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Rapid Changes in NFAT-directed Transcriptional Activity after Muscle Paralysis Induced by a Spinal Cord Injury

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Abstract

In skeletal muscles, the calcineurin - nuclear factor of activated T-cells (NFAT) pathway has been implicated as a positive regulator of the slow muscle phenotype. Spinal cord transection (ST) results in muscle paralysis and a slow to fast shift in muscle phenotype in the normally slow soleus muscle of rats. Therefore, to determine if the NFAT pathway could mediate the slow to fast shift after ST, NFAT-directed transcription was assessed in the soleus muscle of rats after ST. Seven days after ST, a significant down-regulation in the activity of an exogenous NFAT sensor promoter-reporter construct was observed (~20% of control levels). Second, RT-PCR analyses revealed that MCIP1.4 mRNA, an endogenous indicator of NFAT-directed transcriptional activation, was decreased by ~80% relative to controls when normalized to β-actin mRNA levels. Similarly, real time RT-PCR showed a down-regulation in the relative levels of MCIP1.4 mRNA at 1 day (92% reduction) and 7 days (89% reduction) after ST relative to control levels. These data demonstrate that NFAT-directed transcriptional activity is rapidly down-regulated following a reduction in neuromuscular activity. Since ST also results in a slow to fast shift in phenotypic protein expression, the down-regulation of NFAT transcriptional activity is consistent with a role for the calcineurin - NFAT pathway in down regulating slow muscle phenotypic gene expression after ST.

Keywords: Spinal cord injury; Muscle paralysis; Rehabilitation

Introduction

Skeletal muscle provides the power and support for movement. One of the unique features of skeletal muscle is its fiber (cell) type diversity [1]. Fiber type diversity is directly related to the MyHC isoform expressed in a fiber [2], such that four types of MyHC isoforms are expressed in the corresponding fiber types from adult rat limb skeletal muscles [2-4]. These include (in order from slowest to fastest) the slow type I isoform, MyHC I, and three fast type II isoforms, MyHC IIa, MyHC IIx (also known as IId) and MyHC IIb [2-4].

Neuromuscular activity is one factor that plays a large role in muscle fiber type specification [5,6]. Skeletal muscles are capable of transforming from the fast to the slow phenotype under several conditions [1]. For instance, endurance exercise training, which is characterized by repeated bouts of contractile activity over a period of several weeks results in exercise capacity improvement [7] and induces an up-regulation in type I and a down-regulation in type II fibers in the human VL muscle [8].

In contrast, reduced neuromuscular activity causes a reduction in electrical activation and/or load bearing of a muscle and results in a slow to fast fiber type transformation of normally slow muscles [6]. The rodent spinal cord transection (ST) model, an animal model for spinal cord injury, results in a reduction in electrical activation of the hind limb muscles and hind limb muscle paralysis. The muscles of rats and other mammals (including humans) respond to reductions in neuromuscular activity by reducing the expression of slow phenotypic proteins and increasing the expression of fast phenotypic proteins [6]. For example, following ST, MyHC I mRNA in the rat soleus muscle was down-regulated to ~26%, 19%, and 12% of control levels after 3 months, 6 months and 1 year, respectively [9-11]. Changes in MyHC expression at the mRNA level suggest that fiber transformation is regulated at the level of transcription, as changes in the levels of the corresponding protein isoforms follow the changes in the mRNA [9]. In addition to muscle atrophy and a corresponding reduction in maximal force production, the rat soleus muscle also obtains significantly faster contractile properties after ST [11]. Thus, ST strongly induces a slow-to-fast fiber type transformation.

Several cellular and molecular mechanisms may be involved in regulating MyHC isoform expression. One is the calcineurin - nuclear factor of activated T-cells (NFAT) pathway, a cellular signalling mechanism that appears to be highly activated and involved in the fast to slow fiber type adaptation occurring after increases in neuromuscular activity [6]. The calcineurin - NFAT pathway involves two main proteins: calcineurin and its down-stream target, NFAT. Calcineurin, also known as protein phosphatase 2B (PP2B), is a calcium activated serine/threonine phosphatase [12]. NFAT is a gene transcription factor that up-regulates the expression of slow muscle specific genes when present in the nucleus and is also one target of calcineurin. At rest, NFAT remains phosphorylated in the cytoplasm in an inactivated state. Following prolonged elevations in cytosolic Ca^{2+}, such as occur during normal activation and contractions of slow postural muscles like the soleus, calcineurin becomes activated and dephosphorylates NFAT. Following dephosphorylation, NFAT undergoes a conformational change exposing a nuclear localizing signal (also known as an NLS) on NFAT allowing it to interact with proteins known as nuclear importins. Upon interation with the importin NFAT is translocated into the nucleus where it is released from the importin and can now bind to DNA and aid in the initiation of slow muscle gene transcription [13-15].

In the present study, the activation status of NFAT-directed transcription was evaluated after a reduction in neuromuscular activity following a complete ST. NFAT-directed transcriptional activity was assessed using three different techniques. This was accomplished by: (1)assessing the activity of an exogenous NFAT sensor - reporter gene construct containing multiple NFAT response elements (NFAT binding sites also known as NREs) linked to a luciferase reporter gene following direct injection into the soleus muscles of control and ST rats; (2) assessing the expression of MCIP1.4 via conventional RT-PCR, as an endogenous and indirect indicator of NFAT transcriptional activation since the MCIP1.4 gene promoter contains fifteen consecutive NREs and its expression is highly up-regulated by NFAT, and (3) assessing the expression of MCIP1.4 using real time RT-PCR. It was hypothesized that ST would induce: (1) a down-regulation in exogenous NFAT reporter gene activity and (2) a down-regulation in MCIP1.4 mRNA. These experiments allowed for an evaluation of the status of NFAT-directed transcription after muscle paralysis induced by ST.

Methods

Experimental model

Female Sprague-Dawley rats (~200 g) were randomly assigned to two treatment groups; control or spinal cord transected (ST) (n=5-6 per group). The n value was based on our previous observations on the statistical significance of adaptations in MyHC isoform expression after ST [6,9-11]. Rats randomly selected for ST were subjected to a complete transection of the spinal cord at vertebral level T7-T8 using aseptic techniques as previously described [10]. One or seven days after ST, the animals were euthanized via CO2 inhalation, and the soleus muscles were quickly removed, weighed and quickly frozen in isopentane cooled by liquid nitrogen.

At the time of ST surgery, the exogenous NFAT-promoter reporter plasmid was injected directly into the surgically exposed soleus muscles via intramuscular injection and 30 μg of the NFAT-promoter reporter plasmid was co-injected with 7.5 μg α-actin promoter linked to Renilla luciferase (to control for plasmid uptake). The skin was then sutured using ethilon. All use of animals was approved by the Animal Care and Use Committee at California State Polytechnic University, Pomona.

NFAT-reporter and β-actin promoters

The exogenous NFAT-reporter plasmid construct was used to indicate NFAT-induced transcriptional activation was kindly donated by Dr. J. Molkentin (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). The NFAT-reporter promoter contained nine copies of the NFAT binding sites (9x NFAT response elements, NRE) from the IL-4 promoter which were inserted to the 5' end of the α-MHC promoter and linked to a luciferase reporter. The α-actin promoter linked to Renilla luciferase was used as a reference vector for normalization of plasmid uptake efficiency, since its activity is primarily dependent on the amount of uptake.

MCIP1.4 mRNA quantification

One step reverse transcription polymerase chain reaction (RT-PCR): As stated previously the MCIP1.4 gene contains 15 NREs just proximal to the transcription start site at exon 4. Because MCIP1.4 mRNA expression is directly related to NFAT transcriptional activity, MCIP1.4 mRNA levels are indicative of NFAT-induced transcriptional activation. Total RNA was extracted from soleus muscles using the RNeasy Fibrous Tissue Midi Technique (Qiagen, Valencia, CA) and subjected to DNase treatment (Ambion DNA-free™ kit, Austin, TX). The RT-PCR reaction components included 1x PCR buffer, dNTPs (10 μM), 1x Q solution, Taq Polymerase, Omniscript reverse transcriptase (2.0 μl/reaction), 80 ng/μl of RNA template, 10 μM MCIP1.4 forward primer (5'-AAGGAACCTCCAGCTTGGGCT-3') and 10 μM MCIP1.4 reverse primer (5'-CCCTGGTCTCACTTTCGCTG-3'). Additional sets of no primer and no RT reaction controls were performed to ensure the reactions were controlled for any random cDNA synthesis or DNA contamination, respectively. The reverse transcription reaction was set at 50°C for 30 minutes and stopped by incubation at 94°C for 15 minutes (this also served to initiate the PCR reaction). The PCR reaction conditions were a total of 34 cycles under the following conditions; denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1 minute and following the 34 cycles a final extension at 72°C at 10 minutes using the MJ Research Thermal Cycler (MJ Research, Waltham, MA). The same protocol was also applied for analysis of β-actin mRNA, which served as a normalizing gene, using primers from Ambion, Inc., (Austin, TX). The PCR products were electrophoresed using 2% agarose gels. DNA band densities were analyzed using the SYBR green detection method and a FluoroChem™ imager (Alpha Innotech, San Leandro, CA).

Real-time reverse transcription PCR: To ensure that the analysis of MCIP1.4 gene expression via the One Step RT-PCR method was not influenced by product accumulation at the plateau stage, MCIP1.4 gene expression was also assessed by real-time RT-PCR using the ΔΔCt method with 18S rRNA serving as a reference gene (Guide to performing relative quantification of gene expression using real time RT-PCR, Applied Biosystems, Foster City, CA). Total RNA was isolated as described above. Following DNase treatment, RNA was reverse transcribed to cDNA and subjected to real time RT-PCR using SYBR green fluorescence to indicate product accumulation. Each PCR tube contained MCIP1.4 cDNA template, 2x FullVelocity® SYBR® green QPCR master mix (Stratagene, Cedar Creek, TX), 100 μM MCIP1.4 upstream primer and 100 μM MCIP1.4 downstream primer or 18S primer pair containing a forward primer and a reverse primer (Ambion, Inc., Austin, TX).

Real time RT-PCR reactions were set at 39 cycles under the following conditions; initial denaturing step at 95°C for 5 minutes, denaturation at 95°C for 45 seconds, and annealing-extension at 60°C for 1 minute, followed by melting curve analyses to determine the existence of potential primer-dimer formation and any cDNA contaminants using the DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA). Triplicate sets of ST and control samples were run simultaneously along with 18S rRNA analyses in separate PCR tubes under the same amplification reaction as described above. Additionally, negative cDNA controls (i.e., no RT reaction) were run to control for contaminating DNA. A cycle threshold (Ct) was manually set at the point where fluorescence was above the background level, which represented the initial formation of product above background levels. In theory, samples with a lower cycle threshold contained higher starting template.

To determine whether the PCR amplification procedures were not influenced by factors such as PCR efficiency, differences in reagent volumes and pipeting techniques during real time RT-PCR, the

efficacy of the PCR amplification was determined by performing a validation experiment (Applied Biosystems, Foster City, CA). The MCIP1.4 RNA and 18S standard were diluted to the following concentrations; 1:2, 1:5, 1:10, 1:20, 1:50, 1:75, and 1:100. A logarithmic scale of the diluted RNA concentrations were plotted against the average difference between MCIP1.4 and 18S (ΔCt). A slope of<0.01 indicated a similar efficiency regardless of starting template concentration. Relative quantification of MCIP1.4 and 18S mRNAs at 1 and 7 days after ST was calculated using the 2-(ΔΔCt) equation to determine the mean fold change of MCIP1.4 mRNA levels, where ΔΔCt represented: 2-((CtST(MCIP1.4) - (CtST(18s)) - ((Ave. CtControl(MCIP1.4)-Ave. CtControl(18s)).

Statistical analyses

All values were reported as mean ± SEM. Data were analyzed using the SigmaStat statistical program with Student's t-tests. Group differences between control and ST muscle and body weights were performed using the one-way analysis of variance (ANOVA) followed by post Bonferroni post-ANOVA test. Statistical significance was determined by the level of acceptance at p<0.05.

Results

Animal and muscle mass

One day of ST had no impact on the absolute mass of the soleus in ST rats relative to control rats. Absolute and relative (relative to total body mass) soleus mass were significantly reduced by ~30% 7 days after ST (Table 1). The final body masses were not significantly changed at either 1 or 7 days after ST.

NFAT promoter activity

The activity of the exogenous NFAT sensor-reporter gene construct was investigated in soleus muscles of control and ST rats to determine whether NFAT-induced gene transcription was altered after ST. An exogenous gene promoter-reporter construct, containing nine NFAT response elements (gene promoter binding sites for NFAT) linked to the α-MyHC promoter and a firefly luciferase reporter gene, was co-injected with an α-actin promoter linked to renilla luciferase (to control for plasmid uptake) directly into the soleus muscles of control and ST rats. Seven days after ST, NFAT-induced transcriptional activity was reduced by ~80% as measured by the reduction in NFAT-luciferase reporter activity (Figure 1).

MCIP1.4 mRNA

Conventional RT-PCR: Using a one-step RT-PCR method, the levels of MCIP1.4 mRNA were quantified in ST and control rats with β-actin used as a normalizing gene. As early as 1 day after ST, there was an ~85% reduction in MCIP1.4 mRNA compared to controls (ST, average ratio of MCIP/β-actin=3.03 ± 1.10; control, 19.77 ± 3.09, p<0.01) (Figure 2A and 2C). Seven days after ST, MCIP1.4 mRNA was decreased by ~90% in ST rats compared to controls (ST, average ratio of MCIP/β-actin=2.51 ± 0.20; control, 24.25 ± 9.84, p<0.01) (Figure 2B and 2C).

Real time RT-PCR: In order to validate the conventional RT-PCR method, MCIP1.4 mRNA was also quantified using real time RT-PCR methodology according to the ΔΔCt method using 18S rRNA as a normalizing gene. The cycle threshold (Ct) for each reaction was determined and used according to the guide to performing relative quantification of gene expression (Applied Biosystems, Foster City, CA).

Group	Soleus (mg)	Body Mass (g)	Soleus/Body Mass (mg/g)
1 day Control	122 ± 2	263 ± 4	0.465 ± 0.005
1 day ST	119 ± 5	266 ± 7	0.450 ± 0.010
7 day Control	135 ± 3	270 ± 7	0.501 ± 0.012
7 day ST	91 ± 3*	259 ± 5	0.352 ± 0.011*

Table 1: Soleus muscle and final body mass from 1 and 7 days control and ST rats. Values are means ± SEM (n=6/group). *Significant difference from control, p<0.05.

The number of cycles taken to reach the Ct was higher in ST rats relative to control rats (Ave. Ct after 1 day, ST=25.97, control=22.63; Ave. Ct after 7 days, ST=28.41 and control=22.99) indicating a reduction in MCIP1.4 mRNA levels (Figure 3A and 3B). The ΔΔCt method revealed that there was a 92% reduction in the relative levels (mean fold change analysis) of MCIP1.4 mRNA (relative to 18S rRNA) at 1 day after ST relative to control levels (ST, 0.11 ± 0.02; control, 1.41 ± 0.41, p=0.01). Similarly, seven days after ST, there was an 89% reduction in the relative levels MCIP1.4 mRNA (ST, 0.12 ± 0.04; control, 1.13 ± 0.35, p<0.05) (Figure 3C). Thus, the two RT-PCR methods were in agreement.

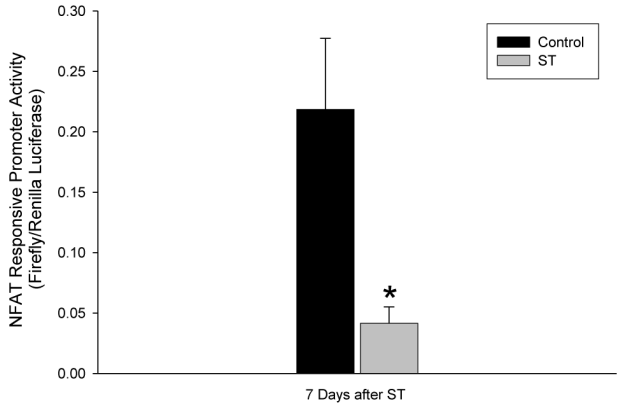

Figure 1: Nuclear factor of activated T-cells (NFAT) responsive promoter activity in control and spinal cord transected (ST) rat soleus. NFAT induced transcriptional activity was measured using an NFAT-responsive promoter containing 9x NFAT response elements (NREs) linked to the firefly luciferase reporter gene. An α-actin promoter linked to renilla luciferase was simultaneously injected into the muscle to normalize the data and control for varying injection efficiencies. Values are presented as mean firefly/renilla ratios ± SEM (n=5/group). * denotes statistical difference between the two groups (p<0.05).

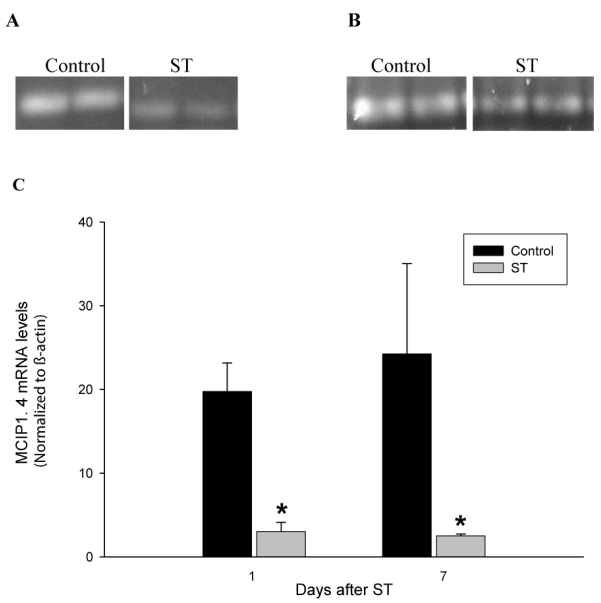

Figure 2: Quantification of MCIP1.4 mRNA levels 1 and 7 days after ST using one-step RT-PCR. (A) Representative MCIP1.4 bands from control and 1-day ST rats. (B) Representative MCIP1.4 bands from control and 7-day ST rats. (C) Normalized levels of MCIP1.4 mRNA for control (n=6) and ST (n=6) groups. Values are represented as mean ± SEM. * denotes statistical difference between the two groups (1 day ST, $p<0.01$; 7 days ST, $p<0.01$).

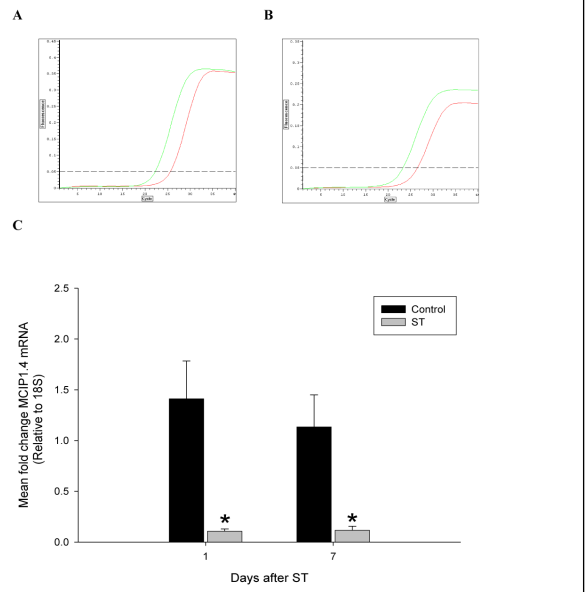

Figure 3: Real-time RT-PCR analysis of MCIP1.4 mRNA. (A) and (B) are amplification plots of MCIP1.4 mRNA from 1 day and 7 day (control and ST) samples, respectively. Controls shown in green and ST shown in red. (C) Quantification of MCIP1.4 mRNA levels relative to 18S using the Delta Delta Ct (2-ΔΔCt) method. Values are represented as mean fold change ± SEM (n=5 - 6 rats per group). * denotes statistical difference between the two groups (1 day ST, $p<0.05$; 7 days ST, $p<0.01$).

Discussion

The primary findings of this study were that muscle paralysis, as induced by spinal cord injury, resulted in a down regulation of NFAT-directed transcriptional activation in the normally slow soleus muscle of rats. This was demonstrated using three different strategies. First, the activity of an exogenous NFAT-responsive promoter was reduced by ~80% after ST. Second, conventional RT-PCR assessment of the expression of the MCIP1.4 gene (normally activated by NFAT) was dramatically reduced (by nearly 90%). Finally, real time RT-PCR assessment of the expression of the MCIP1.4 gene confirmed that MCIP1.4 transcripts were dramatically reduced after ST. Collectively, these data demonstrate that short-term muscle paralysis is a potent stimulus to 'turn-off' NFAT-directed transcription. Since ST also induces a slow to fast transformation in muscle phenotype [9,10,16], these data are consistent with a role for the calcineurin-NFAT in specifying the slow muscle fiber type in the normal rat soleus.

NFAT promoter activity was down-regulated after ST

As expected, ST down-regulated NFAT-directed promoter activity as measured by the exogenous NFAT promoter reporter gene. It is well established that ST causes a severe reduction in slow muscle contractile activity. For example, in the cat soleus muscle, the total daily electromyographic (EMG) activity (a measurement of muscle activity), was reduced by 75% following ST [17], demonstrating that ST causes a profound reduction in the total daily activation of the slow postural soleus muscle.

Contractile activity has been shown to modulate nuclear NFAT levels. The NFATc1 isoform predominantly localizes in the cytoplasm of the fast tibialis anterior (TA) muscle; however, after applying a low frequency electrical stimulation pattern, which mimics the pattern observed for slow muscles, it was found that NFATc1 was translocated to the nucleus [18]. A similar response in NFATc1 translocation was observed in cultured rabbit muscle cells. For instance, when rabbit myotube cultures were electrically stimulated at 10 Hz for 30 minutes, a pattern that mimics prolonged trains of low frequency stimulation characteristic of slow muscles, a rise in NFATc1 levels in the nucleus was observed [19]. These data strongly suggest that the translocation of NFAT into the nucleus is regulated by a motoneuron activity pattern consisting of long trains of low frequency action potentials, such as occur in normal slow muscle and which are reduced after ST.

As stated previously, NFAT activation (i.e., dephosphorylation by calcineurin) is dependent on motoneuron firing patterns and activity. High amount, low-frequency electro-stimulation, such as occurs in normal slow muscles, resulted in the up-regulation of NFAT-directed transcriptional activity [20]. In the fast tibialis anterior (TA) and fast flexor digitorum brevis (FDB) muscles, continuous electrostimulation at 10 Hz (low frequency, high amount, mimics slow muscle activation) resulted in NFATc1 dephosphorylation and nuclear import [18,21-23]. Furthermore, continuous electrical stimulation, longer than 24 hours, resulted in NFAT nuclear translocation in 60% of muscle cells [24]. In vivo and in vitro studies showed that: a) continuous stimulation at 1 Hz for 15 minutes or every 5 minutes with 10 minute rest intervals of rabbit muscle cultures and b) constitutive activation of NFATc1 (caNFATc1) in rat EDL muscle result in an up-regulation of MyHC I

and a down-regulation of MyHC IId(x) isoform expression [19,20]. After cessation of continuous stimulation, MyHC I mRNA was down-regulated and MyHC IId(x) mRNA was up-regulated, suggesting the slow-to-fast fiber transformation among myosin heavy chain proteins is regulated at the transcriptional level [20].

Collectively, these studies show that the elevated expression of slow muscle phenotypic genes can result from enhanced NFAT nuclear translocation induced by chronic low frequency motoneuron activity and NFAT-directed transcriptional activation. Furthermore, there is a direct relationship between higher amounts of dephosphorylated forms of NFAT in the nucleus and slow fiber gene expression, suggesting that NFAT plays a role in fast to slow fiber type transformation in skeletal muscles. As speculated, in the present study the converse sequence of events appears to occur. That is, ST results in reduced activation of soleus muscle fiber contraction, resulting in reduced cellular Ca2+ (i.e., fewer calcium release events associated with contraction). The reduced levels of Ca2+ result in apparently lowered activation of calcineurin and subsequent lowered activation of NFAT, i.e., NFAT remains phosphorylated and in the cytosol where it cannot bind to DNA and impact gene expression, thus lowering the expression of slow fiber-specific genes.

MCIP1.4 mRNA levels were down-regulated after ST

The modulatory calcineurin inhibiting protein (MCIP) is a 22-27 kDa protein and exists as three isoforms (MCIP1, MCIP2 and MCIP3) in humans and mice [25]. MCIP1, also known as Down syndrome critical region 1 (DSCR1), is located on human chromosome 21 and is predominantly expressed in the brain, heart and skeletal muscles [25]. Four different variants (MCIP1.1-1.4) are derived from alternative transcription initiation sites associated with the first 4 exons on the MCIP1 gene. This results in each of the 4 variants having a different initial exon, either 1-4 for MCIPs 1.1-1.4, respectively. Three other exons are uniform components of all MCIP isoforms (exon 5-7). The region located just proximal to the 5' region of exon 4 contains 900 base pairs with 15 consecutive NFAT binding sites and represents an NFAT responsive promoter region that is important for regulating the expression of the MCIP1.4 variant [25]. Thus, MCIP1.4 expression is highly dependent on activated (i.e., nuclear localized) NFAT [26,27].

Our finding of a significant reduction in MCIP1.4 mRNA levels in the rat soleus after ST, as observed with both real time RT-PCR and conventional one-step RT-PCR, is an indirect confirmation that a reduction in NFAT transcriptional activity is caused by ST. Several studies have shown that the calcineurin - NFAT pathway can modulate MCIP gene expression, specifically, MCIP1.4 mRNA expression [26]. For instance, in the heart of transgenic mice expressing constitutively active calcineurin, calcium influx and nuclear NFAT import up-regulated MCIP mRNA levels [26]. Also, a 90-fold up-regulation in MCIP1.4 transcriptional activity was observed in C2C12 myoblast cells when transfected with a plasmid expressing a constitutively active form of calcineurin [26,27]. The above studies suggest that the level of NFAT in the nucleus regulates the transcriptional activity of the MCIP1.4 gene. These previous studies have shown that MCIP1.4 expression is directly regulated by calcineurin activation and NFAT transcriptional activation. The data presented here show that MCIP1.4 transcription is reduced after ST, likely mediated by a reduction in NFAT-directed transcription.

Potential role for the calcineurin-NFAT in specifying the slow muscle fiber type

The hallmark study by Chin et al. [28] showed that slow muscle gene expression could be regulated by the activity of the calcineurin - NFAT signalling pathway. In fact, disruption of the NFAT binding sites in the promoter of the slow muscle-specific proteins, myoglobin and troponin I, dramatically reduced activities of these promoters despite activation of calcineurin [28]. This study [28] suggested that slow muscle specific gene expression was dependent on the direct interaction between NFAT protein and NREs located in slow muscle gene promoters.

Several studies have supported the role of calcineurin activity in promoting slow muscle gene expression [29-31]. The plantaris muscle of transgenic mice expressing a constitutively active form of calcineurin [29] and fibroblasts transfected with a plasmid containing the calcineurin Aα isoform [30] showed an up-regulation in the slow MyHC protein levels. Interestingly, a previous study demonstrated that the calcineurin Aα isoform (expressed in the brain and skeletal muscles) was able to regulate nuclear NFATc1 levels, whereas the calcineurin Aβ isoform (expressed in immune cells) did not have an effect on regulating nuclear NFATc1 levels [31]. Therefore, even though slow-fiber phenotypic gene expression is regulated by calcineurin, a specific isoform of calcineurin, calcineurin Aα, may regulate this process. Additionally, contractile proteins associated with slow fibers, such as slow troponin I, are also up-regulated by calcineurin activation [28]. These studies suggest that muscle fiber phenotype specificity and contractile proteins associated with slow fibers are regulated by calcineurin activation.

The data in the present study showed that muscle inactivity resulted in a down-regulation of calcineurin - NFAT signalling. Since calcineurin - NFAT signalling is important for slow muscle gene expression, as discussed above, a reduction in the activity of this pathway would be predicted to be associated with a slow to fast transformation in muscle phenotype. In fact, previous data from our lab shows a dramatic down-regulation in slow muscle phenotypic gene expression, along with enhanced fast muscle phenotype gene expression after ST [9,10,16,32]. Thus, our data are consistent with a potential role for the calcineurin - NFAT pathway in specifying the slow muscle phenotype and that suppression of this pathway allows for slow to fast fiber transformation that occur after ST.

In summary, ST induced-paralysis of the soleus muscle resulted in a significant reduction in (1) exogenous NFAT-directed gene promoter activity, and (2) MCIP1.4 mRNA levels, an indirect measurement of NFAT-directed transcriptional activation. The data suggest that reduced neuromuscular activity induced by ST prevented or reduced the ability for calcineurin to dephosphorylate NFAT which down-regulated NFAT nuclear import and reduced the availability of NFAT to activate gene transcription. These data are consistent with previous studies showing A) an involvement of the calcineurin - NFAT pathway in specifying muscle fiber type and B) previous data showing that ST results in a fast to slow muscle fiber type transformation.

Implications for rehabilitation following spinal cord injury (SCI). SCI is associated with paralysis of muscles that are innervated by neurons residing at or below the site of injury. This paralysis likely results in lowered muscle cytosolic Ca^{2+} due to a reduction in activation and Ca^{2+} release events. In turn, this results in de-activation of the calcineurin-NFAT signalling pathway and reduced NFAT-directed gene transcription, as shown here in a rodent model. This

would result in suppression of slow muscle-specific gene expression and the subsequent acquisition of fast fiber characteristics as we have shown previously in rodent models [9-11]. Therefore, in order to spare slow muscle fiber characteristics after paralysis, interventions are needed which re-activate the calcineurin-NFAT pathway. Rehabilitative approaches could include some combination of body weight supported treadmill training to retrain locomotor programs and electrical stimulation either with or without resistance to reactivate the calcineurin-NFAT pathway. Unfortunately to date, functional electrical stimulation (FES), FES combined with cycle ergometry, and intraspinal microstimulation have failed to induce the expression of slow MyHC (Type I) following SCI [33-36]. It is possible that this is due to a lack of sufficient activation of the calcineurin-NFAT pathway. In addition, no studies have assessed the activation state of this cellular signalling pathway during rehabilitation from muscular paralysis. Therefore, future studies should directly address the activation state of the calcineurin-NFAT pathway during rehabilitation from muscle paralysis.

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References

1. Pette D, Staron RS (2001) Transitions of muscle fiber phenotypic profiles. Histochem Cell Biol 115: 359-372.
2. Pette D, Staron RS (2000) Myosin isoforms, muscle fiber types, and transitions. Microsc Res Tech 50: 500-509.
3. Schiaffino S, Reggiani C (1994) Myosin isoforms in mammalian skeletal muscle. J Appl Physiol (1985) 77: 493-501.
4. Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, et al. (1989) Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. J Muscle Res Cell Motil 10: 197-205.
5. Talmadge RJ, Roy RR, Edgerton VR (1993) Muscle fiber types and function. Curr Opin Rheumatol 5: 695-705.
6. Talmadge RJ (2000) Myosin heavy chain isoform expression following reduced neuromuscular activity: potential regulatory mechanisms. Muscle and Nerve 23: 661-679.
7. Booth FW, Thomason DB (1991) Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models. Physiol Rev 71: 541-585.
8. Jansson E, Kaijser L (1977) Muscle adaptation to extreme endurance training in man. Acta Physiol Scand 100: 315-324.
9. Talmadge RJ, Garcia ND, Roy RR, Edgerton VR (2004) Myosin heavy chain isoform mRNA and protein levels after long-term paralysis. Biochem Biophys Res Commun 325: 296-301.
10. Talmadge RJ, Roy RR, Edgerton VR (1999) Persistence of hybrid fibers in rat soleus after spinal cord transection. Anat Rec 255: 188-201.
11. Talmadge RJ, Roy RR, Caiozzo VJ, Edgerton VR (2002) Mechanical properties of rat soleus after long-term spinal cord transection. J Appl Physiol (1985) 93: 1487-1497.
12. Olson EN, Williams RS (2000) Remodeling muscles with calcineurin. BioEssays 22: 510-519.
13. Rao A, Luo C, Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 15: 707-747.
14. Kiani A, Rao A, Aramburu J (2000) Manipulating immune responses with immunosuppressive agents that target NFAT. Immunity 12: 359-372.
15. Hogan PG, Chen L, Nardone J, Rao A (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 17: 2205-2232.
16. Talmadge RJ, Roy RR, Edgerton VR (1995) Prominence of myosin heavy chain hybrid fibers in soleus muscle of spinal cord-transected rats. J Appl Physiol (1985) 78: 1256-1265.
17. Alaimo MA, Smith JL, Roy RR, Edgerton VR (1984) EMG activity of slow and fast ankle extensors following spinal cord transection. J Appl Physiol Respir Environ Exerc Physiol 56: 1608-1613.
18. Tothova J, Blaauw B, Pallafacchina G, Rudolf R, Argentini C, et al. (2006) NFATc1 nucleocytoplasmic shuttling is controlled by nerve activity in skeletal muscle. J Cell Sci 119: 1604-1611.
19. Kubis HP, Scheibe RJ, Meissner JD, Hornung G, Gros G (2002) Fast-to-slow transformation and nuclear import/export kinetics of the transcription factor NFATc1 during electrostimulation of rabbit muscle cells in culture. J Physiol 541: 835-847.
20. McCullagh KJ, Calabria E, Pallafacchina G, Ciciliot S, Serrano AL, et al. (2004) NFAT is a nerve activity sensor in skeletal muscle and controls activity-dependent myosin switching. Proc Natl Acad Sci U S A 101: 10590-10595.
21. Shen T, Liu Y, Cseresnyés Z, Hawkins A, Randall WR, et al. (2006) Activity- and calcineurin-independent nuclear shuttling of NFATc1, but not NFATc3, in adult skeletal muscle fibers. Mol Biol Cell 17: 1570-1582.
22. Tomida T, Hirose K, Takizawa A, Shibasaki F, Iino M (2003) NFAT functions as a working memory of Ca2+ signals in decoding Ca2+ oscillation. EMBO J 22: 3825-3832.
23. Liu Y, Cseresnyés Z, Randall WR, Schneider MF (2001) Activity-dependent nuclear translocation and intranuclear distribution of NFATc in adult skeletal muscle fibers. J Cell Biol 155: 27-39.
24. Kubis HP, Hanke N, Scheibe RJ, Meissner JD, Gros G (2003) Ca2+ transients activate calcineurin/NFATc1 and initiate fast-to-slow transformation in a primary skeletal muscle culture. Am J Physiol Cell Physiol 285: C56-63.
25. Rothermel BA, Vega RB, Williams RS (2003) The role of modulatory calcineurin-interacting proteins in calcineurin signaling. Trends Cardiovasc Med 13: 15-21.
26. Yang J, Rothermel B, Vega RB, Frey N, McKinsey TA, et al. (2000) Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. Circ Res 87: E61-68.
27. Sanna B, Brandt EB, Kaiser RA, Pfluger P, Witt SA, et al. (2006) Modulatory calcineurin-interacting proteins 1 and 2 function as calcineurin facilitators in vivo. Proc Natl Acad Sci U S A 103: 7327-7332.
28. Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, et al. (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev 12: 2499-2509.
29. Talmadge RJ, Otis JS, Rittler MR, Garcia ND, Spencer SR, et al. (2004) Calcineurin activation influences muscle phenotype in a muscle-specific fashion. BMC Cell Biol 5: 28.
30. Delling U, Tureckova J, Lim HW, De Windt LJ, Rotwein P, et al. (2000) A calcineurin-NFATc3-dependent pathway regulates skeletal muscle differentiation and slow myosin heavy-chain expression. Mol Cell Biol 20: 6600-6611.
31. Parsons SA, Wilkins BJ, Bueno OF, Molkentin JD (2003) Altered skeletal muscle phenotypes in calcineurin Aalpha and Abeta gene-targeted mice. Mol Cell Biol 23: 4331-4343.
32. Talmadge RJ, Roy RR, Edgerton VR (1996) Myosin heavy chain profile of cat soleus following chronic reduced activity or inactivity. Muscle Nerve 19: 980-988.
33. Andersen JL, Mohr T, Biering-Sorenson F, Galbo H, Kjaer M (1996) Myosin heavy chain isoform transformation in single fibres from m. vastus lateralis in spinal cord injured individuals: effects of long-term functional electrical stimulation (FES). Pflugers Archives 431: 513-518.
34. Bamford JA, Putman CT, Mushahwar VK (2011) Muscle plasticity in rat following spinal transection and chronic intraspinal microstimulation. IEEE Trans Neural Syst Rehabil Eng 19: 79-83.
35. Mohr T, Andersen JL, Biering-Sørensen F, Galbo H, Bangsbo J, et al. (1997) Long-term adaptation to electrically induced cycle training in severe spinal cord injured individuals. Spinal Cord 35: 1-16.

36. Demchak TJ, Linderman JK, Mysiw WJ, Jackson R, Suun J, et al. (2005) Effects of functional electric stimulation cycle ergometry training on lower limb musculature in acute sci individuals. J Sports Sci Med 4: 263-271.