Acute molecular response of mouse hindlimb muscles to chronic stimulation


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Submitted 23 January 2009; accepted in final form 15 July 2009

LaFramboise WA, Jayaraman RC, Bombach KL, Ankra DP, Krill-Burger JM, Sciulli CM, Petrosko P, Wiseman RW. Acute molecular response of mouse hindlimb muscles to chronic stimulation. Am J Physiol Cell Physiol 297: C556–C570, 2009. First published July 22, 2009; doi:10.1152/ajpcell.00046.2009.—Stimulation of the mouse hindlimb via the sciatic nerve was performed for a 4-h period to investigate acute muscle gene activation in a model of muscle phenotype conversion. Initial force production (1.6 ± 0.1 g body wt) declined 45% within 10 min and was maintained for the remainder of the experiment. Force returned to initial levels upon study completion. An immediate-early growth response was present in the extensor digitorum longus (EDL) muscle (FOS, JUN, activating transcription factor 3, and immediate-early growth response) with a similar but attenuated pattern in the soleus muscle. Transcript profiles showed decreased fast muscle-specific mRNA (myosin heavy chains 2A and 2B, fast troponins T3 and I, fast myosin 3y) in the EDL versus soleus muscles. Histological analysis of the soleus muscle revealed glycogen depletion without inflammatory cell infiltration in stimulated versus control muscles, whereas ultrastructural analysis showed no evidence of myofiber damage after stimulation. Multiple fiber type-specific transcription factors (tea domain family member 1, nuclear factor of activated T cells 1, peroxisome proliferator-activated receptor-γ coactivator-1α and -β, circadian locomotor output cycles kaput, and hypoxia-inducible factor-1α) increased in the EDL along with transcription factors characteristic of embryogenesis (Kruppel-like factor 4, SRY box containing 17; transcription factor 15; PBX/knotted 1 homeobox 1; and embryonic lethal, abnormal vision). No established in vivo satellite cell markers or genes activated in our parallel experiments of satellite cell proliferation in vitro (cyclins A2, B2, C, and E1 and MyoD) were differentially increased in the stimulated muscles. These results indicated that the molecular onset of fast to slow phenotype conversion occurred in the EDL within 4 h of stimulation without injury or satellite cell recruitment. This conversion was associated with the expression of phenotype-specific transcription factors from resident fiber myonuclei, including the activation of nascent developmental transcriptional programs.

myofiber; myocyte; fiber type; satellite cell; expression profiling; transcription factor; plasticity; myogenesis; contraction; force; chronic stimulation; histology

ADULT SKELETAL MUSCLES subjected to variations in activity or loading exhibit striking phenotype plasticity in their ability to alter contractile and metabolic protein expression over a broad range of transitional and mature myofiber types (26–28, 37, 62–63, 71–73). This phenotype plasticity was originally described in cross-innervation (9), chronic nerve stimulation (71), and denervation studies (41) conducted over 4 decades ago and led to studies of phenotype conversion during overload via synergist ablation (32), exercise (28), chronic stretch (17), and unweighting by hindlimb or whole body suspension (56, 60). Delineation of fiber type transitions required the detection of histochemical changes in myofibrillar ATPase activity under varying pH conditions (41), enzymatic assays of metabolic enzymes (79), or physiological motor unit analysis (24). However, identification of the specific myosin heavy chains (MyHCs) underlying the physiological properties of muscle fiber phenotypes provided definitive proteins and transcripts as precise indicators of the conversion process (3, 6, 68). Comprehensive expression profiling of muscle fiber transformation has now been performed in rabbit and rodent muscles associated with aging (61), hindlimb suspension (87), microgravity (2), injury (80), regeneration (25), and exercise (13, 53). Many of these transcriptional changes have been individually validated at the protein level, but the application of high-throughput mass spectroscopy assays to chronically stimulated fast muscle (23) has recently added a comprehensive proteomics analysis of the myofiber transition process to this extensive database of muscle fiber type plasticity. While these studies have necessarily focused on the outcomes of myofiber phenotype changes in the days to months after the inductive stimulus, little information is available regarding the onset of this process. Specifically, the early molecular events associated with the initiation of the fast to slow transition induced by a defined physiological stimulus regimen have yet to be extensively examined at the molecular level.

Conversion of a fast muscle to a slow phenotype by chronic low-frequency electrical nerve stimulation (CLFS) has been well established as a model to study muscle phenotype switching in vivo (42, 63) and was the focus of the present study. Unilateral hindlimb stimulation offers several advantages. First, the contralateral muscle from the same animal provides an unstimulated control that is genetically identical and subjected to the same circulating plasma hormones and growth factors. Second, the use of neuronal stimulation rather than intramuscular electrodes markedly reduces the risk of muscle damage since the stimulation is indirectly applied and excitation levels can be adjusted to prevent injury. Third, the application of supramaximal neural stimuli provides uniform activation of all motor units and eliminates the complexities associated with fiber recruitment patterns. Fourth, experiments can be designed to measure physiological performance (force per time unit or duty cycle) so that it can be precisely quantified.
and continuously monitored to control the magnitude of the stimulus and minimize the risk of injury. Finally, the phenotypic characteristics of the mouse extensor digitorum longus (EDL) and soleus muscles have been characterized based on histochemical, immunohistochemical, and physiological assays of the different MyHCs and myosin light chains (MyLCs) that distinguish individual fiber types (8, 45, 47). Because of these advantages, this chronic stimulation model was used in the present study to interrogate the underlying acute transcriptional changes associated with the phenotypic remodeling that occurs with increased contractile activity.

In addition to the remarkable plasticity that mature skeletal muscles display to altered functional demands, remodeling often occurs when muscle tissue undergoes activation of an otherwise quiescent pool of resident progenitor muscle cells called satellite cells. After injury, adult skeletal muscles undergo repair and regeneration by the activation of these myogenic satellite cells, which form new myofibers or fuse with existing fibers (12, 31, 57, 78). The degree to which satellite cell activation contributes to muscle remodeling in the absence of injury remains a source of considerable controversy complicated by the fact that experimental models of myofiber adaptation, e.g., nerve or muscle stimulation, endurance or ballistic exercise, and hindlimb loading or unweighting via synergist ablation, may cause varying degrees of trauma, inflammation, and myogenic cell activation as part of the regeneration response (1, 34, 49, 91).

The central hypothesis of this study was that muscle fiber type transformation can occur in the absence of injury and without the activation of satellite cell nuclei. To test this hypothesis, the chronic stimulation model was used, and high-resolution transcriptional profiling was performed to investigate acute changes (4 h) in the mouse EDL muscle, comprised of fast fiber types (MyHC-2B and 2X), and the soleus muscle, containing predominantly slow and 2A fiber types (MyHC-1β/slow and 2A) (45, 47, 92). Gene expression of the stimulated muscles was directly compared with the unstimulated, contralateral muscles for each animal. Physiological responses were continuously monitored to assess the contractile performance of the hindlimb including both the muscle and nerve. Subsequent histological and ultrastructural analyses were used to evaluate the morphological integrity of the target muscles. To delineate transcripts associated with satellite cell activation, separate mouse EDL and soleus muscles were harvested, and primary satellite cell cultures were purified and expanded in culture. Molecular profiles were generated for these cells via microarray analysis both during proliferation as myoblasts and after differentiation into myocytes and myotubes in vitro for comparison with the transcriptional activity of control and stimulated muscles in vivo.

METHODS

Exercise protocol and muscle physiology. All mouse care and experimental protocols were approved by the All University Committee on Animal Use and Care at Michigan State University. Adult male Swiss Webster mice (30–35 g, Harlan, Indianapolis, IN) were maintained in a controlled environment with a 12:12-h light-dark cycle and food and water administered ad libitum. Experiments were performed on mice anesthetized to a deep plane of surgical anesthesia with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Additional doses were administered as necessary by monitoring respiration and heart rate in each animal using a pressure transducer placed on the chest cavity. Core body temperature was maintained at 37°C using a temperature-controlled environmental chamber and monitored using a thermocouple placed in the rectum of the animal. Surgical preparations comprised a small incision (0.5 cm) proximal to the iliac crest to expose the sciatic nerve as previously described (69). In brief, a pair of bipolar hook electrodes was implanted adjacent to the nerve, and the wound was sealed with cyanoacrylate glue. The hindlimb was subsequently immobilized by positioning the animal prone on a Plexiglas platform fitted with a patellar brace to fix the knee. The animal was positioned with the chest wall over an aperture designed to fit a pressure sensor built into the platform to monitor respiration and heart rate. A length of 2.0 silk suture was attached to the Achilles tendon proximal to the calcaneous process, and this ligature was then connected to a force transducer (Grass FT10, Astromed, West Warwick, RI) to record the performance of the gastrocnemius-plantarulus-solus muscle group throughout the stimulation time course. Before each experiment, the voltage-force relation was determined to establish supramaximal stimulation conditions, and the length-tension relation was determined to set the resting length for maximum twitch tension development. Contractions were induced by sciatic nerve stimulation using a Grass S48 stimulator (0.5-ms duration, 2–5 V). Muscles were allowed to rest for 15 min after these preliminary tests to allow for full metabolic recovery at physiological temperatures (86). Supramaximal stimulation was applied at a rate of 10 Hz for 4 h (42, 63, 73). Force was digitally captured using an AT MIO 16E analog-to-digital board and software (National Instruments, Austin, TX) and analyzed using custom MatLab software (MathWorks, Natick, MA) (39). After cessation of the stimulation period, test twitches were elicited via the sciatic nerve to assess nerve viability and to confirm recovery of muscle force in each animal before tissue harvest. Four hours of stimulation were selected as a time frame in which the animals exhibited full recovery after completion of the stimulation protocol. At the end of each experiment, the EDL and soleus muscles were carefully dissected and either flash frozen in liquid nitrogen and stored at −80°C for later analysis of mRNA expression or fixed at resting length in 10% neutral buffered formalin for histological processing.

Histological analysis. Whole muscle specimens from matching stimulated and contralateral resting muscles (n = 5) were removed and fixed at resting length in 10% neutral buffered formalin, embedded in paraffin, and cut in serial sections to a thickness of 5–8 μm. Alternate sections were stained via a standard hematoxylin and eosin protocol versus periodic acid Schiff (PAS) to detect differences in intracellular glycogen levels as an index of muscle activity (24). PAS staining of both experimental and control muscles was performed concurrently in the same coplin jars, and rinsing times were held constant to control for methodological artifacts. The PAS protocol was performed according to the directions specified by the manufacturer (no. 24200, Polysciences, Warrington, PA). In brief, slides containing the muscle sections were deparaffinized by serial washing with xylene and descending concentrations of alcohol to distilled water. Slides were placed in 0.5% periodic acid for 5 min followed by a distilled water rinse (3 times) and then placed in Schiff’s reagent for 15 min. Slides were then washed in 0.55% potassium metabisulfite for 1 min (2 times) and rinsed under gently running tap water for 10 min to allow the color to develop. Acidified Harris hematoxylin was applied to each slide for 30 s as a counterstain, after which the specimens were dehydrated (95% alcohol, 100% alcohol, and xylene) and a coverslip was applied to each slide.

Electron microscopy analysis. Whole muscles from matched stimulated and control animals (n = 3 pairs) were rapidly dissected, mounted on toothpicks at resting length, and placed in 2.5% glutaraldehyde in PBS for overnight fixation at 4°C. Postfixation was performed in aqueous 1% osmium tetroxide for 60 min. Specimens were dehydrated with ethanol (50% ethanol for 15 min, 70% ethanol for 15 min, 95% ethanol for 15 min, and 100% ethanol twice for 15
min) and propylene oxide (2 × 15 min), slowly infiltrated with epoxy resins (Embed 812/Araldite, Electron Microscopy Microscopy Sciences, Hatfield, PA), embedded, and then heated overnight at 60°C. Thick sections (5–10 μm) were obtained from longitudinal cuts along the length of the muscle and stained with hematoxylin and eosin or toluidine blue O. Thin sections (~90 nm) were cut, stained with uranyl and lead salts, and examined using a transmission electron microscope (FEI Philips CM12). For scanning electron microscopy, the specimens were critical point dried, mounted on aluminum stubs, sputtered with gold coat, and examined under a JEOL 6335F field emission gun scanning electron microscope.

**Satellite cell culture model.** The methods for purification and culture of primary muscle cells have been previously described (45). Briefly, muscles of the anterior and superficial posterior compartments were separately dissected, removed aseptically, pooled, and finely minced with iris scissors to form a suspension that was seeded on multiple 100-mm tissue culture dishes and left undisturbed overnight in an incubator at 37°C. Each plate contained 10 ml of growth media composed of Ham’s F10C (calcium: 1.2 mM, Sigma, St. Louis, MO), bovine basic fibroblast growth factor [bFGF (6.6 ng/ml), Sigma], 200 μl chick embryo extract (CEE; Invitrogen, Carlsbad, CA), and 10 μl Fungizone (GIBCO-BRL, Gaithersburg, MD). The muscle suspension was transferred to a 15-ml conical tube on ice, triturated, and vortexed vigorously for 30 s. Tubes were then centrifuged at low speed (Jouan BH-12, 1,000 rpm for 5 min at 4°C), the supernatant was removed, and the pellet was resuspended in growth media for transfer to a 100-mm dish. Plates were incubated overnight at 37°C and received supplemental feeding after 12 h (66 ng bFGF and 200 μl CEE). The entire procedure, beginning with trituration, was repeated each day for 5 days including preplating on plastic plates to remove fibroblasts. Eventually, a mix of purified myoblast clones remained, which were seeded on denatured collagen (0.07% gelatin, Difco, Kansas City, MO)-coated plates. All subsequent media exchanges (every 12 h) involved complete growth media replacement, but without CEE, and these plates were expanded to a density of 0.8 × 10^6 cells/plate. Myoblasts were either harvested for analysis as proliferating satellite cells or rinsed twice in PBS and “switched” to a differentiation media involved complete growth media replacement, but without CEE, and these plates were expanded to a density of 0.8 × 10^6 cells/plate. Myoblasts were either harvested for analysis as proliferating satellite cells or rinsed twice in PBS and “switched” to a differentiation media.

**RNA purification and processing.** Frozen muscle specimens were homogenized and subjected to total RNA purification using the Qiagen RNaseasy Mini Kit and protocol (Purification of Total RNA from Animal Tissues, Qiagen, Valencia, CA) and suspended in nuclease-free water. Cultured cells were scraped in TRIzol (5 ml/100-mm dish, Life Technologies/GIBCO, Gaithersburg, MD), and RNA was precipitated, washed, and resuspended in nuclease-free water (44). Criteria for the inclusion in subsequent in vitro transcription (IVT) assays was a spectrophotometric absorption ratio of 260/280 > 1.8 (NanoDrop, Wilmington, DE) and a RIN value of >8.0 via electrophoretic analysis (Agilent Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA). In vitro transcription of total RNA used the Ambion MessageAmp Premier kit and protocol (Applied Biosystems/Ambion, Austin, TX) starting with 100 ng of purified RNA. Six bacterial control mRNAs (Applied Microarrays, Tempe, AZ) were amplified in parallel to serve as fiducial markers and for intensity normalization across samples after microarray scanning. Confirmation of cRNA diversity was obtained using the Bioanalyzer 2100 to generate an electropherogram for each IVT reaction regarding sample yield, integrity, and size diversity against a laboratory mouse RNA standard and a Universal Human Reference RNA (Stratagene, La Jolla, CA). Evaluation of bacterial sequences indicated that the IVT assay provided 100- to 200-fold amplification, thereby reducing the variability and potential for errors associated with PCR exponential amplification (11).

**Hybridization procedures.** CodeLink fragmentation buffer was added to the cRNA (5 μl buffer/10 μg cRNA) and incubated for 20 min at 94°C. Fragmented cRNA was suspended in hybridization solution such that 250 μl loading volume/array contained 10 μg cRNA. The solution was vortexed, heated (5 min at 90°C), and transferred to ice before being loaded. Each slide contained a removable plastic hybridization chamber with an infusion port that was sealed immediately after loading. Microarrays were placed in a mixing incubator (Innova 4080 Shaking incubator, New Brunswick Scientific, Edison, NJ) for 18 h and 300 rpm at 37°C. CodeLink arrays (Uniset Mouse 1 Bioarrays, Mouse Whole Genome Bioarrays) were selected because of their high sensitivity and low error rate compared with other platforms, especially regarding transcripts expressed at low copy numbers (11, 76). After hybridization, the array chambers were removed, and the slides were placed in a reservoir containing 0.75× TNT [0.1 M Tris-HCl (pH 7.6), 0.15 M NaCl, and 0.05% Tween 20] for a 1-h high-stringency wash at 46°C. Arrays were incubated in Streptavidin Alexa fluor 647 (Molecular Probes) for 30 min (0.2% Alexa fluor 647 in 0.1 M Tris-HCl, 0.15 M NaCl, and 0.05% NEN blocking reagent-PH 7.6, Perkin-Elmer, Boston, MA). Slides were subsequently washed (1× TNT for 5 min each of 4 washes at room temperature and then 0.1% SSC and 0.05% Tween 20 for 30 s at room temperature) and dried by low-speed centrifugation.

**Scanning and data processing.** Slides were scanned using a 4000B GenePix scanner (Axon Instruments, Foster City, CA) calibrated before use. Laser scanning parameters were set at 635 nm, the photomultiplier tube voltage was 600, and analysis of the array image was configured using the CodeLink Expression Analysis software (version 5.0). Data for each spot were determined as intensity per pixel within the probe zone. Data for each array were then generated as both raw intensity values and normalized for the large dynamic range by dividing each spot by the overall median intensity value for that array. Values derived from questionable spot profiles and all manufacturing errors designated by the CodeLink manufacturing spot report were removed from further analysis. The threshold for each array was subtracted as background from the normalized values for that array, and these values were subjected to statistical analysis. Because of the small size of these muscles, four of seven matched pairs of individual EDL muscles and five of seven matched pairs of soleus muscles passed all of the rigorous requirements (RNA yield and quality, IVT yield and profile, and hybridization and scanning criteria) to undergo statistical analysis. Satellite cells from three mice were separately expanded under proliferation and differentiation conditions and passed all requirements for statistical evaluation. To eliminate batch processing effects across the large number of arrays, each set of EDL, soleus, or satellite cell samples was run from IVT through hybridization and scanning in a separate batch. Thus, a batch contained control and stimulated muscles or proliferating and differentiated satellite cells.

**Statistical analysis.** Data processing followed recommendations for microarrays using paired sample comparisons. The bacterial standards provided internal controls spanning the dynamic range of the array as a basis for comparison of all processing steps from the IVT reaction through the acquisition of final data. Data were subjected to ANOVA testing to determine whether the stimulation protocol had a significant effect on gene expression within each muscle group and whether satellite cell transcripts differed between proliferation and differentiation conditions. Subsequent post hoc analysis was performed on individual transcripts after removal of 1) transcripts that remained “off,” i.e., at threshold levels, in both control and stimulated muscles or in both proliferation and differentiation states for satellite cells and 2) cDNA clones and transcripts lacking a functional gene classification. Correction for multiple testing to minimize the false discovery rate used methods specifically adapted for microarray analysis (66). Differentially expressed genes were classified into functional groups based on the National Institute of Allergy and Infectious Diseases database for annotation, visualization, and integrated discovery.
(DAVID), and the results were crossed with the GeneCards and GENATLAS databases between July 1, 2006 and December 1, 2008 (21, 67) and by manual curation of the primary literature. Ingenuity Pathways Analysis (IPA software version 7.0, Ingenuity, Redwood City, CA) was used to compare the gene expression results against the IPA library of canonical functional biological molecules and pathways. Transcripts were expressed as fold changes to simplify presentation, but statistical testing was performed on the median normalized array intensity values after correction for the individual array background values. The entire compendium of raw intensity and median normalized data for all probes is provided in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/) under the series record GSE14421.

RESULTS

Muscle physiology. The force recorded from the Achilles tendon for a single twitch (0.5-ms stimulation) was $1.4 \pm 0.1$ g force/g body wt. This represented the net contractile activity of anterior and posterior muscle groups contracting in opposition across the ankle joint. The larger cross-sectional area of the posterior compartment resulted in a net positive force vector causing isometric shortening of the posterior compartment of the hindlimb. The force transients are shown in Fig. 1. The exercise protocol used stimulation at 10 Hz, consistent with previous studies using this technique. Under these conditions, the stimulation protocol produced initial twitch peak values of $1.6 \pm 0.1$ g force/g body wt but decreased by $\sim 45\%$ within 10 min and was maintained at this level for the rest of the stimulation time course (Fig. 1A). Concomitant with this early decrease in force output was an increase in half-relaxation time, but this value recovered progressively toward its initial value by the end of the exercise period (Fig. 1A). There was no mechanical activity detected in the contralateral unstimulated limb as determined by both manual palpation and tension recordings in control experiments. Single twitch profiles acquired at the onset and end of stimulation are shown for a representative subject in Fig. 1, B and C.

Histology. Hematoxylin and eosin staining revealed no significant differences in the general morphology or cellular integrity of each pair of matched muscles whether subjected to stimulation or serving as the contralateral control (Fig. 2, A and B). Specifically, there was no visible evidence of myofiber damage, including disruption of sarcomere organization or infiltrating inflammatory cells, in cross sections obtained from either control or stimulated muscles. Differences in PAS staining were evident among the stimulated EDL muscles, which demonstrated a marked decrease in overall staining intensity (Fig. 2D) compared with the contralateral unstimulated muscle from the same animal (Fig. 2C), consistent with decreased glycogen content. In both control and stimulated muscles, there was a mosaic staining pattern that became more pronounced in the stimulated muscles, suggesting that both initial glycogen levels and glycogen depletion rates were not uniform among all

Fig. 1. Contractile characteristics for the stimulation time course. A: temporal changes in twitch force normalized to total body weight (in g/g body wt) and the associated changes in half-relaxation time (in ms) throughout a representative experiment reported in 10-min intervals. There was an initial decline in peak twitch force output and a concomitant prolongation of half-relaxation time after the onset of stimulation. Data are presented as means $\pm$ SE and were averaged for each 10-s period every 10 min over the time course. B and C: twitch at the start of the experiment (second twitch; B) and the next to last twitch at the end of the experiment (C) for the comparison of changes in the twitch profile throughout the duration of the experiment. Normalized force is presented on the ordinate, and time (in ms) is presented on the abscissa.
fast myofiber types, as has been previously demonstrated in other studies (24, 40) in both cats and rats.

**Ultrastructural analysis.** Longitudinal thick sections of stimulated versus unstimulated muscle specimens revealed morphological similarity between the paired muscles of each animal with no obvious signs of inflammation, damage, or necrosis. Electron microscopic analysis of thin sections demonstrated normal membrane and myofibrillar architecture, including intact z-bands from both unstimulated and stimulated EDL muscles (Fig. 3, A and E). There were unusual accumulations of mitochondria near the end of EDL muscle fibers in some but not all animals, but this finding was consistent in both control and stimulated muscles (Fig. 3, B and F). Figure 3C shows a satellite cell from an unstimulated muscle identifiable by the heterochromatic nucleus and absence of myofilaments within the extensive membrane-bound cytoplasm, whereas the satellite cell nucleus from the stimulated muscle shown in Fig. 3G is similarly heterochromatic with minimal cytoplasm contained within its own cell membrane. The resident myofiber nuclei populating these muscle fibers were similar in appearance and typically located in juxtaposition to the sarcolemma, consistent with the morphology of normal skeletal muscle fibers (Fig. 3, D and H).

**Fiber type-specific transcripts.** Four hours of muscle stimulation caused alterations in EDL gene expression in a direction indicative of long-term molecular and protein transformation from a fast to slow phenotype (20, 23, 63). The pattern included decreased MyHC-2B and -2A, fast-phosphorylatable MyLC, fast troponins T3 and I, and muscle creatine kinase in stimulated versus control muscles (Fig. 4). Parvalbumin was also significantly decreased after EDL stimulation [control: 1,161 ± 285 vs. stimulated: 824 ± 1.65 intensity units (IU), means ± SD, P < 0.04], consistent with its role as a calcium-binding protein expressed at highest levels in fast muscles (63). Each of the animals demonstrated a substantial increase in MyHC-1β/slow expression in stimulated EDL compared with control muscles. At the same time, transcripts for troponin C-slow and T1-slow and tropomyosin 3y were increased, consistent with the exercising muscle undergoing conversion toward a slow phenotype within the time frame of the stimulation protocol. These shifts in phenotype-specific transcripts were not significant in the soleus muscle, although the decreases in fast fiber-specific transcripts followed a similar pattern in that muscle. The data indicated that the 4-h stimulation protocol induced phenotype transformation in the muscle fibers of the EDL muscle at the molecular level for transcripts encoding myofibrillar proteins characteristic of slow myofibers, including MyHC and troponin species not typically expressed in EDL fibers.

Unstimulated EDL muscles expressed abundant levels of transcripts for glycolytic enzymes as a signature of fast muscle fibers, as reflected in phosphofructokinase transcript levels that were four times higher than isocitrate dehydrogenase, whereas soleus transcript levels for these two enzymes were nearly identical. Specifically in the EDL, muscle forms of five critical glycolytic enzymes [enolase 3 (ENO3; 966 ± 194 IU), phosphoglycerate mutase 2 (PGAM2; 816 ± 204 IU), phosphofructokinase (PFKM; 603 ± 54 IU), glucose phosphate isomerase 1 (GPI1; 250 ± 38 IU), and glycogen phosphorylase (PYGM; 155 ± 39 IU)] were expressed at levels approaching that of myofibrillar proteins. Stimulation caused a decline in the expression of transcripts for each of these enzymes, but only the changes in PFKM were statistically significant (P < 0.02) despite this common trend. It was interesting to note that in contrast to all other glycolytic enzymes, hexokinase 2 transcripts increased (control: 150 ± 35 vs. stimulated: 306 ± 67 IU, P < 0.006), as has previously been reported to occur transiently using CLFS in fast muscles (85). These data indicated that chronic stimulation of the EDL muscle resulted in a trend toward a reduction in transcripts encoding glycolytic enzymes consistent with a shift from a fast to slow phenotype. Transcripts for these glycolytic enzymes were present in the control soleus muscles at levels approximately half of those measured in the EDL muscles (ENO3: 460 ± 78 IU, PGAM2: 293 ± 32 IU, PFKM: 349 ± 53 IU, GPI1: 172 ± 8, and PYGM: 80 ± 20 IU), as established for slow muscles dominated by oxidative enzymes as a source of metabolic energy production. No significant changes occurred among these enzymes after stimulation.
Early response genes. There was an “immediate-early growth response” or “stress-adaptive response” pattern indicative of a primary reaction to extracellular stimulation particularly within the EDL muscle, where 11 transcripts reached levels 1.5- to ~10-fold of normal values (Fig. 5). The response within the soleus muscle was comparatively smaller, with five transcripts elevated above 1.5-fold. FOS and JUN-B have been defined as canonical genes of the early response pathway and were similarly elevated in both EDL and soleus muscles, whereas Dmel/homer1 (HOMER) and early growth response 2 (EGR2) were significantly increased in the EDL muscle (Fig. 5) (16, 55). In addition, activating transcription factor 3 (ATF3), B cell translocation gene 2 (BTG2), musculoaponeurotic fibrosarcoma oncogene (MAFK), immediate-early response 5 (IER5), ephrin A1 (EFNA1), JUN protooncogene related D1 (JUND), and heterogenous ribonucleoprotein D (HNRPD) have been associated with both an immediate-early gene response as well as a stress response, and these transcripts were all increased in the EDL muscle (Fig. 5).

Transcription factors. Gene ontology (GO) and pathway analysis of differentially expressed genes revealed that “transcriptional regulators” was the highest ranked category under the GO term “molecular function.” The most significant “molecular and cellular functions” among these transcription factors derived from the GO category of “cellular development” (28 genes) with several genes overlapping the classification of “cell growth and proliferation” comprising 39 genes significantly overrepresented among all transcription factors (IPA version 7.0: \( P < 5.97 \times 10^{-8} \) to \( 2.58 \times 10^{-2} \)). Genes within these categories were further classified by manual curation of the primary literature.

Among the altered transcription factors and cofactors were several transcripts that have been previously implicated directly or indirectly in fiber type transformation. Intensity values for these 11 genes were significantly elevated in the EDL muscle, whereas 2 genes were increased in the soleus muscle (Fig. 6). Peroxisome proliferator-activated receptor (PPAR)-\( \gamma \) and its coactivators, PPAR-\( \gamma \) coactivator (PPARGC)-1\( \alpha \) and...
PPARGC-1α, have been found to play a central role in mitochondrial biogenesis, inflammation, and metabolism (65, 88), and a recent study (33) in knockout mice has established PPARGC-1α as a critical factor in the formation and transformation of the myofiber phenotype. All three genes were significantly increased in the EDL muscle by the stimulation paradigm (Fig. 6). Similarly, eyes absent 1 homolog (EYA1) and tea domain family member 1 (TEAD1) have been associated with muscle fiber type development and transformation and were substantially increased in the stimulated EDL muscle (Fig. 6) (29, 30, 83). The calcium/calcineurin nuclear factor of activated T cells (NFATc) transcription complex has been reported to modulate phenotype specification during both muscle development and fiber transformation, and both NFATc1 and NFATc4 were significantly increased in the EDL muscle (43, 52, 54). Hypoxia-inducible transcription factors have been implicated in fiber type transformation through an impact on metabolic genes (74, 81) or through an interaction with circadian clock genes (14, 51). Transcripts for hypoxia-inducible factor (HIF)-1α, a master transcriptional regulator of the hypoxic response, and HIF-3α were significantly increased in the EDL muscle (Fig. 6), whereas circadian locomotor output cycles kaput (CLOCK) transcript levels increased along with its modulator, the basic helix-loop-helix domain containing B2 (BHLHB2) gene (Fig. 6).

A surprising number of the transcription factors significantly elevated in this study have been characteristically associated with functional roles during embryogenesis, mesodermal pattern formation, and somitogenesis under the classification of “growth and differentiation” based on IPA pathway analysis (Fig. 7). For example, Kruppel-like transcription factors (KLF4 and KLF5) (18), SRY box-containing genes (SOX 17) (64), transcription factor 15 (TCF15) (35), embryonic lethal, abnormal vision (ELAV) (15), and tuberous sclerosis factor 1 (TSC1) (59) were significantly increased in the stimulated but not resting EDL (Fig. 7; not all shown). In addition, PBX/
knotted 1 homeobox (PKNOX1) binds the E2A/myogenic factor complex in competition with inhibitor of DNA binding (ID) during myogenesis (22), and both were elevated within the stimulated EDL muscle (Fig. 7). Figure 7 also shows significant changes in CRIPTO and TWISTED as mouse homologs for Drosophila genes critical to dorsal-ventral mesodermal pattern formation during gastrulation, although these gene products are not transcription factors (48, 89). The stimulated soleus muscle also exhibited the activation of a small number of developmental transcripts but within a subset of factors that demonstrated little overlap to the EDL muscle.

Satellite cell gene expression in vitro and in vivo. A previous study (45) has established that primary mouse hindlimb muscle satellite cells differentiated into myocytes and myofibers that coexpressed MyHC-1\(/\)H9252/ -2A in culture by PCR and immunocytochemistry. In the present study, both MyHC-1\(/\)H9252/slow and -2A transcripts were significantly increased in differentiated myocyte cultures along with muscle creatine kinase based on microarray analysis, thereby corroborating previous PCR findings (Table 1). The microarray methodology provided the capability to identify a large number of additional phenotype specific transcripts that were found to be significantly increased in our differentiated myocyte cultures, including fast phosphorylatable MyLC, troponins C, T1 and T3, skeletal muscle actin, and dystrophin (Table 1). Myogenin was also significantly higher after myocyte maturation and myotube fusion compared with proliferating satellite cells in the present study, as has been reported previously (12, 91). Overall, 412 different transcripts were detected at significantly different levels between the proliferating satellite cells and differentiated muscle cell cultures.

Transcripts significantly elevated in the proliferating satellite cell-derived cultures compared with the differentiated myocytes comprised a large number of genes associated with cell cycling, including multiple DNA polymerase-\(\beta\),-\(\epsilon\), cyclin A2, B2, C, and E1, cyclin-dependent kinases 1 and 4, and cell
Table 1. Primary muscle cell cultures

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Mean values indicate the median normalized expression intensity values ± SD for 3 independent satellite cell harvests from the anterior and posterior compartments of the hindlimb. Myoblast cultures were studied under high-serum conditions supporting proliferation and were switched to low-serum conditions to induce myocyte differentiation and myotube formation. CDK, cyclin-dependent kinase; CDC, cell division cycle. All values were statistically significant at the delineated P value including a cutoff of 1.5-fold change.

The results obtained in this study revealed that 10-Hz stimulation of the murine hindlimb for 4 h produced MyHC transcripts in the EDL muscle, reflecting the molecular onset of the conversion from a fast to slow phenotype. To our knowledge, this is the earliest time point in a chronic stimulation model demonstrating the initiation of these phenotype changes in contractile protein transcripts. In contrast to the EDL muscle, the stimulated soleus muscle, with an intrinsically slower myofiber composition, exhibited comparatively few phenotype-specific changes after stimulation. The chronic stimula-
tion protocol produced an increase in the EDL muscle of transcription factors previously implicated in myofiber phenotype conversion in other experimental models, including EYA1, TEAD1, and CLOCK and members of the NFAT, PPAR-γ, and HIF-1 gene families. Surprisingly, multiple genes characteristically associated with morphogenesis and mesoderm pattern formation were concurrently activated in the stimulated EDL muscle. Finally, the transcriptional response of the stimulated muscles bore little similarity to the expression profile of cyclins and myogenic factors detected in parallel proliferating satellite cells, nor were there increases in other established in vivo satellite cell markers (PAX3 and PAX7) (12, 91). The absence of indicators of satellite cell activation and the lack of transcripts associated with injury and inflammation of the stimulated EDL muscles suggested that the molecular response originated within resident nuclei of the differentiated myofibers. Histological and ultrastructural analyses confirmed that the stimulated muscles showed no sign of damage, inflammation, and/or regeneration, adding further support to the hypothesis that mature skeletal muscle nuclei retained the plasticity to re activates otherwise quiescent transcription programs typically associated with development and morphogenesis.

**Chronic stimulation model.** Chronic electrical stimulation has been used broadly for the activity-induced conversion of muscle phenotype, and unilateral sciatic nerve stimulation has been used in acute and long-term studies of both unanesthetized and sedated animals (65, 73). The absence of quantitative information regarding force output and mechanical loads in most of these studies prevented a comparison of the model across laboratories as well as the evaluation of parameters such as energetic demands and calcium handling. The present study continuously monitored mechanical performance from individual Twitches throughout the entire time course with muscles held at resting length and under loaded conditions, thus optimizing the maximal initial force output and standardizing the physiological readout for the working muscle preparation across all of the subjects. Supramaximal stimulation intensities were applied to ensure that all motor units excited by the sciatic nerve were activated during the stimulation protocol. The recovery of initial force after the stimulation protocol indicated that no profound neuronal or mechanical damage occurred within the stimulation paradigm, as confirmed by the histological and ultrastructural analyses. In addition to mechanical output, the physiological homeostasis of each animal was monitored with respect to body temperature, respiration, and heart rate to maintain consistency both within and among all experiments.

Sciatic nerve stimulation at 10 Hz caused glycogen depletion among EDL myofibers based on the diminution of PAS staining versus unstimulated muscles. There was no indication of tissue or motor nerve damage based on poststimulation recovery to initial force values, microscopic evaluation of hematoxylin and eosin-stained specimens, and ultrastructural analysis using scanning electron microscopy. These findings were consistent with the microarray results, which revealed no significant expression of markers associated with injury or inflammation in the muscles. An obligatory role for muscle injury and fiber replacement in the process of fast to slow phenotype conversion remains uncertain. Fiber degeneration and regeneration have been reported in chronically stimulated rabbit fast muscles (49) but did not occur in rat fast muscles subjected to the same protocol (20). In the absence of physiological data from these other studies, we can only speculate as to whether these results were attributable to species differences or differential mechanical demands on the muscles in those studies. In other studies where contractile forces were quite large, significant damage has been noted. For example, Yamasaki et al. (90) demonstrated damage to rat masseter muscles stimulated in the same time frame as the present study. Based on their physiological measurements, the masseter muscles generated five times the net force output compared with the hindlimbs in the present study. Thus, differences in mechanical strain associated with the higher magnitude of force generated by the masseter muscles may account for the injury associated with chronic stimulation in some cases. Precise delineation of the minimal contractile activity and activation time required for the...
induction of phenotype remodeling remains to be defined, but potentially confounding effects of injury, inflammation, myogenic cell activation, and other factors may be eliminated by establishing these parameters.

The electrode was positioned on the sciatic nerve to activate both the anterior and posterior hindlimb muscle compartments containing the EDL and soleus muscles, respectively. While previous studies have demonstrated the efficacy of the stimulation protocol for the transformation of fast muscles, the phenotype conversion of the soleus muscle typically required high-frequency tetanic stimulation (~100 Hz) plus the removal of tonic activity by anatomic or functional denervation (27, 63, 73). Thus, the changes observed in the stimulated soleus muscle may have reflected an adjustment toward a slower phenotype within the limited fast myofiber complement of MyHC-2X fibers (45, 47, 92). However, it is also important to consider that the EDL muscle differs from the soleus muscle in both lower mitochondrial density and capillary-to-fiber ratios (19). Therefore, we cannot rule out that the higher metabolic cost of contraction in the EDL muscle contributed to the observed differences. It is also possible that the soleus muscle response reflects its smaller contribution to the synergistic muscles of the posterior compartment in contrast to the EDL muscle, which contributes relatively more of the output of the anterior compartment. Based on the present results, additional studies will be required to delineate the precise physiological signaling mechanisms underlying the differential response of the EDL and soleus muscles.

**Fiber type transformation.** Prototypical fast to slow phenotype conversion of MyHCs in response to chronic stimulation has been detected in transcripts as early as 1 d after stimulation onset with RNA and protein changes reaching a steady state around 35 d (38, 82). It has been established that the chronic molecular adaptations to spaceflight and hindlimb suspension undergo rapid reversal (3.5–4 h) when exposed to chronic stimulation. It has been established that the changes in myofibrillar proteins were not detected (2). In this study, decreased transcripts for fast muscle contractile proteins (MyHC-2B and -2A, fast phosphorylatable MyLC, and fast troponin isoforms I and T1) were observed after only 4 h of stimulation along with increased slow muscle specific transcripts (MyHC-1β/slow and slow troponin C and T1). The rapidity of this response was reflected in the fact that it occurred within the same time frame as the immediate-early growth response typically observed shortly after a cellular stress. These data emphasized the remarkable lability of the transcriptional programs controlling adult myofibrillar proteins despite their abundant contribution to the overall anatomic muscle hierarchical structure and relatively long half-life within myofibers (7–10 d) (5, 38). There was a concomitant decline in transcripts encoding glycolytic enzymes, consistent with the shift in myofibrillar proteins. While the pattern of these changes in metabolic enzymes was consistent and the magnitude was substantial, the variability was sufficiently large so that few of these changes achieved statistical significance. This may reflect a wider variability in the transcriptional control or functional reserve of these proteins versus that of the myofibrillar apparatus but fits the overall pattern observed in the contractile proteins of a rapid shift in EDL fiber phenotype.

Transcripts associated with phenotype transformation were detected without satellite cell activation as indicated by 1) the lack of overlap of stimulated EDL and soleus expression profiles with transcripts expressed in parallel proliferating satellite cell cultures and 2) the absence of changes in transcripts indicative of in vivo satellite cell activation, including myogenic determination factors (MEF2A, MEF2B, MEF2C, Myf6, and myogenin) and PAX transcription factors (PAX3 and PAX7). These satellite cell markers were present at low levels in both control and stimulated muscles but were not altered by stimulation. Controversy exists regarding the role of satellite cells in phenotype conversion. The application of antigravid irradiation has been shown to inhibit satellite cell proliferation and fusion but did not alter the process of fast to slow phenotype conversion in the EDL muscle of mice subjected to synergist ablation (70), whereas significantly diminished fiber type transformation occurred in gamma-irradiated rat tibialis anterior muscles after chronic stimulation (50). The results of the present study agree with those obtained in the mouse EDL muscle via synergist ablation, albeit in an earlier time frame: that satellite cell activation was not a prerequisite for the induction of transcripts for contractile protein phenotype conversion.

An unexpected finding was the detection of several transcription factors and coactivators previously observed among various cell types associated with morphogenesis, somitogenesis, mesodermal patterning, and lineage differentiation. These transcription factors included multiple members of the Kruppel family (KLF4, KLF5, and GLI5), SOX 17, PKNOX1, ID, TCF15, and ELAV (18, 22, 35, 59, 64) and other transcripts characteristic of tissue formation and organogenesis (CRIPTO, TWISTED, and ZYXIN) (36, 48, 89). All were present at significantly increased levels in stimulated EDL muscles. The presence of these transcripts indicated that unique developmental pathways were reactivated in the myonuclei of otherwise differentiated myofibers by the stimulation paradigm. These results raise other interesting biological questions regarding the capacity for reprogramming of mature myonuclei within differentiated myofibers. For example, is there a unique subset of nuclei apart from the satellite cells but within the mature muscle myonuclear population that may undergo reprogramming associated with the expression of slow muscle transcripts? Mechanistic studies will be required to establish both the role of these transcription factors as well as to locate the precise nuclear domains where they were present. Nevertheless, these data provide evidence of unique plasticity in the nuclei of mature muscles to recapitulate or reprogram a pattern of expression of factors typically associated with early embryogenesis.

**Phenotype conversion transcription factors.** TEAD1, PPARGC1α, and NFATc1 have separately been implicated in direct transcriptional activation of the fast to slow phenotype switch, and each was elevated by 4 h of chronic stimulation. The overexpression of TEAD1 under the control of the muscle creatine kinase promoter in transgenic mice increased the population of MyHC-1β/slow fibers at the virtual exclusion of MyHC-2X fibers (83). TEAD1 binds to an A/T-rich element critical for MyHC-1β/slow transgene expression in response to mechanical overload. PPARGC1α binds with MEF2C to induce a transcriptional cascade including the coactivation of several nuclear factors (nuclear response factors 1 and 2) leading to the activation of mitochondrial transcription factors, e.g., TFAM (33). The overexpression of PPARGC1α in transgenic mice increased mitochondria numbers accompanied by
increased slow and type 2A fiber numbers. The transcriptional basis for an effect of NFATc1 on myofiber transdifferentiation has been obtained predominantly from transfection experiments in cultured myotubes that undergo a calcium ionophore-induced fast to slow phenotype shift. NFATc1 translocated to the nucleus under the influence of calcium transients, where it bound to a consensus sequence in the MyHC-1β/slow promoter in a complex including MEF2D, MYOD, and p300 (54). The finding that each of these factors increased in the stimulated EDL muscle was consistent with the initiation of processes leading to the established end result of chronic stimulation, i.e., wholesale remodeling of the myofiber structure from mitochondria to sarcomeres. The present study indicated that these transcriptional programs were acutely activated within the first 4 h of stimulation.

Transcription factors with indirect effects on myofiber phenotype were significantly increased by chronic stimulation in the EDL muscle, including CLOCK, PPARGC1β, and HIF-1α. The recent discovery of a circadian-regulated muscle-specific transcriptome associated with mitochondrial number and metabolic function (51) suggested that significant changes in the CLOCK gene associated with chronic stimulation play an important role in phenotype conversion. PPARGC1β was one of those circadian-regulated genes but also played a role in the basic energetic balance of muscles expressing MyHC-1β/slow, since PPARGC1β knockout mice demonstrated a reduced mitochondrial complement in both the soleus and heart muscles (46). Thus, increased PPARGC1α and PPARGC1β indicated the activation of pathways leading to the remodeling of oxidative enzyme content and the mitochondrial compartment associated with the availability of ATP. In contrast, members of the HIF family were first discovered in association with hypoxia-induced angiogenesis, where HIF-1α binds to a hypoxic response element within the VEGF promoter (7, 81). We cannot rule out focal ischemia as the stimulus leading to increased HIF-1α and HIF-3α levels during CLFS despite the presence of an intact circulation. However, it has recently been shown that HIF-1α and VEGF play a direct role in embryonic development and wound repair under physiological conditions other than hypoxia (7). HIF-1α transcripts have also been detected bound to glucose transporter 4 E box-binding sites in isolated, incubated soleus muscles subjected to a brief bout of high-frequency stimulation (77), indicative of a role in modulating glucose uptake. Consequently, increased HIF-1α transcript levels may represent a response to changes in energy balance.

The role of increased EYA1 and NFATc4 transcripts in the present study was unclear. The Six1/EYA1 pathway has been implicated in the maintenance of the fast fiber phenotype, and forced expression converts the slow twitch muscle of the soleus to a fast phenotype (30), opposite of the effect in the present study. However, EYA1 has been subsequently demonstrated to be critical for somitogenesis (29), and it may be that this distinct role was enlisted by chronic stimulation in the present study. Similarly, the forced expression of NFATc4 in cardiomyocytes indicated a role in calcineurin-induced hypertrophy (84), and, by virtue of its sequence similarity to NFATc1, it has been implicated in muscle fiber-specific expression pathways through conserved NFAT consensus binding sites. Similarly, knockout of NFATc3 and NFATc4 in mice resulted in embryonic lethality secondary to aberrant mitochondrial bio-

The role of increased transcripts for these two genes in the stimulated EDL muscle remains to be determined regarding their participation in potentially discreet transcriptional programs associated with phenotype conversion versus muscle morphogenesis.

Changes in transcripts associated with signal transduction were surprisingly few, suggesting that signal transduction responses were mediated at nontranscriptional levels during the acute phase of stimulation, such as through posttranslational modifications, e.g., phosphorylation. Elevated MAPK pathways have been associated with myofiber transdifferentiation, as occurred with MAPK6 in this study (75). Also, increased CASK transcripts in the stimulated muscle supported a role for calcium as a specific trigger in the early activation of transcription factors associated with contractile protein remodeling (4).

However, the transduction pathways whereby electrically induced, neural stimulation was translated into transcriptional events were not reflected at the RNA level despite the abundance of these transcripts detected by the arrays. Furthermore, transcripts for critical energy regulators such as AMP kinase, mammalian target of rapamycin, AKT, and transcripts associated with mitochondrial biogenesis (TFAM) were not significantly altered after 4 h of stimulation, suggesting that signals driving the activation of transcription factors may have arisen from extracellular sources such as cytokines, chemokines, metabolic substrates, and growth factors or through changes in the intracellular ionic equilibrium involving calcium, potassium, or other critical electrolytes.

Methodological considerations. The present study used an established chronic stimulation protocol for the induction of fiber type transformation in the hindlimb. We delivered supramaximal sciatic nerve stimulation and adjusted muscle length to obtain consistent, comparable force output in each animal. The contralateral muscles provided identically matched genetic controls for comparison with the stimulated muscles regarding histological properties and transcript profiles. The microarray platform comprised oligonucleotide probes (30-mer) with approximately equivalent target avidity, thereby scaling transcript output levels based on biological abundance. Low array background levels (average background intensity: 0.29 ± 0.15 IU) resulted in a high signal-to-noise ratio from transcription factors to myofibrillar proteins (5- to 5,000-fold background, respectively) (11, 76). Transcript levels for MyHC proteins and myogenic determination factors from control EDL, soleus, and satellite cell cultures correlated with results obtained previously using PCR and immunocytochemical assays (46).

Conclusions. In conclusion, the onset of fast to slow myofiber transformation of critical myofibrillar proteins was detected in the EDL muscle after 4 h of chronic stimulation in the absence of ultrastructural, histological, and molecular signs of muscle damage or satellite cell activation. Multiple fiber type-specific transcription factors were increased in synchrony with the process of phenotype conversion along with a surprising subset of transcription factors characteristic of embryogenesis. Based on these findings, the mature myonuclei of differentiated muscle fibers or a subset of these nuclei were the source of transcriptional plasticity including the recapitulation of developmental morphogenic programs. The distinct subsets of transcription factors activated by chronic stimulation may provide insight into the separate pathways and transductive signals responsible for the delineation of the myofiber phenotype and
phenotype conversion as well as potential new therapeutic targets for muscle repair and regeneration.

ACKNOWLEDGMENTS

The authors express special thanks to Dr. Sheldon Bastacky, Ardhith Reis, and Donna Ziesmer for the excellent work in providing the electron microscopic analysis and Dr. Marcia Ontell for the insightful review of these images.

GRANTS

This work was supported by National Space Biomedical Research Institute Grant MA 00210, Michigan State University Investigator-Initiated Research Grant 41006, National Cancer Institute Cancer Center Support Grant P50-CA-47904, funding through the Pasquerilla Foundation, and with support from Allegheny Heart Institute Grant 49399109.

REFERENCES

Molecular dynamics of the fast muscle response to stimulation


