and noninjured volunteers subjected to cast immobilization. In the ankle fracture patients, muscle biopsies were obtained from the immobilized and contralateral limb within the first 2 wk of cast immobilization, a time period during which muscle atrophy is known to be most prevalent (81, 92). Specifically, biopsies were obtained from the medial gastrocnemius after either 4–5 days (2 subjects) or 9–11 days (2 subjects) of cast immobilization. In two noninjured volunteers subjected to cast immobilization, three biopsies were obtained from the medial gastrocnemius. One biopsy was obtained before immobilization (pre-), and the two other biopsies were obtained from the immobilized and contralateral limb after 5 days of cast immobilization, similar to the patients. The repeat biopsy in the healthy volunteers was collected from an incision site ~1 cm proximal to the previous site. Finally, muscle biopsies from four additional patients (5 days of immobilization) and five nonimmobilized healthy controls were collected for data validation using quantitative RT-PCR and immunohistochemistry. Following excision, all biopsy samples were immediately frozen in 2-methylbutane cooled in liquid nitrogen and subsequently stored at ~80°C for further analysis.

**Expression profiling.** Affymetrix U95A(v2) microarrays containing ~12,000 full-length genes and expressed sequence tags (ESTs) were used for this study. Each muscle biopsy was split into two fragments that were processed independently. Total RNA was isolated from each fragment and processed for production of biotinylated cRNA and hybridization to microarrays, as we have previously reported (16). A total of 20 microarray profiles were created using standard operating and quality control procedures (2, 88a).

The expression level of each gene was calculated using two algorithms, microarray suite (MAS; version 5.0, Affymetrix) and dCHIP (51). During MAS5.0 absolute analysis, the average intensity of the probes on each individual microarray was scaled to the same target intensity. Given that the RNA content per muscle volume has previously been reported to be reduced following unloading (19, 23), this scaling strategy, based on the global intensity of the whole microarray (>260,000 probes), has the advantage that it avoids bias based on changes in total RNA content and experimental variability and allows a comparison across microarrays.

**Data filtering and analysis.** Since our primary goal was to identify genes that are consistently differentially expressed during cast immobilization in patients, we first pooled the data from the immobilized muscles in all four patients (8 profiles) and compared them with the control preimmobilization samples collected in healthy noninjured volunteers (4 profiles). Data filtering was accomplished by retaining only those probe sets that showed significant ($P < 0.05$) gene expression changes in the immobilized samples compared with the preimmobilized samples as determined by the Welch t-test. To reduce potential false positive findings, multiple testing correction was performed using the Benjamini and Hochberg false discovery rates (5% false positive discovery rate) (5, 6). After we obtained the filtered gene list, the average expression level of the two biopsy fragments for each subject was determined, and statistical analysis was performed on the average values. The advantage of using a filtering strategy for microarray analysis is that it reduces the number of genes for multiple testing and increases the statistical power. Welch t-test and the Benjamini and Hochberg false discovery rates (5% false positive discovery rate) were used to analyze the mean expression changes; and genes that reached $P < 0.05$ were reported.

To categorize the large number of genes into functional groups and rank the significance of each group, we used a combination of expression analysis system and expression analysis tools. Gene Expression Omnibus (GEO) databases (2, 88a) and Ingenuity Pathway Analysis (Ingenuity Systems) software. First, EASEv2.1 was implemented to identify gene ontology and enrichment analysis was performed in the gene list. The probability of the number of “list hits” in the “list total” given the frequency of “population hits” in the “population total” was calculated as the Fisher’s exact probability. The EASE score was then calculated to categorize genes of interest. In the gene list, Ingenuity Pathway Analysis was applied to identify over-represented functional groups. All profiles have been made publicly accessible via the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (2, 88a). Online data queries are also publicly accessible, both through a single gene query via PEPR (1, 14) and via the GEO.

Genes with $P < 0.05$ were further analyzed and hierarchically clustered to visualize transcripts showing coordinate regulation across conditions (healthy volunteers and patients) and as a function of immobilization time (4–5 days). GENECLUSTER software was used to cluster genes by performing a hierarchical algorithm and to visualize gene expression clusters. The clusters are reported in Table 1. All primers were tested for specificity by agarose gel electrophoresis and melting curve analysis following the RT-PCR assays. The ΔΔCt value method (where Cx is cycle threshold) was used to determine fold differences as described previously (15).

**Immunohistochemistry.** Serial 4-μm-thick frozen muscle sections with the IEC Minotome cryostat and mounted to the Superfrost Plus Slides (Fisher Scientific). After fixation in cold acetone, rinsing in 0.3% H2O2 in 0.3% normal serum in phosphate-buffered saline (PBS), and blocking with serum buffer, the slides were incubated with the primary antibodies overnight at 4°C, followed by incubation with biotinylated secondary mouse/ rabbit antibody for 2 h at room temperature. Sections were washed three times with PBS, incubated for 30 min with Vectastain ABC reagent (Vector Laboratories), and stained with peroxidase substrate solution. Monoclonal antibodies against APOL (Calbiochem) were diluted at 1:40. Isotype-matched primary antibody and secondary antibody only were performed to verify the specificity of antibody-protein interaction, respectively.
Polyclonal antibodies against LEPR (Calbiochem) were diluted at 1:100. Secondary antibody only was performed.

RESULTS

For an initial overview of the profiling data, we used hierarchical clustering to visualize changes in gene expression patterns in each biopsy sample acquired in patients and healthy volunteers (Fig. 1). All profiles were normalized to the control preimmobilization biopsy samples acquired in healthy volunteers before cluster analysis. As shown in Fig. 1, a large number of transcripts were differently expressed in the immobilized limb of both ankle fracture patients and healthy noninjured volunteers compared with preimmobilization samples. Interestingly, the transcriptomes from muscle samples obtained from the contralateral leg in patients were also dramatically changed. As shown in Figs. 1 and 2, most transcripts differentially regulated in the immobilized legs were also differentially regulated in the contralateral legs of patients but not the contralateral legs of healthy volunteers.

To identify transcripts that were consistently differentially regulated during cast immobilization in patients, we pooled the profiles of the immobilized legs of the patients (4–5 days, 9–11 days) and compared them with the profiles of the control preimmobilization muscle samples. In doing so, we identified 277 shared misregulated transcripts ($P < 0.05$) in all immobilization samples after multiple testing corrections. Most of the transcripts were downregulated (only 26 transcripts were found upregulated) in the immobilized muscles of patients with an ankle fracture.

Among the upregulated transcripts, APOD showed the most dramatic changes in the muscles of the immobilized legs. We also found increased expression in LEPR, a protein that has been shown to interact with APOD. To verify the upregulation of APOD and LEPR, we performed qRT-PCR and confirmed that APOD (5.0-fold, $P < 0.001$) and LEPR (5.7-fold, $P < 0.05$) mRNA levels were significantly higher in immobilized muscles of ankle fracture patients. Since no probe on the
U95Av2 represents the atrogin-1/MAFbx gene, we also performed qRT-PCR for human atrogin-1 and found, as expected, that atrogin-1/MAFbx is 2.4-fold upregulated ($P < 0.005$) in immobilized muscles of patients compared with control muscles (Fig. 3). Immunohistochemistry further showed that both APOD and LEPR were localized in the subsarcolemmal regions of immobilized muscle fibers, whereas no positive signals were observed in healthy control muscles (Fig. 4). Whereas upregulation of the APOD protein was dramatic, with positive staining seen in all myofibers and distributed continuously in the subsarcolemmal regions, the signal of LEPR protein was much less dramatic, with portions of myofibers showing positive staining near the perinuclear region.

To manage the large number of downregulated transcripts, we grouped the genes by functional categories using a combination of EASE, NetAffx, and Ingenuity Pathway Analysis. Table 2 shows the six gene ontology biological processes that received significant EASE scores after correction for false positives. All of them were related to energy metabolism and primarily mitochondrial function. With Ingenuity Pathway Analysis, the top two overrepresented functional groups that were identified were also genes involved in energy metabolism and mitochondrial function. Given the overrepresentation of the energy metabolism category, all energy metabolism genes that were identified are summarized in Table 3.

A summary of the downregulated transcripts identified in the other major categories has been provided in Table 4. They represent genes involved in stress responses, sarcomere structure, protein turnover, and cell growth/death (Table 4). Immobilized patient muscle samples showed decreased expression in several stress response genes, including heat shock protein-70 and glutathione S-transferase. In addition, we noted an approximate twofold downregulation in the genes encoding proteolytic proteins, calpain-3, and calpastatin. Not surprisingly, many genes involved in mediating cell growth and cell death showed lower mRNA levels, including insulin receptor sub-

![Fig. 3. Upregulation (2.4-fold, $P < 0.005$) of atrogin-1 in immobilized muscles of patients vs. control muscles by quantitative RT-PCR.](http://physiolgenomics.physiology.org/)

![Fig. 4. Cellular localization of apolipoprotein D in control muscles (A) vs. immobilized muscles of patients (B) and leptin receptor in control muscles (C) vs. immobilized muscles of patients (D) by immunohistochemistry. Arrows indicate perinuclear staining, while arrow head in B indicates cytoplasm staining. Isotype-matched primary antibody and secondary antibody alone did not show positive signals, respectively (not shown). Scale bar = 50 μm.](http://physiolgenomics.physiology.org/)
strate-1, poly(ADP-ribose) polymerase-1, and cyclin D1. Many of these genes have previously been shown to be differentially regulated during muscle remodeling, supporting the findings of the present study (4, 7, 8, 15, 18, 27, 79, 82, 97, 98). Of interest is that we did not see downregulation of a significant number of extracellular matrix/cell adhesion genes in the immobilized muscles nor did we see the upregulation of genes of the ubiquitin proteasome pathways (except for atrogin-1), as reported in other studies (24, 38, 67, 90, 97).

Finally, to visualize the effect of length of immobilization and condition (healthy volunteer vs. patient) on gene expression, we grouped the profiles of patients immobilized for 4–5 days, patients immobilized for 9–11 days, and healthy immobilized volunteers and then visualized each group separately using GeneSpring software (Fig. 2). As expected, the gene expression changes were most dramatic in patients who were immobilized for a longer length of time. In addition, we found that the changes in the immobilized muscles of healthy volunteers were

<table>
<thead>
<tr>
<th>Gene Category</th>
<th>List Hits</th>
<th>List Total</th>
<th>Population Hits</th>
<th>Population Total</th>
<th>EASE Score</th>
<th>Bonferroni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy pathways</td>
<td>22</td>
<td>227</td>
<td>184</td>
<td>8,095</td>
<td>3.56E-08</td>
<td>3.99E-05</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>8</td>
<td>227</td>
<td>23</td>
<td>8,095</td>
<td>2.02E-06</td>
<td>2.27E-03</td>
</tr>
<tr>
<td>Tricarboxylic acid cycle</td>
<td>7</td>
<td>227</td>
<td>21</td>
<td>8,095</td>
<td>1.56E-05</td>
<td>1.75E-02</td>
</tr>
<tr>
<td>Energy derivation by oxidation of organic compounds</td>
<td>14</td>
<td>227</td>
<td>119</td>
<td>8,095</td>
<td>2.52E-05</td>
<td>2.83E-02</td>
</tr>
<tr>
<td>Main pathways of carbohydrate metabolism</td>
<td>11</td>
<td>227</td>
<td>73</td>
<td>8,095</td>
<td>3.14E-05</td>
<td>3.52E-02</td>
</tr>
</tbody>
</table>

Categories were ranked by significance. EASE, expression analysis systematic explorer.

Table 2. Six gene ontology biological processes received significant EASE scores

Table 3. Summary of shared downregulated genes involved in energy metabolism in the immobilized muscles of ankle fracture patients

Summary of shared downregulated genes (P < 0.05 after multiple testing correction) involved in energy metabolism in the immobilized muscles of ankle fracture patients. The last 3 columns show previous studies related to atrophy/disuse, hypertrophy/activity, and aging, respectively. Parenthetical information indicates upregulation (+) or downregulation (−) of the gene in the cited paper. TCA cycle, tricarboxylic acid cycle.

---

Physiol Genomics • Vol 31 • www.physiolgenomics.org
teers were less dramatic than the changes in patients immobilized for the same length of time. Interestingly, while the contralateral legs of healthy volunteers showed a gene expression profile similar to that of the preimmobilized or “true” control muscles, the profiles from contralateral legs in both patient groups (4–5 days and 9–11 days) were quite similar to the immobilized samples. In fact, 96% (266/277) of the differentially regulated transcripts showed the same directional changes in the contralateral legs of patients. Among those transcripts that did not change in the contralateral leg of patients were cyclin D1, microsomal glutathione S-tranferase-3, and two mitochondrial genes, cytochrome c oxidase subunit VIIc and mitochondrial ribosomal protein L12. Complete gene lists providing a comparison between immobilization and preimmobilization samples as well as contralateral and preimmobilization samples are reported in the Supplemental Table (supplemental data are available at the online version of this article).

**DISCUSSION**

Genome-wide gene expression profiling has created unique opportunities for studying complex biological processes regulated at the transcriptional level. Previous studies have used profiling techniques to describe the molecular responses associated with disuse atrophy in a variety of animal models (7, 68, 82). Although a number of studies using different experimental designs and molecular techniques have also been performed in

---

**Table 4. Summary of shared downregulated genes involved in stress response, sarcomere structures, cell growth/death, and protein turnover in the immobilized muscles of ankle fracture patients**

<table>
<thead>
<tr>
<th>Affymetrix Accession No.</th>
<th>Fold Change</th>
<th>Gene Description</th>
<th>Atrophy/Disuse</th>
<th>Hypertrophy/Activity</th>
<th>Aging</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stress responses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37222_at</td>
<td>−2.6</td>
<td>Glutathione S-transferase theta 1</td>
<td>Ref. 7 (−), Ref. 18 (−), Ref. 15 (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1180_g_at</td>
<td>−1.8</td>
<td>HSP 70 kDa</td>
<td>Ref. 82 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39018_at</td>
<td>−2.2</td>
<td>Glutathione S-transferase 3</td>
<td>Ref. 82 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sarcomere structure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38444_at</td>
<td>−3.9</td>
<td>CSRP3 (LIM protein)</td>
<td>Ref. 82 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32542_at</td>
<td>−1.7</td>
<td>Four and a half LIM domains 1</td>
<td>Ref. 11 (−), Ref. 39 (−), Ref. 83 (−), Ref. 87 (−), Ref. 88 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38461_at</td>
<td>−1.6</td>
<td>Nebulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39002_at</td>
<td>−2.2</td>
<td>Titin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39301_at</td>
<td>−2.2</td>
<td>Calpain 3</td>
<td>Ref. 11 (−), Ref. 39 (−), Ref. 83 (−), Ref. 87 (−), Ref. 88 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35137_at</td>
<td>−2.2</td>
<td>Myomesin 1 (skelemin)</td>
<td>Ref. 87 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41730_at</td>
<td>−2</td>
<td>Myosin, light chain 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41257_at</td>
<td>−2</td>
<td>Calpastatin</td>
<td>Ref. 87 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40059_at</td>
<td>−1.6</td>
<td>PDZ and LIM domain 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Protein turnover</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35211_at</td>
<td>−4.5</td>
<td>Protein phosphatase 2, regulatory subunit B′, alpha</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39127_f_at</td>
<td>−2.9</td>
<td>Protein phosphatase 2A, regulatory subunit B′ (PR 53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39083_at</td>
<td>−2.5</td>
<td>Ubiquitin-conjugating enzyme E2D3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>924_s_at</td>
<td>−2.5</td>
<td>Protein phosphatase 2, catalytic subunit-beta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>237_s_at</td>
<td>−2.2</td>
<td>Protein phosphatase 2 catalytic subunit-alpha</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1160_at</td>
<td>−1.9</td>
<td>Ubiquitin-conjugating enzyme E2N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40109_at</td>
<td>−1.8</td>
<td>c-fos serum response element-binding transcription factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cell growth/death</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35818_at</td>
<td>−1.7</td>
<td>Cytochrome c, somatic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2081_s_at</td>
<td>−2.5</td>
<td>Protein kinase C, theta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014_s_at</td>
<td>−2.5</td>
<td>Mitogen-activated protein kinase 6</td>
<td>Ref. 42 (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36179_at</td>
<td>−1.6</td>
<td>Mitogen-activated protein kinase 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>851_y_at</td>
<td>−2.1</td>
<td>Insulin receptor substrate-1</td>
<td>Ref. 33 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1953_at</td>
<td>−2</td>
<td>VEGF</td>
<td>Ref. 7 (−), Ref. 82 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40109_at</td>
<td>−1.8</td>
<td>Sarcoma viral oncogene homolog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38418_at</td>
<td>−1.8</td>
<td>Cyclin D-1</td>
<td>Ref. 98 (−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1287_at</td>
<td>−1.9</td>
<td>Poly (ADP-ribose) polymerase 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1706_at</td>
<td>−1.4</td>
<td>v-raf murine sarcoma 3611 viral oncogene homolog</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Summary of shared downregulated genes (P < 0.05 after multiple testing correction) involved in stress response, sarcomere structures, cell growth/death, and protein turnover in the immobilized muscles of ankle fracture patients. The last 3 columns show previous studies related to atrophy/disuse, hypertrophy/activity, and aging, respectively. Parenthetical information indicates upregulation (+) or downregulation (−) of the gene in the cited paper.
humans, to our knowledge, this is the first genome-wide profiling study examining molecular responses associated with a clinical model of disuse in humans. Using gene expression profiling, we identified 277 transcripts that were differentially regulated in immobilized muscles of ankle fracture patients, of which the majority were downregulated. Only 26 transcripts were upregulated, including genes encoding APOD and LEPR. Hierarchical clustering further revealed strong similarities between samples acquired from immobilized muscles in orthopedic patients and those from noninjured healthy subjects. Interestingly, the majority of transcripts (266/277) differentially regulated in immobilized limbs also expressed the same trend of changes in the contralateral legs of patients but not the contralateral legs of healthy volunteers. These data suggest that, in an orthopedic clinical patient population, such as the one studied herein, inactivity and bed rest may induce transcriptomic changes reflective of muscle disuse atrophy in both the involved and uninvolved limb.

We performed pathway-based analyses using EASE, Affymetrix NetAffx, and Ingenuity Pathway Analysis to describe the downregulated transcriptional pathways associated with muscle remodeling during immobilization. The functional groups that were identified as being overrepresented in the muscles of immobilized legs in patients included genes involved in energy metabolism, mitochondrial function, sarcomere structure, cell growth regulation, and stress response. There was a dramatic downregulation of genes involved in energy metabolism, with a decreased expression of 36 energy metabolism-related genes. Represented pathways included mitochondrial metabolism, glycolysis/glucoseogenesis, and tri-carboxylic acid cycle as well as glycogen, lipid, and fatty acid metabolism (Table 3). A large number of the identified energy metabolism genes have previously been reported to be downregulated in animal models of disuse atrophy or upregulated in conditions leading to muscle growth (7, 64, 69, 78, 79, 82, 95, 97).

As can be expected during an active phase of muscle atrophy, we observed decreased expression in a number of genes linked to structural proteins. Sarcomere structure genes that were downregulated include myomesin-1, myosin light chain-3, titin, nebulin, and troponin T1. In addition, we noted a decreased expression in the genes encoding proteolytic proteins, calpain-3 and calpastatin. Although the exact function of calpain-3 is not well understood, several cytoskeletal proteins have been identified as substantial substrates for calpain-3 (45). In addition, mutations in the gene encoding calpain-3 are responsible for limb girdle muscular dystrophy type 2A and sarcomere disassembly (46, 73). Calpain-3 has previously been reported to be downregulated in two models of cachexia (11, 88), during denervation atrophy (83) and cast immobilization (39). Consistent with our data, Jones et al. (39) reported that human muscle-specific calpain-3 was downregulated by 36% after 2 wk of immobilization. In this study, we found a twofold decreased expression in the genes encoding both calpain-3 and calpastatin in immobilized muscles of ankle fracture patients compared with preimmobilized muscle samples. Calpain-3 has been speculated to contribute to muscle atrophy by deregulation of an anti-apoptotic response of the NF-κB pathway or by altering the proteasome activity, although in this study we did not observe an increase in members of the NF-κB pathway.

The coordinated downregulation of calpain-3 and calpastatin is particularly intriguing. Calpastatin is a specific endogenous inhibitor of calpain-1 and -2, and overexpression of calpastatin in transgenic mice has been shown to reduce muscle atrophy during unloading (87). In addition, calpastatin overexpression prevents the shift from slow to fast fibers that normally occurs during unloading. In contrast to our study, Jones et al. (39) did not find a decrease in the expression of calpastatin during 2 wk of cast immobilization but showed a twofold increase 24 h after cast removal and one bout of exercise training. The coordinated downregulation of genes encoding both calpain-3 and calpastatin in our study provides additional evidence that the calpain-calpastatin system plays a significant role in inducing muscle atrophy during cast immobilization.

Among the various systems involved in muscle protein degradation, the ubiquitin proteasome system is thought to degrade the bulk of skeletal muscle proteins (74). This system is characterized by the concerted action of ubiquitin-conjugating enzymes that link chains of the polypeptide co-factor ubiquitin onto proteins for degradation (28, 61). Atrogin-1/MAFbx is a component of an SCF-type (Skp1/Cdc53/F-box complex) E3 ubiquitin ligase that determines substrate specificity for proteasome degradation (25). The protein is specifically expressed in cardiac and skeletal muscle and has previously been identified as a marker of muscle atrophy (9, 29). In this study, we found a 2.4-fold upregulation (P < 0.005) in muscle samples collected from immobilized limbs of ankle fracture patients relative to nonimmobilized controls, thus providing additional support for the protein’s role as a regulator of muscle atrophy. Interestingly, our profiling data demonstrated downregulation in several other genes belonging to the ubiquitin proteasome pathway during immobilization. Jones et al. (39), using qRT-PCR, found a 62% increase in MAFbx expression in human skeletal muscle after 2 wk of immobilization but no change in mRNA expression in E3a ligase and only a small increase in 20S proteasome subunit a7. The more modest changes in the genes involved in the ubiquitin proteasome pathway in human studies compared with reports in animal disuse models may reflect species-related differences. It also stresses the importance of complementing animal studies with studies in clinical patient populations. We should point out that, because of our study design, we may have missed early or transient increases in the expression of individual members of the ubiquitin proteasome pathway.

Myostatin and IGF-I have been identified as negative and positive regulators of muscle growth, respectively. Myostatin is a member of the TGF-β superfamily and is known as an inhibitor of skeletal muscle cell proliferation and differentiation. Blocking of the myostatin signaling transduction pathway by specific inhibitors and genetic manipulations has been shown to lead to both muscle hypertrophy and hyperplasia (31, 59, 102). Increased levels of myostatin have also been reported in human immunodeficiency virus patients suffering from muscle wasting and during administration of high doses of glucocorticoids, indicating that myostatin contributes to cachexia-related atrophy (30, 55). However, the role myostatin plays during disuse-induced atrophy is more ambiguous, with some studies demonstrating increases in myostatin expression during disuse, whereas others failed to find a link between myostatin expression and muscle atrophy during unloading (12, 71, 96). In our study, we did not detect an upregulation in...
myostatin expression but found a twofold decreased expression in cyclin D1. D-type cyclins play a crucial role in the progression through G1 in the cell cycle, and in many cell types TGF-β has been shown to cause growth inhibition via the downregulation of D cyclins (43, 57, 76). Yang et al. (99) in a recent paper provided strong experimental evidence that, in specific, cyclin D1 is a crucial target for the growth inhibitory effects of myostatin. C2C12 cells overexpressing cyclin D1 not only grew faster than control cells but also showed more resistance to the proliferative effect of myostatin. In addition, treatment with IGF-I blocked myostatin-induced inhibition of cell proliferation by stabilizing cyclin D1 protein and increasing Akt and glycogen synthase kinase-3β (GSK-3β) phosphorylation. These findings led the authors to suggest that IGF-I may antagonize myostatin-regulated cyclin D1 degradation and its inhibitory growth effect through the phosphatidylinositol 3-kinase/Akt/GSK-3β pathway. In our study, the twofold downregulation in cyclin D1 expression in the immobilized muscles of ankle fracture patients was accompanied by decreased expression of several members of the IGF-I pathway, including sarcoma viral oncogene homolog, insulin receptor substrate-1, and MAPK-2 and -6. These data may provide additional evidence that inhibition of muscle growth, specifically, inhibition of cell proliferation, contributes to disuse-induced muscle atrophy in humans.

Although the preponderance of genes that were differentially expressed in immobilized muscles were downregulated, several genes were upregulated in the immobilized muscle samples, including APOD, LEPR, and atrogin-1/MAFbx. The combined upregulation of APOD and LEPR in immobilized muscles is novel and deserves special attention. Leptin is a fat-derived cytokine regulating food intake and energy expenditure, primarily through the hypothalamus (50, 56, 91, 101). Circulating leptin levels have been shown to correlate with fat mass, and lack of leptin or LEPRs is associated with morbid obesity in both mice and humans (17, 26, 56, 62, 101). Interestingly, skeletal muscles express both long (ObRb) and short (ObRa) forms of the LEPR. Previous studies have demonstrated that administration of recombinant leptin results in activation of AMP-activated protein kinase, which plays a key role in the regulation of energy control, including promotion of fatty acid oxidation (60, 80). The upregulation of LEPR in immobilized muscles in the present study suggests that atrophic muscles increase their sensitivity to leptin signaling in an attempt to reduce fatty acid deposition in muscle cells. This phenomenon might result from an overall downregulation of energy supply pathways specifically involved in lipid metabolism or other factors activated during muscle disuse.

Among the upregulated genes, APOD showed the most dramatic changes in the muscles of immobilized legs of both patients and healthy volunteers. APOD is a protein component of high-density lipoproteins in human plasma (58). The primary structure of this protein suggests that it is closely related to lipocalins, a group of proteins that serve to transport small hydrophobic ligands. APOD has been shown to bind cholesterol, progesterone, bilirubin, and arachidonic acid (58, 63, 70). In addition, studies have shown that APOD may also form a complex with lecithin:cholesterol acyltransferase (LCAT) and is associated with stabilization and activation of this ligand (22). Although many potential functions of APOD have been proposed, the true function of this gene is not yet clear. APOD is reported to be upregulated during regeneration after neuronal injury as well as in neurodegenerative disease states (10, 72, 85, 86). In vitro studies also suggest that the expression of APOD is accompanied by inhibition of cell proliferation and growth arrest (54, 77). Although there is no obvious relationship between APOD and lipoprotein metabolism, upregulation of APOD has been associated with a number of diseases with defects in lipoprotein function (52, 84). Polymorphisms in APOD have also been linked to non-insulin-dependent diabetes mellitus and obesity and hyperinsulinemia in syndrome X (3, 94). Interestingly, one study has shown ObRb to be localized in the perinuclear region of hypothalamic cells and demonstrated direct interaction between ObRb and APOD (53). This same study also showed that expression levels of APOD can be regulated by leptin and are associated with the deposition of body fat. The upregulation of APOD and LEPR in our study suggests that both proteins might be involved in the same pathways regulating the homeostasis of fat in muscle cells during disuse following immobilization. Whether this pathway is actively participating in the atrophic process of skeletal muscle or just compensatory in response to the changes in metabolic flux within these tissues warrants further investigation.

A secondary goal in this study was to visualize the effect of the duration of immobilization as well as the condition (patients vs. healthy) on gene expression. For this purpose, we grouped the profiles of patients immobilized for 4–5 days, patients immobilized for 9–11 days, and healthy immobilized volunteers and visualized each group separately using GeneSpring software. We found that the gene expression changes were most dramatic in immobilized muscles of patients who were immobilized for 9–11 days and least dramatic in immobilized muscles of healthy volunteers. In addition, we noted that, whereas contralateral legs of healthy volunteers displayed a gene expression profile similar to that of preimmobilization muscle samples, the contralateral limbs in both patient groups shared a large number of genes (266/277, or 96%) with the immobilized muscles. These data indicate that, in an orthopedic clinical patient population such as studied herein, inactivity and bed rest may induce transcriptional changes reflective of muscle disuse atrophy in both the involved and uninvolved limb. We anticipate that this observation may be of considerable value to future clinical studies, since, in many studies, the contralateral limb serves as a control.

Last, there are some shortcomings in our study. Gene expression profiling measures the amount of mRNA in samples, which can be regulated by transcriptional and posttranscriptional mechanisms. Therefore, changes in gene expression ultimately may not result in activation of a pathway. Second, in an attempt to identify consistent, robust changes in skeletal muscle during disuse, profiles of immobilized muscles (4–5 days of immobilization and 9–11 days of immobilization) were combined and compared with preimmobilization control samples from healthy volunteers. By implementation of this strategy, only genes that are consistently differentially expressed between 4 and 11 days of immobilization are revealed. Consequently, changes that occur transiently or biphasically or that fluctuate during the period of immobilization are not identified in this study. Finally, the sample size in this study was small, and a detailed analysis of the gene expression change with the progression of atrophy was not performed.
In conclusion, this is the first study implementing gene expression profiling to examine the transcriptional pathways involved in mediating muscle remodeling during disuse atrophy in a clinical patient population. A total of 277 genes were differentially regulated in immobilized muscles of patients with an ankle fracture compared with healthy muscles, of which the majority were downregulated. Among the upregulated genes, the upregulation of APOD and LEPR was confirmed by use of qRT-PCR and immunohistochemistry. The combined upregulation of APOD and LEPR has not been reported previously in the context of disuse atrophy and is a novel finding. Downregulated genes were categorized into the following functional groups: energy metabolism, mitochondrial function, cell cycle regulation, stress response, sarcomere structure, cell growth/death, and protein turnover. A large proportion of the downregulated genes (36 genes) in immobilized muscle of ankle fracture patients was involved in energy metabolism. We also found decreased expression in the genes encoding proteolytic proteins, calpain-3, and calpastatin, providing further evidence that the calpain-calpastatin pathway plays a critical role in mediating muscle atrophy during disuse. Immobilized muscles also displayed decreased expression in several genes involved in muscle growth and cell proliferation and differentiation. Specifically, we found a twofold downregulation in the myostatin target, cyclin D1, as well as in several members of the IGF-I pathway (sarcoma viral oncogene homolog, insulin receptor substrate-1, and MAPK-2 and -6). Finally, many transcripts that were differentially regulated in immobilized muscles were also differentially regulated in the contralateral limb of patients but not the contralateral limbs of healthy volunteers. Information obtained in this study complements findings in animal models of disuse and provides important feedback for future clinical studies targeting the restoration of muscle function following limb disuse.

GRANTS

This work was supported in part by National Center for Medical Rehabilitation Grant 1R01-HD-048051 to K. Vandeborre and Y.-W. Chen and National Institutes of Health Grant 5R21-AR-048318 to Y.-W. Chen.

REFERENCES


Physiol Genomics • VOL 31 • www.physiolgenomics.org

Downloaded from http://physiolgenomics.physiology.org/ by 10.220.33.1 on November 6, 2017


91. Wallis MG, Appleby GJ, Youd JM, Clark MG, Penchow JD. Reduced glycogen phosphorylase activity in denervated hindlimb muscles of rats is related to muscle atrophy and fibre type. Life Sci 64: 221–228, 1999.


