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Altered mRNA expression after long-term soleus electrical stimulation training in humans with paralysis

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Abstract

Introduction—In humans, spinal cord injury (SCI) induces deleterious changes in skeletal muscle that may be prevented or reversed by electrical stimulation muscle training. The molecular mechanisms underlying muscle stimulation training remain unknown.

Methods—We studied two unique SCI subjects whose right soleus received >6 years of training (30 minutes/day, 5 days/week).

Results—Training preserved torque, fatigue index, contractile speed and cross-sectional area in the trained, but not the untrained leg. Training decreased 10 mRNAs required for fast twitch contractions and mRNA that encodes for myostatin, an autocrine/paracrine hormone that inhibits muscle growth. Conversely, training increased 69 mRNAs that mediate the slow twitch, oxidative phenotype, including PGC-1 α , a transcriptional co-activator that inhibits muscle atrophy. When we discontinued right soleus training, training-induced effects diminished slowly; some persisted >6 months.

Discussion—Training of paralyzed muscle induces localized and long-lasting changes in skeletal muscle mRNA expression that improve muscle mass and function.

Keywords

atrophy; hypertrophy; myostatin; myosin; actin

INTRODUCTION

Spinal cord injury (SCI) induces severe atrophy of paralyzed muscles as well as an increase in contractile speed and a reduction in peak torque, fatigue index and torque-time integral.^{1,2} These changes are central features of the chronic musculoskeletal deterioration that contributes to morbidity and mortality in SCI subjects.³

Muscle training, accomplished via transcutaneous electrical stimulation of paralyzed muscle, can prevent or reverse the effects of SCI on muscle mass and function.^{1,2} However, the molecular mechanisms of electrical stimulation-mediated muscle training remain unknown, as do the majority of post-SCI muscle molecular adaptations.^{4–8} To address this question,

we studied two subjects from a previously described cohort that received electrical stimulation training to the right soleus for > 3 years. In this cohort, muscle training preserved right soleus mass, strength, and slow twitch physiology. Conversely, the left (untrained) soleus displayed the atrophy, weakness and fast twitch physiology characteristic of chronic SCI.⁹⁻¹¹

The two subjects in this study continued to train their right soleus muscles with the prescribed dose of exercise for an additional 3 years after the initial report, with no hiatus in training. Here, we report that >6 years of electrical stimulation training maintained right soleus cross-sectional area, peak torque, and fatigue index, whereas the left soleus remained atrophic, weak and fatigable. These subjects, while limited in number, offered an unprecedented opportunity to understand if and how electrical stimulation training might alter skeletal muscle mRNA expression. Their left soleus served as a powerful within-subject control for the effects of long-term muscle training in the right soleus. Moreover, within-subject controls eliminate many intra-subject variables (including nutrition, medications, activity, endocrine factors, and genetic predisposition) that render direct comparisons between separate independent cohorts challenging. In this report, we studied these unique subjects to test the hypothesis that the physiological effects of muscle training might be accompanied by and perhaps explained by differential expression of skeletal muscle mRNAs.

METHODS

Subjects and Training History

Two males with T4 complete paraplegia (ages 28 and 30) participated in the study. The protocol was approved by the University of Iowa Human Subjects Institutional Review Board. Subjects provided written informed consent before participating.

The subjects had performed electrical muscle stimulation training of the right lower leg for 6.0 years (subject 1) or 7.2 years (subject 2). Details of the training regimen may be found in a previous report, where SCI subjects 1 and 2 are described as subjects 7 and 6, respectively.⁹ In brief, the subjects performed at least 4000 soleus contractions per month using a custom designed stimulator equipped with a data logging compliance system. The dose of muscular load transmitted to the tibia was ~ 1.5 times body weight (BW). For this study, the subjects underwent two baseline bilateral biopsies of the trained and untrained limbs. The subjects returned their stimulator systems after the second biopsy and began a period of detraining. The subjects returned for bilateral biopsies after 2 and 6 months of detraining (Figure 1).

Muscle Physiology Testing

Subjects underwent muscle physiology testing before the first baseline biopsy and after 6 months of detraining. The ankle was stabilized in a system which measured isometric plantar flexion torque, as described previously.^{9,12} The knee was positioned at ninety degrees of flexion, and the ankle was in neutral joint position. The test position (knee flexed) minimized the contribution of the gastrocnemius to the plantar flexor torque.¹³ Electrical current was delivered via adhesive carbon electrodes placed over the posterior aspect of the plantar flexor muscles. Stimulation was controlled by digital pulses from a data-acquisition board (Metrabyte DAS 16F, Keithley Instruments Inc., Cleveland, OH) housed in a microcomputer under custom software control. The microcomputer output was conveyed via shielded cabling to a muscle stimulator unit (Digitimer model DS7A, Digitimer Ltd., Welwyn Garden City, Hertfordshire, England). The stimulator was set to deliver 200 μ sec pulses at 400 V and 200 mA. This intensity was sufficient to elicit supramaximal contractions in both subjects, indicating full recruitment of all plantar flexor muscle fibers.⁹

The stimulator was programmed to deliver a 10 pulse train (15 Hz; train duration 667ms) every two seconds. A bout of exercise consisted of 125 trains.

We identified three muscle physiology variables of interest. Torque was defined as the peak torque achieved from the torque-time curve for any given contraction. Fatigue index (FI) was computed according to the following formula: $100 * (\text{minimum torque in a bout} / \text{maximum torque in a bout})$. Higher values indicate greater resistance to fatigue. In each torque train, torque rise time was defined as the elapsed time in milliseconds between 20% and 80% of peak torque for that train. Because torque rise time is influenced by the overall torque achieved, we normalized torque rise time values to the peak torque of each contraction.

Magnetic Resonance Imaging (MRI)

Axial-plane spin-echo, T1 weighted, fat suppressed images were acquired from the level of the ankle joint to the tibial plateau (1.5T Siemens Avanto scanner). A three-dimensional gradient-echo-based sequence was used for high-resolution imaging. The acquisition parameters included repetition time (TR) of 15.0ms and echo time (TE) of 6.7ms, 512×256 matrix covering a field of view of $36\text{cm} \times 18\text{cm}$ with 2.5mm slice thickness. A water-excitation pulse was used to suppress signal from fat. MIPAV¹⁴ version 4.3.1 (National Institutes of Health) was used to manually trace the single-joint plantar flexors (soleus, tibialis posterior, flexor digitorum, flexor hallucis longus) as a region of interest in each axial section. (The bent-knee training position limited the mechanical tension, and therefore the hypertrophy stimulus,¹⁵ for the gastrocnemius; it was thus excluded from the region of interest). Plantar flexor cross-sectional area (CSA) was obtained at 66% of tibia length (measured from the distal end), as denoted by the location of a fat capsule taped to the skin.

Muscle Biopsy and mRNA Analysis

Percutaneous muscle biopsies were taken from both soleus muscles of each subject using a Temno biopsy needle (T1420, CardinalHealth) under ultrasound guidance. Following harvest, muscle biopsy samples were immediately placed in RNAlater (Ambion) and stored at -80°C until further use. Total RNA was extracted using TRIzol solution (Invitrogen) according to the manufacturer's instructions. Microarray hybridizations were performed at the University of Iowa DNA Facility. Briefly, 50 ng total RNA was converted to SPIA amplified cDNA using the WT-Ovation Pico RNA Amplification System, v1 (NuGEN Technologies, San Carlos, CA, Cat. #3300) according to the manufacturer's recommended protocol. The amplified SPIA cDNA product was purified through a QIAGEN MinElute Reaction Cleanup column (QIAGEN Cat #28204) according to modifications from NuGEN. Four μg of SPIA amplified DNA were used to generate ST-cDNA using the WT-Ovation Exon Module v1 (NuGEN Technologies, Cat #2000) and again cleaned up with the Qiagen column as above. Five μg of this product were fragmented (average fragment size = 85 bases) and biotin labeled using the NuGEN FL-Ovation cDNA Biotin Module, v2 (NuGEN Technologies, Cat. #4200) per the manufacturer's recommended protocol. The resulting biotin-labeled cDNA was mixed with Affymetrix eukaryotic hybridization buffer (Affymetrix, Inc., Santa Clara, CA), placed onto Human Exon 1.0 ST arrays (Part No. 900650), and incubated at 45°C for 18 h with 60 rpm rotation in an Affymetrix Model 640 Genechip Hybridization Oven. Following hybridization, the arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes, Inc., Eugene, OR), signal amplified with antistreptavidin antibody (Vector Laboratories, Inc., Burlingame, CA) using the Affymetrix Model 450 Fluidics Station. Arrays were scanned with the Affymetrix Model 3000 scanner with 7G upgrade and data were collected using the GeneChip operating software (GCOS) v1.4.

In Affymetrix Human Exon 1.0 ST arrays, the log₂ hybridization signal reflects the mean signal intensity of all exon probes specific for an individual mRNA, and is proportional to the level of that mRNA. In our studies comparing the four sets of untrained and trained biopsies from SCI subjects 1 and 2, we excluded mRNAs that did not possess a mean log₂ hybridization signal ≥ 5 in either the right or left soleus ($n = 4$) in order to restrict our analysis to those mRNAs with reasonably high levels of expression. We then used paired *t*-tests to determine which of the remaining mRNAs were present at a significantly different level ($P \leq 0.05$) in the right soleus relative to the left soleus. All microarray data have been deposited in the GEO database.

Quantitative real-time RT-PCR (qPCR) analysis was performed using human TaqMan Gene Expression Assays (Applied Biosystems). First strand cDNA was synthesized from 1 μ g of RNA using random hexamer primers and the TaqMan reverse transcription kit (catalog no. N808-0234; Applied Biosystems). The real time PCR contained, in a final volume of 20 μ l, 20ng of reverse transcribed RNA, 1 μ l of 20X TaqMan Gene Expression Assay, and 10 μ l of TaqMan Gene Expression Master Mix (catalog no. 4369016; Applied Biosystems). PCR was carried out using a 7500 Fast Real-Time PCR System (Applied Biosystems) in 9600 emulation mode. All qPCR reactions were performed in triplicate, and the cycle threshold (Ct) values were averaged to give the final results. To analyze the data, we used the Δ Ct method, with level of 36B4 mRNA serving as the invariant control.

RESULTS

Effect of Long-term Electrical Stimulation Training on Soleus Size and Physiology

Our electrical stimulation training protocol consisted of 30 minutes of training at a stimulation intensity sufficient to generate tibia loads of ~ 1.5 times BW for 5 days a week. Importantly, the dose of muscle stress was quantified, and compliance was measured by digital logging of delivered doses of electrical current (> 4000 contractions per month). Representative examples of soleus torque-time curves (generated in response to transcutaneous electrical stimulation) appear in Fig. 2A. In both subjects torque and fatigue index (FI) were higher in the trained leg than the contralateral, untrained leg (Figure 2B). Training also prevented the appearance of contractile slowing during repetitive stimulation, a hallmark of fatigue, as shown in Figure 2C.

To assess the effect of sustained long-term training on soleus cross-sectional area, we used MRI to examine subject 2. As shown in Fig. 2D, training increased the soleus and deep flexor CSA by $> 40\%$. Subject 1 did not undergo MRI due to retained metal fragments in his body; however, the circumferential diameter of this subject's right calf (33.9 cm) was substantially larger than the untrained left calf (29.9 cm). These data are consistent with our previous descriptions of these subjects after 3 years of soleus training. Thus, as a result of long-term training (6 years), the right soleus muscles maintained mass and slow twitch properties. In contrast, the untrained left soleus muscles possessed the atrophy and fast twitch properties characteristic of chronic SCI.

Global Effect of Training on Soleus mRNA Expression

To determine the molecular effects of muscle training in our SCI subjects, we performed bilateral soleus muscle biopsies on both subjects at two time points six months apart. We then subjected RNA from the biopsy samples to exon expression arrays that measure levels of $> 17,000$ mRNA transcripts. Relative to levels in the left (untrained) soleus, the right (trained) soleus samples contained significantly lower levels of 330 mRNAs and significantly higher levels of 806 mRNAs ($P \leq 0.05$). Thus, in total, soleus training altered levels of approximately 6% of soleus mRNAs.

Fig. 3A and B show the ten mRNAs that exhibited the largest decreases and the largest increases, respectively, in the right (trained) soleus. Four of the ten most repressed mRNAs encode established markers of fast twitch muscle: *ACTN3* (encoding α -actinin 3), *PVALB* (encoding parvalbumin), *AQP4* (encoding aquaporin 4) and *MYLK2* (encoding myosin light chain kinase 2). Conversely, four of the ten most induced mRNAs in the right soleus encode sarcomeric proteins that mediate the slow twitch phenotype: *MYH7* (encoding slow twitch myosin heavy chain), *MYL3* (encoding slow twitch alkali myosin light chain), *MYL2* (encoding slow twitch regulatory myosin light chain) and *TNNC1* (encoding slow twitch troponin C). Interestingly, the 3rd most highly repressed mRNA in the right soleus encoded myostatin (*MSTN*), an autocrine/paracrine hormone that inhibits muscle growth¹⁶ (Fig. 3B).

mRNA Expression in Pathways Regulating Fiber Type

We used qPCR to validate the differential expression of key genes regulating fiber type: *ACTN3*, *MSTN*, *MYH7* and *MYL3*. With our laboratory techniques, qPCR is a more sensitive method for detecting changes in mRNA expression than exon expression arrays. As predicted by the exon expression arrays, the trained soleus muscles of SCI subjects contained reduced levels of *ACTN3* and *MSTN* mRNAs (Fig. 3C), and increased levels of *MYH7* and *MYL3* mRNAs (Fig. 3D).

Several other mRNAs encoding fast twitch proteins were also significantly reduced in the right soleus, albeit to a lesser degree (Fig. 4A). These repressed mRNAs included *MYH1* (encoding fast twitch myosin heavy chain type IIx/d), *ATP2A1* (encoding fast twitch sarcoplasmic reticulum Ca^{2+} -ATPase, a.k.a. SERCA1), *TNNC2* (encoding fast twitch troponin C), *MYLPF* (encoding fast regulatory myosin light chain), and *MYPBC2* (encoding fast type myosin binding protein C). The right soleus also contained reduced levels of *MYL5* and *MYL6* mRNAs (encoding regulatory and alkali myosin light chains atypical of adult skeletal muscle) and an increased level of *MYH6* mRNA (encoding cardiac α -(atrial) myosin heavy chain) (Figs. 4A and 4B). Importantly, we did not identify any examples of fast twitch-specific mRNAs that were increased in the right soleus, nor did we identify any slow twitch-specific mRNAs that were decreased in the right soleus. Together, these results indicate that muscle training coordinately repressed genes that mediate the fast twitch phenotype, while coordinately inducing genes that mediate the slow twitch phenotype.

mRNA Expression in Pathways Regulating Metabolism

The right (trained) soleus also contained higher levels of many mRNAs that encode proteins involved in glucose utilization and oxidative phosphorylation (Figs. 4C and 4D). Proteins encoded by these mRNAs include 2 enzymes that mediate glycogen synthesis, 3 enzymes that mediate glycolysis, 5 subunits of the pyruvate dehydrogenase complex, 11 proteins involved in the TCA cycle (including 2 isoforms of succinate dehydrogenase), and 43 subunits of the electron transport chain. Similarly, mRNA transcripts that encode creatine kinase (*CKMT2*) and myoglobin (*MB*) were increased in the right soleus (2.17 ± 0.28 and 1.24 ± 0.08 –fold, respectively; $P \leq 0.02$) (not shown in Figure C or D). Previous work demonstrated that the transcriptional co-activator PGC-1 α promotes the slow twitch, oxidative phenotype in skeletal muscle.¹⁷ Indeed, the right soleus contained an increased level of mRNA that encodes PGC-1 α (*PPARGCA*) (Fig. 4C).

mRNA Expression in Other Pathways

In addition to promoting the slow twitch phenotype, muscle training reduced muscle atrophy (Fig. 2). Consistent with increased protein synthesis, the right (trained) soleus contained significantly higher levels of mRNAs that encode 7 aminoacyl-tRNA synthetases, 7 translation initiation factors and 27 ribosomal subunits. The majority of differentially expressed mRNAs did not have obvious or established roles in adult skeletal muscle

metabolism, physiology or growth. These include some of the most highly repressed mRNAs (such as *TSPAN8*, *SH3RF2*, *HCN1*, *SH2D1B*) (Fig. 3A) and some of the most highly induced mRNAs (such as *PRUNE2*, *RSPO3*, *AGBL1*, *ENPP5* and *MRAP2*) (Fig. 3B).

Effect of Detraining on Soleus mRNA Expression

To determine if the differential mRNA expression that we observed in the soleus was dependent on training, we discontinued training the right soleus of both SCI subjects. We obtained bilateral soleus muscle biopsies from both subjects after two months and six months of detraining and then analyzed levels of *ACTN3*, *MSTN*, *MYH7* and *MYL3* mRNAs using qPCR (Figure 5). Two months of right soleus detraining was not sufficient to increase *ACTN3* or *MSTN* mRNAs, nor was it sufficient to reduce *MYL7* or *MYH3* mRNAs. Six months of detraining tended to normalize levels of these mRNAs, however, all four remained differentially expressed in the right vs. left soleus. These data indicate that muscle training was required for differential mRNA expression in the right soleus muscles of our SCI subjects. Moreover, these results indicate that the effects of muscle training on mRNA expression are long lasting and detectable several months after discontinuation of training.

Effect of Detraining on Soleus Size and Physiology

The finding that detraining had a slow and incomplete effect on right soleus mRNA expression prompted us to examine the physiological characteristics of the right-sided muscles after 6 months of detraining. In both subjects, the right limb torque-time curves indicated a sustained effect of training (Fig. 6A). In the previously trained right leg, torque and FI remained higher (Fig. 6B), and the soleus contraction rise time remained lower after repetitive stimulation (i.e. less contractile slowing due to fatigue) than the untrained limb (Fig. 6C). This indicates that, while a reversion of the detrained limb toward a fast-fatigable contraction profile occurred, the process remained incomplete by 6 months. Moreover, in subject 2, the soleus and deep flexor CSA remained elevated (by 21%) after six months of detraining (Fig. 6D). Although all training-induced changes were diminished by detraining (compare Fig. 2 and 6), these results indicate that physiological and morphological effects of training were still evident after six months of detraining.

DISCUSSION

Long-term soleus training is known to preserve soleus size, strength and slow twitch physiology in humans with SCI. However, the molecular mechanisms were not known. In these studies, we examined two unique SCI subjects whose paralyzed right soleus had been subjected to muscle training for > 6 years. In these subjects, long-term training preserved right soleus function and mass, whereas their left soleus remained fatigable and atrophic. By comparing global mRNA expression in the right vs. left soleus, we found that training induced many localized changes in skeletal muscle mRNA expression.

Training Adaptations in Pathways Regulating Fiber Type

The most notable training-induced pattern we detected was a reduction in 10 mRNAs that mediate fast-twitch muscle contractions and an increase in 69 mRNAs that mediate the slow twitch, oxidative phenotype. A few of these changes were predicted by earlier studies. Paralyzed skeletal muscle from patients with chronic SCI contains an increased proportion of fast twitch, glycolytic myofibers¹⁸⁻²⁰ that possess increased amounts of fast twitch myosin heavy chain type IIx (MyHC-IIx/d), increased amounts of fast twitch sarcoplasmic reticulum Ca²⁺ ATPase (SERCA2), and reduced amounts of succinate dehydrogenase.^{1,20-24} By inhibiting the effects of SCI-induced paralysis, training decreased mRNAs that encode MyHC-IIx/d (*MYH1*) and SERCA2 (*ATP2A1*), and increased mRNA that encodes succinate dehydrogenase (*SDHA* and *SDHB*) (Fig. 4). Our data also elucidate many

additional fiber type-specific mRNAs not previously known to be regulated by training in paralyzed human muscle (Fig. 4).

A key determinant of skeletal myofiber type is PGC-1 α , which promotes the slow twitch, oxidative phenotype.^{17,25} PGC-1 α expression is increased by exercise in human soleus and vastus lateralis muscles^{26,27} and by electrical stimulation training in mouse extensor digitorum.²⁸ Perhaps not surprisingly, we found that training increased the level of mRNA that encodes PGC-1 α (Fig. 4). This suggests that training induces the fast to slow twitch fiber type transformation in paralyzed human muscle by increasing PGC-1 α expression.

Adaptations in Atrophy/Hypertrophy Pathways

In addition to promoting the slow twitch phenotype, muscle training reduced muscle atrophy. In mice, PGC-1 α overexpression reduces skeletal muscle atrophy.²⁹ Coupled with our data, this suggests that training may reduce atrophy in paralyzed muscle by increasing PGC-1 α expression. A second potential mechanism of decreased atrophy in our subjects' trained muscles was reduced expression of mRNA that encodes myostatin (*MSTN*), a locally acting hormone that inhibits muscle growth and decreases PGC-1 α expression.³⁰ Indeed, the fast-twitch, atrophic untrained soleus muscles in our subjects resembled a synthesis of fast-twitch mouse muscles that lack PGC-1 α ²⁵ and atrophic mouse muscles that overexpress myostatin.³¹ Conversely, the slow-twitch, non-atrophic trained soleus muscles in our subjects resembled a synthesis of slow-twitch mouse muscles that overexpress PGC-1 α ¹⁷ and hypertrophic mouse muscles that lack myostatin.³² These considerations lead us to speculate that increased PGC-1 α expression and reduced myostatin expression are key events that mediate the effects of muscle training on paralyzed muscle.

At first glance, our data concerning *MSTN* mRNA levels vary from an earlier study that reported a significant reduction in *MTSN* mRNA in the vastus lateralis muscle of humans with chronic SCI when compared to able-bodied subjects.⁵ Although we cannot provide a definitive explanation for the discrepancy between that study and ours, differences in the muscles that were examined (soleus vs. vastus lateralis) or the experimental design may be responsible.

Many differentially expressed mRNAs did not have obvious or established roles in skeletal muscle. These include some of the most highly repressed mRNAs and some of the most highly induced mRNAs. The finding that training induced significant changes in the levels of these mRNAs suggests that they may play a role in fiber type determination, excitation-contraction coupling, hormonal signaling, and/or skeletal muscle metabolism. This is an important area for future investigation.

In the chronically trained right soleus, we did not detect reduced or elevated expression of mRNAs that encode the proteolytic E3 ubiquitin ligases atrogen-1/MAFbx (*FBXO32*) and MuRF1 (*TRIM63*). In humans, these ubiquitin ligases are induced at the transcriptional level in the acute period following SCI,⁴ and they are required for muscle atrophy in animal models of muscle disuse.^{33,34} Of note, when we use the same exon expression arrays, we find that *FBXO32* and *TRIM63* mRNAs are significantly increased by prolonged fasting in human skeletal muscle (unpublished data). The finding that *atrogen-1/MAFbx* and *MuRF1* mRNAs were not altered by training in our chronic SCI subjects likely reflects the fact that their muscles were chronically untrained or trained. This is consistent with the notion that the muscle atrophy in chronic SCI patients represents a steady-state marked by neither growth nor atrophy.^{5,35} Likewise, our long-term training protocol likely induced a steady-state in paralyzed skeletal muscle. This study represents the first within-subject analysis of SCI subjects who possess both a chronically untrained state and a chronically trained state.

Effects of Detraining

To confirm that the differential mRNA expression in the right (trained) soleus muscles of our subjects was due to muscle training, we discontinued training and re-assessed soleus mRNA levels after two and six months. Remarkably, differential mRNA expression between the previously trained (detrained) and untrained soleus muscles was maintained after two months of detraining. After six months of detraining, this differential expression was greatly reduced, but it was still detectable and was accompanied by physiological and morphological evidence of sustained training-induced effects. These data suggest that long-term training with electrical stimulation has long lasting effects akin to the formation of a memory. We speculate that the underlying molecular mechanism may involve epigenetic effects on skeletal muscle gene expression, and this is an important area for future investigation.

Study Limitations and Future Directions

This study offers a first-look examination of molecular adaptations to training using the unique and critical feature of within-subject controls. Many more subjects with various SCI durations must be studied before the molecular adaptations to electrical stimulation training can be completely characterized. While our sample size prevents us from commenting on the absolute differences in mRNA levels between SCI and control subjects, the within-subject training design provided a level of control that clearly demonstrates the robust influence of periodic muscle training (30 minutes per day, 5 days per week) in paralyzed muscle.

The deep flexor group (tibialis anterior, flexor digitorum, flexor hallucis longus) demonstrated hypertrophy (Figure 2) and thus contributed to the physiologic variables (torque, fatigue index, torque rise time) observed in the trained limb. By the same token, the atrophied deep flexors of the untrained limb contributed to that limb's physiologic performance. Thus while the molecular results pertain to a single muscle (soleus), the physiology results reflect a composite of several muscles. This was an unavoidable limitation of the study; neither biopsy of the deep flexors nor exclusion of deep flexor activation during training was possible. The small physiologic cross-sectional area of the deep flexors minimized their contribution to the physiologic measures; however, it is likely that the trained soleus fatigue index in particular was "blunted" by inclusion of fast-fatigable deep flexors during testing. The observed fatigue index would have likely been even higher in the trained limbs if the slow-oxidative soleus could have been separately assessed. Similarly, normalized rise time of the soleus would have been even lower if it could have been isolated during testing. In addition to studying a larger number of subjects, future studies should examine the acute effects of training, the effects of acute and chronic training on other muscle groups, and the relationship of differential mRNA expression to both epigenetic changes in these gene loci and the levels of proteins encoded by these mRNAs. Mechanistic studies may require the development of new animal models that replicate SCI and muscle training in an authentic manner. It will also be interesting to determine the optimal dose of exercise required to prevent atrophy and physiologic dysfunction of paralyzed muscle in humans with SCI, and to determine if training-induced effects on mRNA expression and muscle physiology require exercise or merely periodic electrical stimulation. Such information will facilitate the development of electrical stimulation protocols and perhaps pharmacological therapies that target the musculoskeletal deterioration that severely limits the health of people with SCI.

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Abbreviations

SCI	(spinal cord injury)
FI	(fatigue index)
MRI	(magnetic resonance imaging)
CSA	(cross-sectional area)
BW	(body weight)

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Figure 1.

Study design. Both subjects were paraplegic secondary to SCI and began training their right lower legs within six weeks of the acute injury. They continued to train the right lower legs for > 6 years prior to this study, which began at 0 months. At 0 and 6 months, we obtained bilateral soleus biopsies from each subject. We then discontinued right soleus training and obtained bilateral soleus biopsies two months and six months later.

Figure 2.

Effect of right lower leg training on muscle physiology and cross-sectional area (CSA). (A-C) Soleus physiologic parameters for subject 1 (left column) and subject 2 (right column). (A) Representative examples of contractions 1 and 125 in trained and untrained conditions. (B) Soleus torque, with fatigue index (FI) values listed. (C) Normalized soleus torque rise time. (D) MRI images of trained and untrained lower legs from subject 2. The graph on the right quantifies plantar flexor CSA. Because of their single-joint attachment sites, the deep flexors also received the training stimulus.

Figure 3.

Effects of long-term soleus training on soleus mRNA levels. (A-B) Global analysis of mRNA levels by exon expression arrays. Each right (trained) soleus mRNA level was normalized to the mRNA level in the paired left (untrained) soleus biopsy sample, which was arbitrarily set at 1. (A) The 10 most highly repressed mRNAs in the right soleus. (B) The 10 most highly induced mRNAs in the right soleus. (C-D) qPCR analysis of *ACTN3*, *MSTN*, *MYH7* and *MYL3* mRNA levels. Each right (trained) soleus mRNA level was normalized to the mRNA level in the paired left (untrained) soleus biopsy sample, which was arbitrarily set at 1 and indicated by the dashed lines. (A-D) Data are means $\pm$ SEM for both subjects from repeat baseline biopsy samples, and $P \leq 0.05$ for all mRNAs shown.

Figure 4.

Effects of soleus muscle training on levels of mRNAs that encode proteins involved in fast twitch muscle contraction (A), proteins involved in slow twitch muscle contraction (B), enzymes that promote glucose utilization (C), and subunits of the electron transport chain (D). Each right (trained) soleus mRNA level was determined using exon expression arrays, and normalized to the mRNA level in the paired left (untrained) soleus biopsy sample, which was set at 1. Data are means $\pm$ SEM for both subjects from repeat baseline biopsy samples. $P \leq 0.05$ for all mRNAs shown.

Figure 5.

Effect of two and six months detraining on right soleus mRNA levels for ACTN3 and MSTN (A) and for MYH7 and MYL3 (B). Each right (trained) soleus mRNA level was normalized to the mRNA level in the paired left (untrained) soleus biopsy sample, which was set at 1. Data are the mean for both subjects at each time point.

Figure 6.

Effect of six months of right leg detraining on muscle physiology and cross-sectional area (CSA). (A-C) Soleus physiologic parameters for subject 1 (left column) and subject 2 (right column). (A) Representative examples of contractions 1 and 125 in trained and detrained conditions for the right limb. (B) Soleus torque, with fatigue index (FI) values listed. (C) Normalized soleus torque rise time. (D) MRI images of untrained (left) and detrained (right) lower legs from subject 2. The graph on the right quantifies plantar flexor CSA.