Denervation in murine fast-twitch muscle: short-term physiological changes and temporal expression profiling

Anna Raffaello,1,* Paolo Laveder,1,* Chiara Romualdi,1 Camilla Bean,1 Luana Toniolo,2 Elena Germinario,2 Aram Megighian,2 Daniela Danieli-Betto,2 Carlo Reggiani,2 and Gerolamo Lanfranchi1

1Centro di Ricerca Interdipartimentale per le Biotecnologie Innovative (CRIBI) Biotechnology Center and 2Department of Anatomy and Physiology, University of Padova, Padua, Italy

Submitted 28 February 2005; accepted in final form 22 December 2005

Denervation in murine fast-twitch muscle: short-term physiological changes and temporal expression profiling. Physiol Genomics 25: 60–74, 2006. First published December 27, 2005; doi:10.1152/physiolgenomics.00051.2005.—Denervation deeply affects muscle structure and function, the alterations being different in slow and fast muscles. Because the effects of denervation on fast muscles are still controversial, and high-throughput studies on gene expression in denervated muscles are lacking, we studied gene expression during atrophy progression following denervation in mouse tibialis anterior (TA). The sciatic nerve was cut close to trochanter in adult CD1 mice. One, three, seven, and fourteen days after denervation, animals were killed and TA muscles were dissected out and utilized for physiological experiments and gene expression studies. Target cDNAs from TA muscles were hybridized on a dedicated cDNA microarray of muscle genes. Seventy-one genes were found differentially expressed. Microarray results were validated, and the expression of relevant genes not probed on our array was monitored by real-time quantitative PCR (RQ-PCR). Nuclear- and mitochondrial-encoded genes implicated in energy metabolism were consistently downregulated. Among genes implicated in muscle contraction (myofibrillar and sarcoplasmic reticulum), genes typical of fast fibers were downregulated, whereas those typical of slow fibers were upregulated. Electrophoresis and Western blot showed less pronounced changes in myofibrillar protein expression, partially confirming changes in gene expression. Isometric tension of skinned fibers was little affected by denervation, whereas calcium sensitivity decreased. Functional studies in mouse extensor digitorum longus muscle showed prolongation in twitch time parameters and shift to the left in force-frequency curves after denervation. We conclude that, if studied at the mRNA level, fast muscles appear not less responsive than slow muscles to the interruption of neural stimulation.

Denervation; cDNA microarray; mitochondria; myosin

*MUSCLE ATROPHY IS AN ADAPTIVE RESPONSE TO REDUCED CONTRACTILE ACTIVITY, REduced MECHANICAL LOAD, or MODIFIED ENVIRONMENTAL CONDITIONS (HYPOXIA, UREMIA, ENDOtoxin ACTION, etc.). THE HALLMARKS OF ANY TYPE OF ATROPHY ARE DECREASEd MUSCLE MASS AND REDUCED CONTRACTILE FORCE. IN THE LAST FEW YEARS, SEVERAL GLOBAL EXPRESSION STUDIES CARRIED OUT IN VARIOUS MODELS OF MUSCLE ATROPHY HAVE REVEALED THE MOLECULAR CHANGES THAT ACCOMPANY THE MUSCLE ALTERATIONS IN RESPONSE TO FOOD DEPRIVATION (33), AGING (71), DISUSE (4, 62), SYSTEMIC DISEASES (40), OR MICROGRAVITY (51). INTERESTINGLY, A SMALL SUBSET OF GENES IS IMPLICATED IN ALL ATROPHY MODELS, WHEREAS MOST DIFFERENTIALLY EXPRESSED GENES ARE SPECIFIC TO VARIOUS FORMS OF ATROPHY (40).

IT IS WEI KNOWN THAT NEURAL STIMULATION IS ONE OF THE MOST IMPORTANT DETERMINANTS OF GENE EXPRESSION IN SKELETAL MUSCLES (FOR A REVIEW, SEE Refs. 19 AND 54) AND THIS MAKES IT VERY INTERESTING TO STUDY HOW GENE EXPRESSION CHANGES WHEN NEURAL CONTROL IS REMOVED. IN SLOW POSTURAL MUSCLES SUCH AS SOLEUS, THE LOW-FREQUENCY NEURAL DISCHARGE IS THE ESSENTIAL FACTOR IN DETERMINING AND MAINTAINING THE PHENOTYPE, WHEREAS IN FAST MUSCLES, THE ROLE OF NEURAL CONTROL IS MORE CONTroversIAL (54). Actually, fast muscle fibers receive ~100-fold less neural stimuli than slow muscle fibers do (25).

Denervation causes atrophy and change in fiber phenotype in fast as well as in slow muscles. The molecular mechanisms responsible for these changes are only partially understood; it is, however, clear that atrophy in response to denervation is different in slow and fast muscles. In denervated rat soleus, both slow and fast fibers undergo a very rapid atrophy (17), which is associated with a marked transformation of the muscle toward a fast phenotype (28, 41). In denervated extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of adult rats, atrophy is not less severe, but it affects specifically fast fibers, whereas slow fibers seem more resistant (9, 17). In the same muscles, a progressive shift of myosin heavy chain (MyHC) isoform expression along the transition pathway 2B → 2X → 2A → 1 has been reported (28). In a recent study on rat EDL denervation (21), we have observed early changes in twitch time course that were accompanied by changes in sarcoplasmic reticulum Ca2+-ATPase (SERCA), parvalbumin, and calsequestrin expression. Electron microscopy has shown in rat fast muscle a proliferation of the junctional structures (triads and diads) that occurs a few days after denervation (58, 63). Thus, despite the limited amount of nervous stimuli received, fast muscles also respond to denervation, and the excitation-contraction coupling mechanism seems to be more sensitive than the contractile mechanisms to removal of neural control. Mitochondria and energy production systems are also affected by denervation in fast as well as in slow muscles (43, 72, 79). Evidence is, however, still fragmentary, as each study has focused on a single specific functional or structural aspect.

The development of the adaptive response requires several days or even weeks, and this makes necessary the identification of the precise time frame in which gene expression is studied. For instance, it is known that denervation of fast muscles produces a rapid upregulation of myogenic regulatory factors (MRFs) (6, 69), whereas the gradual transition in fiber type...
occurs more slowly (74). It is also important to take into account that data on denervation are mostly derived from studies on young adult rat muscles, and results obtained in other species (e.g., rabbit) suggest that the denervation-induced changes may be species, muscle, or developmental stage specific (15).

Many genes differentially expressed in denervated muscle have been identified so far (6, 64). However, only one high-throughput study on gene expression in denervation has been published and is focused on comparing gene expression in denervated neonatal rat muscles (16 days after denervation) with gene expression in space flight atrophy (51). A more complete mapping of the expression changes in an appropriate time window might provide information relevant to understanding the mechanisms leading to atrophy and fiber phenotype change after denervation in muscles.

In this study, we extended our previous work (21) on early changes induced by denervation in fast muscles. By means of real-time quantitative PCR (RQ-PCR) and cDNA microarray expression profiling, we followed changes in the expression of ~2,000 genes in murine TA for 2 wk after surgical denervation. In particular, we focused on genes implicated in contrac-
tile and metabolic functions. A few essential genes were further analyzed at the protein level, and the possible functional relevance was also tested with physiological experi-
ments. The results provided the first general timeline of changes in gene expression after denervation in a fast muscle and revealed that removal of neural control determines co-
ordinated and sequential changes in gene expression.

METHODS

Animals. Denervation was performed in adult (3-mo old, n = 32) CD1 mice anesthetized by an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (20 mg/kg). The sciatic nerve was cut unilater-
ally at the level of trochanter. About 0.5–1 cm of the peripheral nerve stump was removed, and the proximal stump was sutured into a superficial muscle to avoid reinnervation and obtain a permanent denervation of the lower hindlimb. At 1, 3, 7, and 14 days after the injury, animals were killed by cervical dislocation. The TA and EDL muscles from the denervated leg were dissected out and utilized for physiological experiments or gene expression studies, the control muscles being the contralateral innervated muscles. The experiments were approved by institutional review boards.

General design of microarray experiments. Microarray experi-
ments were carried out with cDNA targets from whole TA muscles, which were directly labeled during reverse transcription to preserve abundance relationships among messenger RNAs. Total RNA from denervated and contralateral TA was extracted from five mice for each time point. RNA from each muscle was prepared separately, and competitive hybridizations were carried out between denervated and contralateral samples of the same animal. To correct for biases due to the different incorporation rate and emission signal intensity of each fluorophore, we used reversed labeling of denervated and contralateral samples in each experiment, thus carrying out at least two separate hybridizations for each time point and for each animal. For each time point, the best experiments were selected for analysis. Criteria for selection included low background noise and absence of artifacts. The use of contralateral leg muscles is widely accepted in denervation studies. There is, however, evidence of effects of peripheral nerve section on contralateral motoneurons with possible influence on mus-
cles (reviewed by Koltzenburg et al., Ref. 35). To evaluate whether contralateral muscles would undergo significant changes in gene expression during the time course of the experiment, competitive hybridization experiments were performed between day 1 and day 14 contralateral TA samples. Only 23 spots (each probe is represented on the array by 4 spots) and only 1 probe showed variations greater than two times.

Extraction of RNA. Muscles were removed, weighed, cut into pieces, and frozen in liquid nitrogen immediately after each animal was killed. Frozen tissues were dispersed in 5 vol of Trizol (Invitro-
gen, Carlsbad, CA) and homogenized with an ultra-turrax-T8 blender (IKA-Werke, Staufen, Germany). Total RNA was purified according to the standard protocol. We checked for the absence of significant degradation by running an aliquot of RNA in an Agilent Bioanalyzer 2100, using the RNA 6000 LabChip kit (Agilent Technologies, Palo Alto, CA).

Microarray fabrication. The tissue-specific microarrays used for this work (Mouse MuscleArray 1.0) consisted of 9,216 spots representing 2,061 cDNA probes, 1,402 isolated from cultured C2C12 myoblasts and 659 from adult hindlimb skeletal muscles. About six thousand muscle-specific expressed sequence tags (ESTs) were produced at CRCRI from specially designed cDNA libraries, following a strategy already applied to study human muscles (12, 38, 39). This strategy resulted in a nonredundant collection of cDNA clones. Before cloning, cDNAs were fragmented by sonication, and only the very 3'–end of the mRNAs was selected (38). This distinctive feature of our cDNA probes has two main advantages: the cDNA inserts are uniform in size (300–700 bp) and can be amplified with high efficiency; furthermore, hybridization to targets is more specific. All cDNAs considered in this work were sequence verified. Details on microarray printing and postprocessing have been deposited at the Gene Expres-
sion Omnibus (GEO) database (platform accession no. GPL1523).

Microarray hybridization. Total RNA for each sample (8 µg) was reverse transcribed with Superscript II (Invitrogen) and oligo-dt(21) as primer in the presence of either Cy3-dCTP or Cy5-dCTP (Amersham Biosciences, Uppsala, Sweden). Labeled cDNAs were further purified with a GenElute PCR Clean-Up kit (Sigma). Target cDNAs destined for paired analysis (i.e., denervated vs. contralateral) were mixed and precipi-
tated with ethanol and salts. Competitive hybridizations were carried out in a dual slide chamber (HybChamber; GeneMachines, San Carlos, CA). Pellets of purified, labeled cDNAs were dissolved in 40 µl of hybridization buffer (Northern Max, Ambion) and applied on the mi-
croarrays, covered with a 22 × 22-mm glass coverslip. Hybridization proceeded overnight at 42°C by submersion of the chamber in a high-
precision water bath (W28; Grant, Cambridge, UK). At the end of hybridization, slides were dived for 5 min sequentially in buffer A (1X SSC-0.1% SDS) at 65°C, in buffer B (0.1X SSC-0.1% SDS) at room temperature, and finally in 0.1X SSC.

Analysis of expression data. Digital images were generated in a GSI Lumonics LITE dual confocal laser scanner (ScanArray Microarray Analysis software) and processed with QuantArray Analysis software (GSI Lumonics, Ottawa, Canada). Intensity values were processed at the SNOMAD web site (http://pevsnerlab. kennedykrieger.edu/snomadinput.html). Normalized values were calculated for each spot and converted to a logarithmic scale. Final values correspond to log2 ratio of the normalized intensities; positive numbers correspond to RNA overexpressed in denervated muscles. The microarray data have been deposited in the GEO database (series accession no. GSE1893). To identify differentially expressed genes, we performed comparison statistical tests implemented in significance analysis of microarrays (SAM) (68). SAM analyses were performed at the level of cDNA probes, thus grouping replicate data of the same probe (4 spots on the array). The following criteria were adopted to filter the raw data. 1) After visual inspection of MA scatter plots generated by the SNOMAD software, spots with a weak hybridization signal (normalized meanlogint values < −5, which corresponded approximately to absolute intensities <700 in at least 1 channel) were considered not reliable. 2) Empty spots and cDNA probes with a defective signal were not analyzed. The multiclass SAM analysis was performed on a selected set of 2,056 cDNA probes as follows: four
different classes have been set, one for each time point, and genes differentially expressed across all classes (namely, genes differentially expressed in at least 1 class) were selected with a false discovery rate (FDR) < 1%. On the other hand, a one-class SAM analysis allowed selection of genes up- and downregulated at each single time point. Results of the two tests were consistent. Probes associated with higher FDRs are shown in Supplemental Materials (see below).

**Real-time quantitative PCR.** Real-time quantitative PCR (RQ-PCR) based on the SYBR Green chemistry (Applied Biosystems, Foster City, CA) was carried out as described in a previous study (12). Total RNA (pooled individuals) from each time point was reverse transcribed with SuperScript II and oligo-dT(21). Diluted cDNA was amplified in 10 μl of PCR reactions in a GeneAmp 9600 thermocycler, coupled with a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Gene-specific primers were selected with Primer 3 software; sequences of distinct exons were chosen to avoid amplifying contaminant genomic DNA. For each time point, we amplified samples and controls (denervated and contralateral TA) from multiple serial dilutions of the cDNA input. Differences in gene expression were evaluated by a relative quantification method, as described by Pfaffl (55). Values were normalized to the expression of the β2-microglobulin internal reference, whose abundance did not change under our experimental conditions. Normalized ratios were converted in logarithmic scale, and standard deviation was calculated according to the mathematical methodologies described by Marino et al. (42).  

**Supplemental data.** The cDNA sequences, as well as more information about the Mouse MuscleArrays, are available as Supplemental Materials (available at the Physiological Genomics web site). All information relative to this study is available as Supplemental Materials: 1) RQ-PCR data; 2) a full list of differentially expressed genes detected by cDNA microarray analysis; 3) Supplemental Fig. S8 (nuclear genes with mitochondrial functions); 4) additional tables showing expression data of genes with biological functions relevant to the present study; these genes have been grouped according to Gene Ontology categories, and we noted that several muscle-specific genes have incomplete annotation.

In the Supplemental Tables, genes have been ranked according to their FDR: the lower the FDR associated, the higher the significance of the expression data. We identify five different classes: extremely significant genes (*****) with a probability of being a false positive < 1%; moderately significant genes (****) with probability < 5% or (***)<10%; and low significant genes (**) with a probability <15% or (*)<20%. Only genes of the first class (*****) are shown (see Fig. 3 and Table 2).

**Gel electrophoresis and Western blot.** Small muscle fragments from control and denervated TA [3 for each time point, also used for succinate dehydrogenase (SDH) staining and single-fiber dissection] were shattered with a ceramic pestle in liquid nitrogen and dissolved at a concentration of 2 mg/ml in SDS-PAGE solubilization buffer (62.5 mM Tris, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol). MyHC isoform composition was determined by SDS-PAGE on a 4% stacking gel and on an 8% separating gel. Electrophoresis was run for 42 h at low temperature (70 V constant). After the gel run, the protein bands were revealed by Coomassie brilliant blue or silver staining, as specified. MyHC isoform percentage composition was determined by densitometry with a Bio-Rad Imaging Densitometer (GS-670). For the analysis of low-molecular-weight proteins, 12% electrophoresis gels with 4% stacking gels were used. Densitometer (GS-670). For the analysis of low-molecular-weight position was determined by densitometry with a Bio-Rad Imaging blue or silver staining, as specified. MyHC isoform percentage composition was determined by SDS-PAGE and single-fiber dissection. As described by Pfaffl (55), values were normalized to the expression of a standard reference gene, whose abundance did not vary among samples. 2-microglobulin, 1%; moderately significant genes (****) with a probability of being a false positive < 20%; and low significant genes (**) with a probability < 50% or (**) < 100%. The results are expressed as means ± SE unless stated differently. Nonparametric Mann-Whitney test was used for paired comparison between control and denervated muscles at various times. Pearson correlation coefficient was estimated by a least-squares fitting procedure. Myosin composition was assessed by single-fiber gel electrophoresis (66).

**Physiological analysis on intact EDL.** Intact EDL muscles were dissected from the denervated and contralateral legs of seven mice for each time point (7 and 14 days after denervation). Isometric properties were tested in vitro at 37°C. Details of the setup and the registration system have been given previously (47). Muscle responses were recorded via an AT-MIO 16 AD card, and data were analyzed by a virtual instrument created with the LabView computer program (National Instruments, Austin, TX). Twitch parameters, tension (P), contraction time (CT), and half-relaxation time (HRT), were measured. Force-frequency curves were determined by stimulating EDL muscles at 30-60-100-120-130 Hz for 500 ms. Force recorded at each stimulation frequency was normalized to the value at 130 Hz. Fatigue was induced with a 6-min-long protocol consisting of short tetani at low frequency (60 Hz for 300 ms) delivered every 3 s. Force recorded during the fatigue protocol was normalized to the initial value.

**Statistical analysis.** Data are expressed as means ± SE unless stated differently. Nonparametric Mann-Whitney test was used for paired comparison between control and denervated muscles at various times. Pearson correlation coefficient was estimated by a least-squares fitting procedure.
RESULTS

Denervation atrophy in mouse TA muscle. Murine TA is almost entirely composed of fast-twitch fibers, predominantly expressing 2B and 2X MyHC isoforms with a minor component of MyHC 2A, as shown by SDS-PAGE analysis (Fig. 1A). Slow fibers, although identified by cryosection immunostaining in the deep part of the TA (data not shown), did not contain a sufficient amount of MyHC to reach the resolution level of SDS-PAGE (~3%, see Ref. 10). After resection of the sciatic nerve, there was a progressive and considerable atrophy of the denervated TA muscles, reaching ~34% of the contralateral muscles after 14 days (Table 1). The cross-sectional area of the single fibers containing MyHC 2B isolated for mechanical studies showed an even more dramatic reduction (see Fig. 4C).

To study changes in gene expression, total RNA was extracted from denervated and contralateral TA at four time points, namely 1, 3, 7, and 14 days after the surgical treatment. The total RNA content of muscles was not reduced by denervation in the time interval examined (Table 1), as reported by others (72). RQ-PCR experiments clearly demonstrated that muscle atrophy was associated with a strong early induction of the best-characterized muscle-specific ubiquitin ligases, atrogin-1/MAFbx and MuRF-1, although their expression declined (Fig. 2A). Atrogin-1/MAFbx and MuRF-1, although their expression declined (Fig. 2A). Also, the slow MyHC 1 isoform appeared slightly expressed. Also, the slow MyHC 1 isoform appeared slightly expressed. Moreover, the expression of MyHC 2A and myogenin, whereas Myf5 did not change (Fig. 2B).MRF expression. Because expression of myogenin in adult fast muscles is strongly regulated by neural activity (30, 37), we tested by RQ-PCR the expression of all MRFs. Myogenin was in fact the most strongly induced MRF on denervation; mRNAs for MyoD and MRF4 were also upregulated, although not as strongly as myogenin, while expression of Myf5 did not change (Fig. 2B).

MyHC genes. Initially, all mRNAs appeared equally reduced (Fig. 2C). Later on, MyHC 2B and, to a lesser extent, MyHC 2X remained downregulated, whereas MyHC 2A became overexpressed. Also, the slow MyHC 1 isoform appeared slightly induced (Fig. 2C). The relative amount of each MyHC isoform at the protein level was quantified by SDS-PAGE (Fig. 1B). Corresponding to the transitions observed at the mRNA level, MyHC 2A expression was significantly increased, whereas only minor and insignificant decreases of the MyHC 2B isoform were observed during the time interval examined (Fig. 1, A and B).

MyHC isoform composition of denervated TA. RQ-PCR was further used to analyze the expression of the adult sarcomeric MyHC genes. Initially, all mRNAs appeared equally reduced (Fig. 2C). Later on, MyHC 2B and, to a lesser extent, MyHC 2X remained downregulated, whereas MyHC 2A became overexpressed. Also, the slow MyHC 1 isoform appeared slightly induced (Fig. 2C). The relative amount of each MyHC isoform at the protein level was quantified by SDS-PAGE (Fig. 1B). Corresponding to the transitions observed at the mRNA level, MyHC 2A expression was significantly increased, whereas only minor and insignificant decreases of the MyHC 2B isoform were observed during the time interval examined (Fig. 1, A and B).

Microarray and differentially expressed genes. To extend our analyses to other genes relevant for muscle functions, we took advantage of the microarray technology. The Mouse Muscle Arrays used in this study are muscle-specific microarrays that contain a total of 2,061 cDNA probes, mostly representing mRNAs expressed at high or intermediate levels in skeletal muscle (see METHODS). Due to the composition of the probe set, this microarray provided a reliable description of genes coding for proteins involved in contractile and metabolic functions, whereas less information was available about regulatory genes controlling transcription, cellular growth, proliferation, and signaling cascades (see DISCUSSION). Total RNAs from denervated and contralateral TA muscles of the same animal were directly labeled during reverse transcription, and the cDNA target was subjected to competitive hybridizations.

After processing normalized data with a multiclass comparison statistical test, we found extremely significant expression changes (i.e., with FDR <1%; see METHODS) in 83 cDNAs, which were assigned to 71 unique gene products (Fig. 3). The genes relevant for muscle contractile and metabolic functions are listed in Table 2, whereas a full list of all differentially expressed genes can be found in the Supplemental Materials (see METHODS). To confirm the general validity of the microarray results, the expression of several differentially expressed genes was tested by RQ-PCR, with the finding of a good correlation with microarray data (Table 3).

Table 1. Muscle mass and total RNA

<table>
<thead>
<tr>
<th>Time</th>
<th>Muscle mass, mg</th>
<th>Total RNA, μg/mg muscle</th>
<th>Muscle mass, mg</th>
<th>Total RNA, μg/mg muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>63.3±3.8</td>
<td>0.80±0.15</td>
<td>65.8±4.6</td>
<td>0.75±0.06</td>
</tr>
<tr>
<td>3 days</td>
<td>54.5±7.0</td>
<td>1.01±0.16</td>
<td>71.8±8.6</td>
<td>0.60±0.11</td>
</tr>
<tr>
<td>7 days</td>
<td>57.5±7.9</td>
<td>1.02±0.15</td>
<td>73.0±9.1</td>
<td>0.84±0.14</td>
</tr>
<tr>
<td>14 days</td>
<td>45.3±7.1</td>
<td>1.19±0.34</td>
<td>68.8±2.5</td>
<td>0.90±0.10</td>
</tr>
</tbody>
</table>

Muscle mass and total RNA extracted from denervated tibialis anterior (TA) muscles and innervated, contralateral controls ($n = 5$). A pairwise nonparametric test (Mann-Whitney test) has been performed to test the significance of the difference of means among time points. Muscle weight was significantly reduced since 3 days after nerve resection; the yield of total RNA extracted from denervated TA muscles did not change significantly compared with contralateral controls.

Fig. 1. Myosin heavy chain (MyHC) isoform composition of mouse tibialis anterior (TA). A: electrophoretical separation of MyHC isoforms in control (CTRL) and denervated muscles. B: percent distribution of MyHC isoforms in control and denervated muscles obtained from densitometry of the corresponding electrophoretical bands. Values are means and SE; $n = 5$. *$P < 0.05$. 

Physiol Genomics • VOL 25 • www.physiolgenomics.org
Muscle fiber type-specific genes. Microarray analysis showed a significant downregulation of the gene (Myh4) coding for MyHC 2B at day 7, in agreement with results of RQ-PCR (see above). At the same time point, some genes involved in Ca\textsuperscript{2+} kinetics regulation of fast-twitch fibers, such as calcium ATPase-1 (Atp2a1 alias SERCA1) and parvalbumin, showed a reduced expression (see Fig. 3 and Table 2). By contrast, the expression of troponin isoforms did not vary significantly during the first 2 wk after denervation, with the exception of the slow troponin I, which was overexpressed at late stages. Among myosin-binding proteins (MyBPs), MyBP-H was persistently upregulated, whereas MyBP-C isoforms were instead downregulated: the fast isoform progressively, the slow isoform only around day 3. Myomesin 2 was also significantly downregulated.

To complete the analysis of the changes affecting the contractile mechanism, the expression of the Ca-calmodulin-dependent kinase Mlk, which is responsible for regulatory myosin light chain phosphorylation, was assessed by RQ-PCR and found strongly reduced after 7 days (Supplemental Materials). Calmodulin expression was also generally reduced (Supplemental Materials).

To assess whether the changes in myofibrillar gene expression were accompanied by functional changes, the contractile performance of single skinned fibers was analyzed. Despite the dramatic reduction of cross-sectional area (see Fig. 4C), tension (force/cross-sectional area) developed during maximal activation by fast fibers containing MyHC 2B was not reduced (Fig. 4A). The response to activator calcium was, however, decreased, as indicated by the shift to the right of the pCa-tension curve (Fig. 4B). No changes in troponin I and T expression at the protein level were found (see Fig. 4D, III, for troponin I; data not shown for troponin T). Therefore, the reduced calcium sensitivity might be explained by a lower phosphorylation of myosin light chain in view of the decreased expression of calmodulin and myosin light chain kinase.

Because our previous data (21) were derived from denervated rat muscles, and no information was available on denervated murine fast muscles, it was necessary to examine changes in physiological properties after denervation on fast murine muscles. Murine EDL muscles were chosen as more suitable than TA for in vitro studies (see DISCUSSION) and showed that, after denervation, twitch time parameters were progressively prolonged (see Fig. 5A). This variation was similar to that seen in denervated rat EDL (21). Accordingly, the force-frequency curve was shifted to the left, indicating that fusion occurred at lower frequencies (Fig. 5B). Taken together, these functional changes suggest that the altered expression of genes implicated in calcium kinetics (e.g., SERCA1 and parvalbumin) has immediate effects at the protein level and corresponding effects on function. Interestingly, fatigue curves show changes in the initial days after denervation (Fig. 5C). The transitory increase in tension development during the initial phases of repetitive stimulation disappears, and this can be explained by the reduced expression of calmodulin and myosin light chain kinase genes. Actually, the transient enhancement in tension development at the start of a repetitive stimulation is typical of fast muscles and is attributed to phosphorylation of myosin light chain-2 (22).

The atrophy-related genes, called “atrogenes” by Goldberg and co-workers (40, 59), include only a very few muscle-specific proteins: myosin light chain peptides, parvalbumin, and Zasp/Ldb3, a LIM domain-binding protein (40). Our study revealed that genes coding for other muscle-specific proteins including desmin, lamin A/C, myomesin, titin, and tropomyosin-β might be considered differentially expressed, if higher FDRs were accepted in the statistical test (see Supplemental Materials). Among genes with highly reliable changes in expression (FDR <1%), we found some muscle-specific genes...
whose roles in muscle are still controversial, as they share a nuclear and myofibrillar localization. For example, four-and-half LIM domain 1 (Fhl1 or SLIM1), B-crystallin and cysteine and glycine-rich protein 3 (Csrp3 or muscle LIM protein), which are generally more expressed in slow fibers, showed upregulation starting from day 3, whereas myozenin 1 (alias FATZ-1, calsarcin-2), a protein typical of fast fibers, showed downregulation at all time points (see Fig. 3 and Table 2).

Energy production. Reduced transcription of genes involved in ATP synthesis or glucose utilization is a common feature of the rapid atrophy induced by fasting or systemic diseases (40). Ten differentially expressed genes involved in energy production are listed in Table 2, and all were downregulated after denervation. Five genes (Atp5b, mt-Co1, mt-Cytb, mt-Nd2, mt-Nd5) encode for proteins of the inner mitochondrial membrane (4 of them are mitochondrial encoded), taking part in electron transport and/or ATP synthesis, and three genes (Mdh2, Cs, Aco2) encode for enzymes participating in the tricarboxylic acid (TCA) cycle. The mRNAs of several glycolytic enzymes were reduced, with the muscle-specific isoforms of phosphofructokinase (Pfkm) and enolase (Eno3) reaching significant levels (Supplemental Materials). Adenylate kinase and mitochondrial creatine kinase (Ckm2) were downregulated as well (Table 2). Interestingly, Pfkm, enolase, and adenylate kinase (Ak1) localize to the M band, where myomesin is also localized, pointing to the M band as a specific target for denervation atrophy. Myoglobin gene expression also showed significant changes, decreasing at 3 days and increasing at 14 days after denervation. A clear decrease was detected also at the protein level (Fig. 4D, II).

Mitochondrial enzymes. In mammals, genes on the H-strand of the mitochondrial genome are transcribed as a polycistronic precursor molecule that is later processed to give individual mRNAs for 12 different polypeptides. Because all genes in the mitochondrial genome were represented on the array (in most cases with >1 probe), we checked whether the probes that did not reach statistical significance according to the most severe criteria (FDR <1%) also exhibited the same expression profile observed for the four genes described above (mt-Co1, mt-Cytb, mt-Nd2, mt-Nd5). The mRNAs of all 12 mitochondrial products shared a common expression profile, being progressively reduced up to 7 days after denervation (Fig. 6). At 14 days, a
Table 2. Differentially expressed genes identified by cDNA microarrays (partial list)

<table>
<thead>
<tr>
<th>Gene Symbol and Name</th>
<th>Gene ID</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Gene Ontology</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy production</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cox1: cytochrome c oxidase 1, mitochondrial</td>
<td>17708</td>
<td>0.064±0.103</td>
<td>-1.086±0.465</td>
<td>-1.964±0.093</td>
<td>-1.167±0.186</td>
<td>mitochondria, i.m.</td>
<td></td>
</tr>
<tr>
<td>Cytb: cytochrome b, mitochondrial</td>
<td>17711</td>
<td>0.128±0.213</td>
<td>-0.739±0.220</td>
<td>-1.354±0.475</td>
<td>0.188±0.088</td>
<td>electron transport mitochondria, i.m.</td>
<td></td>
</tr>
<tr>
<td>Nd2: NADH dehydrogenase 2, mitochondrial</td>
<td>17717</td>
<td>0.232±0.124</td>
<td>-0.474±0.245</td>
<td>-1.879±0.428</td>
<td>-0.598±0.146</td>
<td>electron transport mitochondria, i.m.</td>
<td></td>
</tr>
<tr>
<td>Nd5: NADH dehydrogenase 5, mitochondrial</td>
<td>17721</td>
<td>0.380±0.225</td>
<td>-0.304±0.325</td>
<td>-1.316±0.440</td>
<td>0.035±0.234</td>
<td>electron transport mitochondria, i.m.</td>
<td></td>
</tr>
<tr>
<td>Atp5b: ATP synthase, beta subunit</td>
<td>11947</td>
<td>-0.087±0.253</td>
<td>-1.370±0.224</td>
<td>-1.181±0.168</td>
<td>0.211±0.236</td>
<td>ATP synthase mitochondria</td>
<td>(33a)</td>
</tr>
<tr>
<td>Aco2: aconitase 2</td>
<td>11429</td>
<td>0.066±0.066</td>
<td>-1.231±0.168</td>
<td>-1.286±0.097</td>
<td>-0.270±0.153</td>
<td>cytosol, TCA cycle</td>
<td></td>
</tr>
<tr>
<td>Cs: citrate synthase</td>
<td>12974</td>
<td>0.218±0.115</td>
<td>-1.085±0.177</td>
<td>-1.244±0.203</td>
<td>-0.498±0.320</td>
<td>cytosol, TCA cycle</td>
<td></td>
</tr>
<tr>
<td>Mdh2: malate dehydrogenase 2, alias Mor1</td>
<td>17448</td>
<td>-0.124±0.167</td>
<td>-1.269±0.497</td>
<td>-1.194±0.407</td>
<td>-0.824±0.215</td>
<td>cytosol, TCA cycle</td>
<td></td>
</tr>
<tr>
<td>Eno3: enolase 3, beta muscle</td>
<td>13808</td>
<td>-0.274±0.093</td>
<td>-2.981±0.230</td>
<td>-1.974±0.185</td>
<td>-2.285±0.180</td>
<td>cytosol, M-band glycolysis</td>
<td></td>
</tr>
<tr>
<td><strong>Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akl1: adenylate kinase 1</td>
<td>11636</td>
<td>0.098±0.102</td>
<td>-1.625±0.321</td>
<td>-1.348±0.259</td>
<td>-0.899±0.175</td>
<td>cytosol, M-band purine metabolism</td>
<td>(38a)</td>
</tr>
<tr>
<td>Clm2: creatine kinase, mitochondrial 2</td>
<td>76722</td>
<td>-0.237±0.115</td>
<td>-1.850±0.568</td>
<td>-1.590±0.356</td>
<td>-1.471±0.302</td>
<td>mitochondria amino acid metabolism</td>
<td></td>
</tr>
<tr>
<td><strong>Muscle-specific proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mybpb: myosin-binding protein H</td>
<td>53311</td>
<td>-0.214±0.205</td>
<td>1.357±0.350</td>
<td>2.094±0.340</td>
<td>1.866±0.242</td>
<td>thick-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Myosin-binding protein C, slow type (8030451F13Rik)</td>
<td>109272</td>
<td>-0.018±0.113</td>
<td>-1.553±0.390</td>
<td>-0.214±0.287</td>
<td>0.621±0.436</td>
<td>thick-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Mybpc2: myosin-binding protein C, fast type</td>
<td>233199</td>
<td>-0.029±0.082</td>
<td>-0.422±0.157</td>
<td>-0.975±0.216</td>
<td>-1.602±0.427</td>
<td>thick-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>MyH4: myosin, heavy polypeptide 4 (MyHC 2B)</td>
<td>17884</td>
<td>0.062±0.145</td>
<td>-0.876±0.378</td>
<td>-2.863±0.426</td>
<td>-0.979±0.646</td>
<td>thick-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Atp2a1: ATPase, Ca++/ Mg++ transporting, fast twitch 1 (SERCA1)</td>
<td>11937</td>
<td>0.183±0.253</td>
<td>-0.757±0.427</td>
<td>-1.777±0.238</td>
<td>-0.523±0.188</td>
<td>sarcoplasmic reticulum regulation of muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Cad1: caldesmon</td>
<td>109624</td>
<td>0.293±0.152</td>
<td>-0.668±0.273</td>
<td>-1.730±0.443</td>
<td>-0.307±0.209</td>
<td>membrane fraction regulation of muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Tnnl1: troponin I, skeletal, slow 1</td>
<td>21952</td>
<td>-0.100±0.162</td>
<td>-0.197±0.311</td>
<td>2.691±3.416</td>
<td>2.888±2.778</td>
<td>thin-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Mhc: myoglobin</td>
<td>17189</td>
<td>0.010±0.392</td>
<td>-0.928±0.475</td>
<td>0.451±0.326</td>
<td>1.594±0.413</td>
<td>cytosol muscle development</td>
<td>(33b,51a)</td>
</tr>
<tr>
<td>Smnp: small muscle protein, X-linked</td>
<td>66106</td>
<td>-0.180±0.110</td>
<td>-0.222±0.214</td>
<td>0.413±0.308</td>
<td>1.573±0.513</td>
<td>M-band, I-band, nucleus muscle contraction nucleus, focal adhesions</td>
<td></td>
</tr>
<tr>
<td>Fhl1: four and a half LIM domains 1</td>
<td>14199</td>
<td>-0.573±0.146</td>
<td>-1.099±0.389</td>
<td>2.051±0.165</td>
<td>2.556±0.274</td>
<td>muscle development</td>
<td>(57a)</td>
</tr>
<tr>
<td>Myoz1: myozin I alias calasin-2 (FATZ-1)</td>
<td>59011</td>
<td>-0.222±0.140</td>
<td>-1.902±0.192</td>
<td>-2.113±0.105</td>
<td>-1.536±0.239</td>
<td>Z-band muscle development</td>
<td>(14)</td>
</tr>
<tr>
<td>Cryab: crystallin, alpha B</td>
<td>12955</td>
<td>-0.946±0.225</td>
<td>1.766±0.241</td>
<td>1.307±0.275</td>
<td>1.652±0.140</td>
<td>Z-disc muscle development</td>
<td>(14)</td>
</tr>
<tr>
<td>Cxrp3: cysteine and glycine-rich protein 3 (MLP)</td>
<td>13009</td>
<td>-2.154±0.121</td>
<td>1.008±0.373</td>
<td>3.430±0.470</td>
<td>2.420±0.323</td>
<td>Z-disc, nucleus myogenesis</td>
<td>(14)</td>
</tr>
</tbody>
</table>

Gene ID, Entrez Gene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene). Expression, mean log₂ ratio ± SD of technical replicates. Values that were significant according to 1-class significance analysis of microarray (SAM) are shown in bold. Gene Ontology, cellular localization (top; i.m., inner membrane) and biological process (bottom). Ref. No., for more information, see REFERENCES.

partial recovery of mitochondrial gene expression occurred. To explain this, we must take into account that variations of mitochondrial mRNAs might reflect not only changes in gene transcription but also the overall density of mitochondria in the muscle fibers. Of note, SDH staining revealed that denervation induced an altered intracellular distribution of mitochondria. In particular, subsarcolemmal mitochondria were reduced compared with controls, while the SDH activity became higher in the intermyofibrillar region (Fig. 7A). A similar pattern of mitochondria location had been observed also in muscles of spaceflight rats (51). Using a well-characterized antibody that reacts only with the 13-kDa component of the complex III.

Physiol Genomics • VOL 25 • www.physiolgenomics.org

Downloaded from http://physiolgenomics.physiology.org/ by 10.220.33.3 on June 20, 2017
Table 3. Validation of cDNA microarrays data by RQ-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene ID</th>
<th>Method</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Probe</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enolase 3, beta muscle</td>
<td>NM_007933</td>
<td>Microarray</td>
<td>$-0.274 \pm 0.093$</td>
<td>$-2.981 \pm 0.230$</td>
<td>$-1.974 \pm 0.185$</td>
<td>$-2.285 \pm 0.180$</td>
<td>MM1-014A09</td>
<td>0.929</td>
</tr>
<tr>
<td>Growth arrest and DNA damage-inducible 45 alpha (Gadd45a)</td>
<td>NM_007836</td>
<td>RQ-PCR</td>
<td>$-0.903 \pm 0.097$</td>
<td>$-3.849 \pm 0.065$</td>
<td>$-3.086 \pm 0.046$</td>
<td>$-3.917 \pm 0.001$</td>
<td>MM1-017A08</td>
<td>0.971</td>
</tr>
<tr>
<td>Myosin-binding protein C, fast type</td>
<td>NM_178067</td>
<td>Microarray</td>
<td>$0.419 \pm 0.197$</td>
<td>$3.831 \pm 0.206$</td>
<td>$2.322 \pm 0.466$</td>
<td>$5.272 \pm 0.270$</td>
<td>MM1-017A08</td>
<td>0.971</td>
</tr>
<tr>
<td>Myosin-binding protein H</td>
<td>NM_016749</td>
<td>Microarray</td>
<td>$2.303 \pm 0.071$</td>
<td>$5.380 \pm 0.001$</td>
<td>$4.648 \pm 0.189$</td>
<td>$7.615 \pm 0.691$</td>
<td>MM1-012A10</td>
<td>0.759</td>
</tr>
<tr>
<td>Myozin 1 alias calsercin-2 (FATZ-1)</td>
<td>NM_021508</td>
<td>Microarray</td>
<td>$-0.222 \pm 0.140$</td>
<td>$-1.902 \pm 0.192$</td>
<td>$-2.113 \pm 0.105$</td>
<td>$-1.536 \pm 0.239$</td>
<td>MM1-002B05</td>
<td>0.911</td>
</tr>
</tbody>
</table>

*Top*: comparison of results obtained with microarray and real-time quantitative PCR (RQ-PCR) for 5 representative genes. Pearson correlation coefficient ($R^2$) has been calculated by comparison between pairs of cDNA microarray (specified probe) and RQ-PCR gene expression values. Scatter plots of RQ-PCR vs. cDNA microarray data are shown at [http://muscle.cribi.unipd.it/microarrays/atrophy](http://muscle.cribi.unipd.it/microarrays/atrophy). All $R^2$ values show a good level of correlation between the 2 methodologies. Gene ID, GenBank database. Microarray, mean log$_2$ ratio ± SD of technical replicates. RQ-PCR, log$_2$ ratio ± intra-assay SD, relative to β2-microglobulin mRNA used as internal reference (see METHODS). Experiments were performed with pooled RNA samples. *Bottom*: comparison of results of RQ-PCR obtained with RNA samples from single individuals and pooled from several individuals (comparison between pooled samples and average of individual samples). Standard RQ-PCR curves were first generated for the nonregulated reference gene (β2-microglobulin) and the target gene (Eno3) from serial dilutions of PCR templates. The mRNA abundance was estimated by interpolating each quantity from the corresponding standard curve. Values are log$_2$ ratio ± SD of 3 replicate experiments. Values in pooled samples are close to the arithmetic average of values exhibited by single individuals. Similar results were obtained with the other 2 genes (adenylatekinase and aconitase).
proteins (7), the proteins of the mitochondrial fraction were examined by Western blot. A reduction of complex III proteins was apparent during the first week but did not reach statistical significance (Fig. 7B).

DISCUSSION

This study represents the first high-throughput analysis of the expression changes after denervation in fast muscles. Although the effects of denervation on skeletal muscles have been the object of many studies over the last 40 years, only one global expression study on denervation (51) has been published. In that case, only one time point was considered, whereas in the present study, the time course of the expression changes of ~2,000 genes was monitored during 2 wk after denervation. Denervation has distinct and, in some cases, opposite effects on slow and fast muscles. Available evidence (see Refs. 19, 54) suggests that the neural stimulation is essential to induce the slow type of gene expression, whereas it is controversial how much it is relevant to determine gene expression in a fast muscle. The present study was focused on the changes induced by the removal of nerve stimulation in fast muscles, and the TA of the mouse was chosen as a model. Before the current study, the response of murine muscles to denervation was not widely studied. Our analysis of the changes induced by the removal of nerve stimulation in fast muscles, and the TA of the mouse was chosen as a model. Before the current study, the response of murine muscles to denervation was not widely studied. Our analysis of the changes induced by the removal of nerve stimulation in fast muscles, and the TA of the mouse was chosen as a model.

Before a discussion of the expression data, some important features of our approach need to be pointed out. First, total RNA was extracted from whole muscle homogenates, and therefore we could not discriminate among changes occurring in specific cell types. This problem is common in expression studies on muscle tissue (24) and cannot be overcome until single-fiber analyses are feasible. Second, the cDNA microarrays (Mouse MuscleArray) chosen for the expression analysis are derived from muscle cDNA libraries, and therefore the probe set is particularly reliable for assessing changes of transcripts typical of muscle cells. Thus the emerging view of muscle plasticity is centered on contractile and metabolic functions, whereas less information can be extracted about other important classes of proteins, like components of the proteasome subunits, membrane channels implicated in electrical activity, or families of transcription factors (see Supplemental Materials). The peculiar features of the cDNA probes on Mouse MuscleArray (see METHODS) make hybridizations more specific compared with arrays using full-length cDNAs. In fact, we found a good correlation between results of microarrays and RQ-PCR for all the transcripts tested (Table 3). To aid in the comparison of our data with other global gene expression studies, the microarray data have been deposited in the GEO database (series accession no. GSE1893).

Within the time window explored, denervated TA showed clear signs of a transition from fast to slow phenotype involving several myofibrillar proteins. Combined microarray and
RQ-PCR analysis indicated a decrease in MyHC 2B mRNA and an increase in MyHC 2A mRNA, in agreement with previous results of ribonuclease protection assays in TA muscles of rats (28). In substantial agreement with changes in gene expression, a significant increase in MyHC 2A protein expression and little variation of MyHC 2B protein were detected 2 wk after denervation. It is not surprising that changes at the protein level are less pronounced than changes at the mRNA level, particularly in view of the long half-life (14.7 days) of MyHC 2B (65). A generally accepted scheme of reversible fiber type transitions assumes that fast-to-slow transforming muscles undergo a gradual replacement of the fast isoform MyHC 2B by MyHC 2A through the intermediate MyHC 2X (53, 60). The RQ-PCR results indicating overexpression of the slow MyHC isoform should be interpreted with caution, as the expression of MyHC 1 is highly dependent on the neural
discharge [see Spangenburg and Booth (61) for a review]. In the rat, a substantial increase in MyHC 1 expression has been observed only 5 wk after denervation (46). Slow fibers in the deep part of TA are more resistant to atrophy than fast fibers in the superficial part (5, 9, 17). Taking into account that, in the denervated soleus, type 1 fibers undergo a massive atrophy (17), one might speculate that slow fibers in fast muscles such as TA have a different sensitivity to neural stimulation compared with slow fibers in soleus muscle, because they are committed during early myogenesis in a nerve-independent way (50), and for this reason they remain relatively insensitive to neural influences. The greater resistance to atrophy of slow fibers might explain why genes typical of slow fibers appear overexpressed without the need to assume a change in gene expression in fast fibers. Aside from MyHC isoforms, the expression of other genes coding for myofibrillar proteins differentially expressed in fast and slow fibers also suggests a fast-to-slow transition: for example, troponin I slow is overexpressed, whereas MyBP-C fast is downregulated.

The fast muscle phenotype is not only determined by the expression of fast isoforms of myofibrillar proteins but also by specific expression of genes implicated in cytosolic Ca\[^{2+}\] regulation. The transition toward a slower muscle phenotype is evident also at this level, as parvalbumin and SERCA1 expression decreases. Among genes coding for proteins involved in excitation-contraction coupling, the gene coding for the γ1-subunit of dihydropyridine receptor (DHPR) showed a significantly increased expression (see Supplemental Materials), whereas mRNA coding for ryanodine receptor (RyR) or other triad components did not vary significantly. The lack of variation of RyR expression is in contrast to the expectations based on electron microscope observations of a rapid doubling of the contact areas between T tubules and terminal cisternae in denervated muscles (58, 63). These ultrastructural findings contrast with the lack of changes in RyR expression (52) in EDL after long-term denervation (25–50 days). A recent paper by Radzyukevich and Heiny (56) shows an increase of mRNA of DHPR in various models of disuse, including denervation of fast muscle. Other studies (57), however, do not report any variations in DHPR expression. A possible explanation of the ultrastructural findings is that triad morphological changes represent a reorganization in response to the early reduction of myofibrillar mass that likely results in only minor transcriptional changes. The changes in gene expression are, on the whole, suggestive of slower kinetics of intracellular calcium, which is consistent with the prolonged time to peak and the leftward shift of the force-frequency curve observed in denervated murine EDL (Fig. 5), a finding that is in agreement with the previous observations in rat denervated EDL (21). The choice to analyze contraction kinetics in vitro in EDL and not in TA was due to the great thickness and the lack of a proximal tendon of the TA, which make impossible a reliable analysis in vitro. Also, a determination of contractile performance of TA in vivo is made difficult when the nerve supply is interrupted and direct electrical stimulation of the muscle is needed. Because of these difficulties, twitch time parameters, force-frequency curve, and fatigue curve were determined in EDL muscles of the same animals used for RNA preparation. EDL
can be considered a good model of TA because it is very similar to TA in its function (they are both foot dorsal flexor muscles without much of a postural role), innervation (they are both innervated by peroneus nerve), and fiber type composition: MyHC isomorph analysis shows that predominant isomorphs were 2X and 2B in control TA with 35 and 65%, respectively (see Fig. 1), and in control EDL with 16 and 81%, respectively.

A dramatic reduction in mitochondrial enzyme activities in denervated muscles has been reported in previous studies (26) (72). Our microarray data also show changes consistent with the view that denervation has pronounced effects on mitochondrial function and point to the transcriptional nature of these effects. In the first 3 days after denervation, all genes encoded by the mitochondrial DNA and several nuclear-encoded mitochondrial genes involved in energy production were markedly downregulated. However, during the progression of atrophy, we observed a tendency to rescue mitochondrial gene expression. After performing a cluster analysis of all genes present on the Mouse Muscle Arrays with proven mitochondrial localization, we selected 50 genes that exhibited similar expression profiles even if they did not reach statistical significance (Supplemental Fig. S8). Among those genes, soluble enzymes (particularly of the TCA cycle) and membrane complexes involved in energy production through oxidative phosphorylation were particularly represented. Interestingly, some molecular components of the permeability transition pore complex, like voltage-dependent anion channel (VDAC) or adenine nucleotide translocase (ANT), were also identified by these criteria.

Distinct gene expression programs may be activated during the progression of atrophy to achieve a coordinated variation of mitochondrial functions. We noted that nuclear-encoded genes are mostly underexpressed 3 days after denervation, while the mitochondrial-encoded genes reach the lowest expression levels after 1 wk (Table 2). Analysis by computational approaches of the promoter region of the selected genes is underway, to identify putative transcription factor-binding sites and modules involved in energy production through oxidative phosphorylation. These were particularly represented. Interestingly, some molecular components of the permeability transition pore complex, like voltage-dependent anion channel (VDAC) or adenine nucleotide translocase (ANT), were also identified by these criteria.

Distinct gene expression programs may be activated during the progression of atrophy to achieve a coordinated variation of mitochondrial functions. We noted that nuclear-encoded genes are mostly underexpressed 3 days after denervation, while the mitochondrial-encoded genes reach the lowest expression levels after 1 wk (Table 2). Analysis by computational approaches of the promoter region of the selected genes is underway, to identify putative transcription factor-binding sites and modules involved in energy production through oxidative phosphorylation. These were particularly represented. Interestingly, some molecular components of the permeability transition pore complex, like voltage-dependent anion channel (VDAC) or adenine nucleotide translocase (ANT), were also identified by these criteria.

In conclusion, the present results show that denervation of murine TA is followed by marked changes in gene expression that appear to be coordinated in the direction of a fast-to-slow transformation and of a reduced metabolic activity, particularly in denervated laryngeal muscles, might regulate but not inhibit satellite cell activation.

TA denervation was followed by upregulation of genes like Csrp3 (see Fig. 3) and Ankrd2 (RQ-PCR data not shown), which, in accordance with previous studies (2, 67), are involved in transition toward slow phenotype. These proteins are normally expressed in slow muscles (13, 75) and can mediate protein-protein interactions through LIM or ankyrin domains, respectively (48, 70). Their possible role, however, remains controversial, because of their dual localization. While in the nucleus, the CRP3/MLP protein may influence the myogenic activities of the MRFs through a direct physical interaction, as demonstrated for MyoD and myogenin (36). On the other hand, CRP3/MLP can bind several cytoskeletal proteins (14), and these interactions could be important for the rearrangement of the Z-disc during redifferentiation of adult muscle fibers (73). More recently, a multiple role has been suggested for the ANKRD2 protein, based on its ability to bind Z-disc proteins (e.g., telethonin) and to act as gene regulator (34). TA denervation also caused upregulation of αB-crystallin, which is known to be more expressed in slow than in fast muscles (13). This small heat shock protein displays chaperone-like properties and might be involved in the remodeling of myofibrillar structures (18). On the whole, the upregulation in denervated TA of genes expressed at nearly undetectable levels in fast muscles supports the view that a real transformation of fast fibers in slow fibers is on the way.
affecting mitochondrial and aerobic-oxidative metabolism. Our
findings that removal of nerve supply has a great impact on
gene expression in fast muscles were unexpected and open
important questions concerning the signals and their intracel-
lar mediation. Electrical activity, neurotrophic factors, and
load might be the signals relevant to explain transcriptional
changes.

1) The membrane electrical activity is an important regulator
of the activity of Ca\(^{2+}\)-dependent transcription factors via
calcium/calmodulin-regulated enzymes such as calcineurin
(phosphatase 2B), CaMK, and myosin light chain kinases. In
particular, activated calcineurin promotes nuclear translocation
of nuclear factor of activated T cells (NFAT), which in turn
controls the expression of slow fiber type genes (44). Cal-
cineurin activity is not only influenced by intracellular calcium
but also by a family of calcineurin-interacting proteins called
filamin-actinin-telothinin-binding protein of the Z disc
(FATZ)/calsarcins (14). Gene silencing of the slow isoform of
FATZ (calsarcin-1) resulted in constitutively enhanced calcineurin
signaling and an excess of type I fibers in skeletal muscles (20). Similarly, the marked downregulation of the
Myoz1 gene (see Fig. 3 and Table 2), encoding the fast isoform
of FATZ (calsarcin-2), might eventually lead to increased
calcineurin activity in denervated TA muscles. Marked slow-
to-fast transformation occurs after denervation in the soleus
muscle (28), whereas only partial fast-to-slow transitions occur in
the denervated TA. The huge difference in the amount of
neural stimulation delivered to a slow compared with a fast
muscle (~100-fold, see Ref. 25) might account for this dis-
crepancy. Interestingly, there are also some common effects,
for example the upregulation of MyHC 2A in both denervated
slow muscles (28) and fast muscles (this study). Common to
fast and slow muscles is the appearance of a new type of
electrical activity: fibrillation appears in denervated rat TA
after 54–55 h (45) and might cause an increase in electrical activity above that experienced by fast muscle fibers in phys-
iological conditions. Among fast MyHC isoforms, the MyHC
2A promoter was shown to be by far the most responsive to
intracellular calcium (1).

2) Neurotrophic factors might contribute to the control of
transcription. The neuromuscular junction is a potential source
of neurotrophic factors (for a review, see Ref. 23). Actually,
recent studies have shown clear differences between the lack of
nerve-muscle contact and the lack of the mere nerve electrical
activity, suggesting a role for the ciliary neurotrophic factor to
blunt muscle atrophy (32).

3) The load experienced by the leg muscles is dramatically
reduced after sciatic nerve interruption. Both flexors and ex-
tensors of the foot were paralyzed, and the load applied by
tonic and phasic activity of ankle extensors on TA disappeared.
In this respect, it is paradoxical to note that several genes
typical of slow muscles found upregulated in the present study
(e.g., Ankrd2, Cryab, Csrp3) were induced in the TA muscle
even after a single bout of eccentric contraction (3). It is
tempting to speculate that those genes are implicated in a
load-related intracellular signaling that would cause remodel-
ing of myofibrillar structures, although the molecular mecha-
nisms by which muscle cells are sensing such mechanical stimuli remain elusive at present. It must be underlined, how-
ever, that reduction of mechanical load is known to shift gene expression toward the fast and not the slow phenotype (29).

ACKNOWLEDGMENTS

We thank Beniamina Pacchioni for management of printing and postpro-
cessing of the Mouse MuscleArrays at CRIIB. We thank Professor Roger A.
Sabbadini for critical reading of the manuscript.

GRANTS

Microarray instruments were purchased thanks to a generous con-
tribution of the Fondazione della Cassa di Risparmio di Padova e Rovigo, Italy,
and a specific grant of the University of Padova (“Attrezzature Scientifiche
collegate a Progetti di Ricerca-2002”). This work has been partially supported by
the Italian Ministry of University and Scientific Research (Grant FIRB to G.
Lanfranchi and C. Reggiani and Grant PRIN to D. Danielli-Betto) and by a
sabbatical year from the National Institutes of Health (HL-63903) to D. Danielli-
Betto. Construction of the Mouse MuscleArrays (TSP B57-3) and studies on
denervated muscles (GSP04289-2A) have been sponsored by the Fondazione
Telethon Onlus, Italy.

REFERENCES

1. Allen DL and Leinwand LA. Intracellular calcium and myosin isoform
transitions. Calcineurin and calcium-calmodulin kinase pathways regulate
preferential activation of the Ila myosin heavy chain promoter. J Biol

regulator of myogenesis, promotes myogenic differentiation. Cell 79:

3. Barash IA, Mathew L, Ryan AF, Chen J, and Lieber RL. Rapid
muscle-specific gene expression changes after a single bout of eccentric
contractions in the mouse. Am J Physiol Cell Physiol 286: C355–C364,
2004.

MT. Patterns of global gene expression in rat skeletal muscle during
unloading and low-intensity ambulatory activity. Physiol Genomics 16:

5. Bobinac D, Malnar-Dragojevi D, Bajek S, Šoi-Vrani T, and Jerkovi R.
Muscle fiber type composition and morphometric properties of denervated

Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valen-
zuela DM, DeChiara TM, Sött TN, Yancopoulos GD, and Glass DJ.
Identification of ubiquitin ligases required for skeletal muscle atrophy.

S-nitrosylation in protein targeting: NADH-cytochrome b5 reductase
requires myristic acid for association with outer mitochondrial but not ER

8. Borisov AB, Dedkov EI, and Carlson BM. Differentiation of activated
satellite cells in denervated muscle following single fusions in situ and in

9. Borisov AB, Dedkov EI, and Carlson BM. Interrelations of myogenic
response, progressive atrophy of muscle fibers, and cell death in dener-

10. Bottinelli R, Canepari M, Pellegrino MA, and Reggiani C. Force-
velocity properties of human skeletal muscle fibres: myosin heavy chain

on cell cycle control in laryngeal muscle. Arch Otolaryngol Head Neck

Trevisan S, Laveder P, De Pitta C, Pegoraro E, Hayashi YK, Valle G,
Angelini C, and Lanfranchi G. Gene expression profiling in dysferli-
nopathy by 10.220.33.3 on June 20, 2017 http://physiolgenomics.physiology.org/ Downloaded from
13. Campbell WG, Gordon SE, Carlson CJ, Pattison JS, Hamilton MT,
and Booth FW. Differential global gene expression in red and white

14. Clark KA, McElhinny AS, Beckerle MC, and Gregorio CC. Striated
muscle cytoarchitecture: an intricate web of form and function. Annu Rev

15. d’Albis A, Goubel F, Couteaux R, Jannot C, and Mira JC. The effect of
denervation on myosin isoform synthesis in rabbit slow-type and
fast-type muscles during terminal differentiation. Denervation induces
differentiation into slow-type fibres. Eur J Biochem 223: 249–258,
1994.
GENE EXPRESSION PROFILE IN DENERVATED MURINE MUSCLE


REFERENCES