Effects of inactivity on fiber size and myonuclear number in rat soleus muscle

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Zhong, Hui, Roland R. Roy, Boonclaire Siengthai, and V. Reggie Edgerton. Effects of inactivity on fiber size and myonuclear number in rat soleus muscle. J Appl Physiol 99: 1494–1499, 2005. First published June 30, 2005; doi:10.1152/japplphysiol.00394.2005.—The effects of short-term (4 days) and long-term (60 days) neuromuscular inactivity on myonuclear number, size, and myosin heavy chain (MHC) composition of isolated rat soleus fibers were determined using confocal microscopy and gel electrophoresis. Inactivity was produced via spinal cord isolation (SI), i.e., complete spinal cord transections at a midthoracic and a high sacral level bilateral deafferentation between the transection sites. Compared with control, there was an increase in the percentage of fibers containing the faster MHC isoforms after 60, but not 4, days of SI. The mean sizes of type I and type I+IIa fibers were 41 and 27% and 66 and 56% smaller after 4 and 60 days of SI, respectively. Thus atrophy occurred earlier than the shift in myosin heavy chain (MHC) profile. The number of myonuclei was ~30% higher in type I than type I+IIa fibers in control soleus, but after 60 days of SI these values were similar. The number of myonuclei per millimeter in type I fibers was significantly lower than control after 60 days of SI, whereas there was no change in type I+IIa fibers. Thus myonuclei were eliminated from fibers containing only type I MHC Because the magnitude of the loss of myonuclei was less than the level of atrophy, the myonuclear domains of both type I and type I+IIa fibers were significantly lower than control. Thus chronic (60 days) inactivity results in smaller, faster fibers that contain a higher than normal amount of DNA per unit of cytoplasm. The absence of activation of muscle fibers that are normally the most active (pure type I fibers) resulted in most, but not all, fibers expressing some fast MHC isoforms. The results also indicate that a loss of myonuclei is not a prerequisite for sustained muscle fiber atrophy.

spinal cord isolation; myosin heavy chain isoforms; fiber phenotypes; disuse; fiber cross-sectional area

ALTHOUGH IT IS CLEAR THAT activity plays an important role in modulating the size of skeletal muscle fibers (6, 13, 32), the degree that this modulation is dependent on activity remains obscure. Similarly, the role of activity in determining muscle fiber phenotype has not been well defined. Although the effects of “decreased” levels of skeletal muscle activity have been studied, the models used have been limited by representing changed, but not well-defined, absolute levels of activity (14, 41).

Our laboratory previously has shown that spinal cord isolation (SI), i.e., complete transections of the spinal cord at a midthoracic and a high sacral level plus bilateral deafferentation between the transection sites, results in almost complete inactivation of the hindlimb muscles in cats (28, 29) and rats (47). Because the contact between the nerve and muscle remains intact, this preparation has provided a model for defining the effects of activity-independent neural influences on the muscle and muscle fiber properties (15–18, 20, 28, 29, 33, 36, 46). Recently, our laboratory has identified some of the molecular mechanisms underlying the atrophic response to chronic inactivity and concluded that the rapid and marked atrophy associated with inactivity can be attributed to changes in transcription, translation, and protein degradation (17, 18).

Cellular-level mechanisms of adaptation also seem important in defining activity-dependent responsiveness. For example, skeletal muscle fibers are multinucleated cells offering the possibility for the regulation of protein expression not only by a differential change in the pattern and/or amount of gene expression but also by alterations in the total number of myonuclei available for gene expression. Several lines of evidence indicate that when muscle fibers hypertrophy, the number of myonuclei increases (2, 24, 35), and it is generally assumed that satellite cells provide a source for these new myonuclei. A chronic decrease in neuromuscular activity results in a decrease in fiber size and myonuclear number, and it appears that this occurs, at least in part, via apoptotic mechanisms (1, 3). Furthermore, it has been suggested that this modulation in the amount of available DNA might be a factor in regulating the cytoplasmic volume of muscle fibers in response to chronic changes in neuromuscular activity (13).

The purposes of the present study were to determine and compare the effects of short-term (4 days) and long-term (60 days) inactivity on the size, phenotype, and myonuclear domain, i.e., cytoplasmic volume/myonucleus (10, 19), of rat soleus single muscle fibers. On the basis of our laboratory’s observations of a more rapid decrease in fiber size compared with myonuclear number (and thus a decrease in myonuclear domain) in the earlier stages of decreased activity (4, 26), we hypothesized that the mean size of the myonuclear domains would be reestablished to control values after a prolonged period of inactivity.

METHODS

Experimental design and surgical procedures. The muscles used in the present study were derived from a subset of animals used previously for different analyses (16). Adult female Sprague-Dawley rats (242 ± 2 g body wt) were assigned randomly to either a control or a SI group (n = 5/group per time point). The spinal cord of SI rats was completely transected at a midthoracic and a high sacral spinal cord level and then bilaterally deafferented between the two transection sites. The SI and animal care procedures have been detailed previously (16, 34). At either 4 (SI-4d) or 60 (SI-60d) days after SI surgery, the rats were weighed, anesthetized with pentobarbital sodium (100 mg/kg body wt ip), and decapitated. For the control group in the present study, we randomly selected five control rats across the time points. This procedure was deemed appropriate because there was no significant increase in mean body weight of these adult female rats.
across the time points (16). The soleus muscle was excised, cleaned of excess connective tissue and fat, and wet weighed. The muscles then were frozen at approximately their in situ physiological length in melting isopentane cooled in liquid nitrogen and stored at −70°C until further analysis.

**Single-fiber isolation.** The fiber segments analyzed in the present study were isolated randomly from the proximal one-third of the muscle. Segments of single fibers were mechanically isolated from the muscle sample as described previously (2). Briefly, the muscle was thawed gradually in a −20°C freezer for 5 h, then placed in a −20°C 50% glycerol-50% low-calcium relaxing solution (12), and immediately transferred to a −5°C freezer where it was kept overnight. The following day, the muscle was transferred to a refrigerator at 4°C for 1 h, and then one end of the sample was pinned in a 100% low-calcium relaxing solution in a Sylgard-coated culture dish. Single-fiber segments (40–60/muscle; 3–6 mm in length) were mechanically isolated from each muscle sample with microdissection forceps under a dissection scope. Fibers were placed on gelatin-coated slides and stored at −5°C.

**Confocal microscopy.** Slides were removed from the −5°C freezer, air-dried, and stained with acridine orange and propidium iodide as described previously (4). This combination was found to produce the best staining of, and contrast between, the cytoplasm and the myonuclei (Fig. 1). A Sarastro 2000 confocal microscope with an argon laser (Molecular Dynamics, Sunnyvale, CA) and calibrated measurement software (Silicon Graphics, Salt Lake City, UT) were used to analyze fiber cross-sectional area (CSA), myonuclear number, and myonuclear domain. First, the fibers were optically sectioned in steps of 1.0–1.5 μm to create a stack of images encompassing the entire fiber thickness. The stack of images was optically rotated orthogonal to the long axis of the fiber, and the CSA was measured for two different regions within each viewing field. Total myonuclear number was determined from these projections for the portion of the fiber in the field of view (field size = 173 × 173 μm). Sarcomere length was measured for 3 different sets of 10 consecutive sarcomeres to obtain an average sarcomere length for the field. The myonuclear domain for each region of the fiber was determined by multiplying the fiber CSA by the region length (173 μm) and then dividing by the number of myonuclei per field. For each fiber, three separate regions were selected randomly for analysis and then averaged to produce a single value for each fiber. In all cases, the fiber ends, and any fiber region containing fiber damage or adhering connective tissue, were omitted. To correct for differences in the state of stretch, values for myonuclei per millimeter and fiber CSA were multiplied by the average sarcomere length, and divided by 2.5 to normalize to a 2.5-μm sarcomere length.

**Gel electrophoresis.** After confocal analysis, the fibers were destained and dehydrated in 50% ethanol, scraped off the slide, and placed into a microcentrifuge tube containing 12 μl of 1× electrophoresis sample buffer (21). Electrophoresis was carried out with a Bio-Rad (Richmond, CA) Mini-Protein II dual-slab electrophoresis unit essentially as described by Talmadge and Roy (40). The separating gel contained 8% acrylamide and 30% glycerol. The single-fiber samples were heated for 2 min at 80–90°C before being loaded. On each gel, the entire 12-μl sample was loaded in a single lane: in addition, a “standard” lane, i.e., a mixture of rat soleus and medial gastrocnemius homogenate, was loaded to facilitate the accurate determination of MHC isoform expression. Gels were run at 80 V for 24 h in an ice-packed cooler and then stained with rapid Coomassie (Diversified Biotech, Boston, MA). The percentage of each MHC isoform for each fiber was determined by quantitative densitometry of each band. Densitometry was performed using a IS-1000 Alph Innotech densitometer (San Leandro, CA), and the values for each isoform are reported as a percentage of total MHC expression. These procedures resulted in seven MHC combinations (Fig. 2). Fibers coexpressing both type I and type II MHCs were considered hybrid fibers.

**Statistical analyses.** All values are reported as means ± SE unless indicated differently. For each muscle, a linear mixed model (multivariate ANOVA, SAS software PROC MIXED procedure; SAS Institute, Cary, NC) was used to compare the outcomes among the three study groups, the four types of fibers (only those that are observed in all 3 groups), and the difference among the three groups within each type of fiber. A linear mixed model two-way ANOVA was used to assess the main effects of MHC type and experimental procedures resulted in seven MHC combinations (Fig. 2). Fibers coexpressing both type I and type II MHCs were considered hybrid fibers.

**RESULTS**

**Body and muscle weights.** Mean initial body weights for the control and SI rats were 243 ± 2 and 241 ± 2 g, respectively. After 4 and 60 days of SI, mean body weight was 19 and 7% (P > 0.05) lower in SI than control rats (Table 1). Mean body weight was 15% greater in SI-60d than SI-4d rats, indicating that the SI rats were growing. After 4 and 60 days of SI, mean absolute soleus weights were 27 and 64% smaller than in control rats, whereas the relative weights (mg/kg) were 10

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Fig. 1. Representative confocal microscope images of a fiber segment from a control (A), a 4-day spinal cord-isolated (SI-4d; B), and a 60-day spinal cord-isolated (SI-60d; C) rat. Fibers were stained with a combination of propidium iodide and acridine orange to label fiber myonuclei and cytoplasm. Scale square in the bottom left corner, 10 μm².
Mean absolute soleus weights were 49% smaller in SI-60d than SI-4d rats. MHC composition of single fibers. There were seven MHC combinations expressed in the soleus fibers. Only four of these combinations were observed in the control and SI-4d groups: 90% pure type I and 0.5 to 5% type I/IIa, I/IIa/Ix, and IIa fibers in both groups. The SI-60d rats showed all seven MHC combinations: pure type I (37%), I/IIa (32%), I/IIa/Ix (10%), I/Ix (16%), Ix (2%), Ix+IIx (~1%), and IIx (~2%) fibers (Fig. 2). Thus the most dramatic adaptations in the SI-60d soleus muscles were a marked decrease in pure type I fibers, an increase in I/IIa, and a de novo appearance of fibers containing only Ix MHC. All of the hybrid fibers (fibers coexpressing type I and II MHC isoforms) in control and SI-4d rats contained >50% type I MHC. In the SI-60d group, however, 21% of the hybrid fibers contained <50% type I MHC.

Fiber CSA. In control rats, the pure type I fibers were ~36% larger than the type I+IIa fibers (Fig. 3). The mean size of type I fibers was affected the most by inactivity: mean fiber CSA was decreased by 41 and 66% after 4 and 60 days of SI, respectively. Type I+IIa and I+IIa+Ix fibers were 55 and 63% smaller in SI-60d than control rats. Furthermore, type I fibers were 42% smaller in SI-60d than SI-4d rats. Interestingly, the mean size of each fiber type was reduced to ~750 μm² after 60 days of inactivity.

Myonuclear number. The mean myonuclear number of pure type I fibers in control rats was 130/mm of fiber length: this value was significantly higher than for all other fiber types (Fig. 4). Four days of inactivity had no significant effect on the myonuclear number in any fiber type. The number of myonuclei in pure type I fibers, however, was lower (25%) in SI-60d rats.
than control rats. In general, myonuclear number was similar across all fiber types after 60 days of SI.

**Myonuclear domain.** The mean myonuclear domain was similar in pure type I and I+IIa fibers of control rats (Fig. 5). Inactivity resulted in a decrease in the myonuclear domain of pure type I fibers after 4 (36%) and 60 (55%) days and of types I+IIa, (58%) and I+IIa+IIx (67%) fibers after 60 days of SI. After 60 days of inactivity, the mean myonuclear domain was similar among all fiber types. The overall large decreases in myonuclear domain reflect the rapid and more marked decreases in fiber size than in myonuclear number.

**Interrelationships between fiber CSA and myonuclear number across groups.** There was a significant correlation between fiber size and myonuclear number \( (r = 0.49) \) for all fibers across all groups (Fig. 6). The correlation was similar for fibers from either only the control \( (r = 0.47) \) or only the SI-60d \( (r = 0.45) \) groups, but it was weaker for the fibers from only the SI-4d \( (r = 0.31) \) group. The lower correlation for the 4-day time point reflects the earlier onset of atrophy relative to the decrease in myonuclear number.

**DISCUSSION**

More than two-thirds of slow muscle fiber mass can be attributed to activity-dependent factors. SI eliminates the neurally mediated influences on the muscle that are dependent on activation to include the mechanical events associated with activation, while leaving intact the neurally mediated influences that are independent of activation, such as activity-independent neurotrophic influences. The SI model provides a baseline for activity-independent influences on skeletal muscle properties. The present study shows that, after a prolonged period of inactivity (60 days), the mean fundamental morphological property, i.e., CSA, of a slow fiber in a slow muscle was \( 750 \mu m^2 \), which is 34% of normal control levels. Thus it appears that the baseline size for rat soleus fibers after 60 days of inactivity is \( 330% \) of normal. In other words, the normal physiological neural activity-dependent signals, and the associated activation of the muscle, imposed on a slow muscle fiber account for \( 70\% \) of its size.

Myonuclear number per millimeter fiber length is higher in slow than fast fibers in muscles of control animals. We observed that fibers containing only slow MHC have significantly more myonuclei than fibers that contain some fast MHC isoforms in the soleus of control rats (Fig. 4). This observation is consistent with previous results from our laboratory; i.e., our group has reported an 18% \( (4) \) and 54% \( (43) \) higher myonuclear number in slow than fast fibers in male and female rats, respectively. Burleigh (8) also noted that myonuclear density was generally higher in rat and rabbit red (presumably slow) than white (presumably fast) muscles. Others have come to the same conclusion for the soleus and tibialis anterior muscles in rats (38), the anterior and posterior latissimus dorsi muscles in chickens (23), and the soleus muscle in cats (2). All of these data also are consistent with earlier work using stereological techniques that reported that the number of nuclei per unit volume of muscle fiber is higher in slow than fast fibers in the rat semitendinosus muscle (5). Thus there is a general consensus that slow fibers have more myonuclei per millimeter of fiber length.

These differences in nuclear density among the fiber types, both within and across muscles, may be associated with varying fiber properties. For example, slow fibers (and slow motor units) have a relatively high oxidative capacity and resistance to fatigue, and they are normally activated (recruited) before the recruitment of fast fibers (7, 30, 31). Some evidence indicates that the nature of the cytoplasmic proteins, specifically the amount of mitochondrial protein, is a strong determinant of muscle fiber nuclear number (43). When protein synthesis is expressed per unit of DNA, the rate of synthesis is comparable between slow and fast muscles, suggesting that the amount of protein synthesis per nucleus is similar among fiber types (25). The absolute protein synthesis and turnover rates, however, are higher in slow than fast fibers (22), consistent with the findings presented here.

**Fig. 5.** Bar graphs of the mean (± SE) myonuclear domain size for each fiber type of control, SI-4d, and SI-60d rats. *Significantly different from control, \( P < 0.05 \). †Significantly different from SI-4d, \( P < 0.05 \).

**Fig. 6.** Relationship between fiber cross-sectional area and myonuclei per millimeter fiber length for all fibers sampled for the control (a), SI-4d (b), SI-60d (c), and across all (thick solid line) groups. The regression equations and correlation coefficients are the following: control: \( y = 0.022x + 75.233, \ r = 0.47; \) SI-4d: \( y = 0.022x + 93.146, \ r = 0.31; \) SI-60d: \( y = 0.049x + 56.011, \ r = 0.45; \) and overall: \( y = 0.021x + 83.928, \ r = 0.49 \). Inset: plot of mean ± SD values for the 3 groups.
with the higher myonuclear density in slow than fast fibers. In effect, it appears that slow fibers have a smaller myonuclear domain, i.e., a smaller volume of cytoplasm regulated by a single myonucleus, than fast fibers, resulting in the total protein synthesis load on individual myonuclei in slow and fast fibers being similar. Thus assuming that the total metabolic cost of the protein load is a factor that drives protein expression, one might expect a greater number of myonuclei in slow than fast muscle fibers.

**Myonuclear domain size is relatively constant among mammalian species.** The myonuclear domain size is quite similar across mammalian species, regardless of muscle weights. For instance, using the same procedures described in the present study, the mean myonuclear domain sizes (cytoplasmic volume/myonucleus) of slow fibers are as follows: \(~7,500\ \mu m^3\) in mouse soleus (39), \(~18,000\ \mu m^3\) in rat soleus (present study), \(~14,000\ \mu m^3\) in rat plantaris (35), \(~21,000\ \mu m^3\) in cat soleus (2), \(~14,000\ \mu m^3\) in cat plantaris (2), \(52,000\ \mu m^3\) in rhesus soleus (37), \(11,000\ \mu m^3\) in human soleus (27), and \(~18,000\ \mu m^3\) in human vastus lateralis (11). There is an approximately fourfold difference in the mean CSA of slow fibers across these same muscles, i.e., from \(~600\ \mu m^2\) in the mouse soleus (39) to \(~2,700\ \mu m^2\) in the human vastus lateralis (11). A recent study also reported a similar myonuclear domain size, i.e., \(~15,000\ \mu m^3\) for slow fibers in the rat diaphragm muscle (44). Thus, although mean fiber size differs by approximately fourfold, the myonuclear domain size differs by approximately threefold across this wide variety of species and by less than twofold in most species studied. Combined, these data suggest a common regulatory principle that links the amount of DNA and the amount of cytoplasm in skeletal muscle.

**Significance of a lower rate of loss of myonuclei than cytoplasmic volume with chronic inactivity.** Combining the results of our laboratory’s previous time course study (16) and the present study, it is clear that, with inactivity, muscle atrophy occurs earlier and at a higher rate than the loss of myonuclei. Recently, our laboratory reported marked decreases in both total RNA content and concentration in the soleus muscle after only 4 days of SI (17). Because total RNA consists of \(~85\%\) ribosomal RNA (a major component of the protein translational machinery), this observation most likely reflects a limitation in the capacity for protein translation and is consistent with the rapid decrease in fiber size observed in muscles of SI rats (Refs. 16, 33, present study). Haddad et al. (17) also observed an increase in DNA concentration and a decrease in DNA content after SI surgery. The increase in DNA concentration is consistent with the larger decrease in muscle fiber size relative to myonuclear number (thus decrease in myonuclear domain) seen in the present study (Figs. 3–5). The decrease in DNA content is consistent with the net loss in myonuclei observed in inactive muscles (Refs. 2, 45, present study), a process that appears to involve apoptotic mechanisms (1, 3). Haddad et al. also reported a progressive decrease in the whole protein-to-DNA ratio in the soleus muscle of SI rats, consistent with a decrease in fiber size and myonuclear domain. Combined, these findings provide a cellular basis for the adaptations in muscle size and myonuclei content in inactive skeletal muscles and indicate that pretranslational, translational, and, perhaps, posttranslational mechanisms may be involved.

**MHC composition of hybrid fibers reflects whole muscle MHC adaptations.** A prevalent view is that hybrid fibers are transforming from one phenotype to another. On the basis of the staining pattern of a battery of MHC type-specific monoclonal antibodies, however, Talmadge et al. (42) reported that the rat soleus muscle was composed of \(~80\%\), 30, and 10\% hybrid fibers 90, 180, and 360 days after a complete low-thoracic spinal cord transection. Moreover, Caiozzo et al. (9) showed the presence of fibers expressing more than one MHC (including hybrid fibers as defined in the present study) in a variety of muscles in control rats. These data suggest that hybrid fibers may not be merely transitional fibers but may represent an alternative homeostatic condition for prolonged periods. The presence of \(~60\%\) hybrid fibers after 60 days of inactivity is consistent with this view.

One question addressed in the present study is the extent that the proportion of MHC isoforms in individual fibers is activity dependent. We found that all hybrid fibers in the soleus of control and SI-4d rats contained \(>50\%\) type I MHC. These data are consistent with previous results from the soleus of control rats (9). After 60 days of inactivity, however, \(~20\%\) of the hybrid fibers contained \(<50\%\) type I MHC. Whether this trend would continue with more prolonged periods of inactivity is unknown. On the basis of the observation that after 90 days of SI the mRNA and protein levels for MHC I are reduced, respectively, to only \(~4\%\) and \(16\%\) of the total MHC pool (20), however, it is highly likely that the proportion of type I fibers will be lower than type II in the hybrid fibers at this time point and thereafter.

**Summary and conclusions.** Chronic inactivity resulted in the rat soleus muscle fibers becoming smaller, becoming faster, and containing a higher than normal complement of DNA, i.e., having a smaller than normal myonuclear domain. The adaptations in fiber size were more rapid than those in myosin type and myonuclear number. The mean number of myonuclei per fiber was lower in SI than control rats, indicating that the adaptation of skeletal muscle fibers to neuromuscular inactivity is modulated not only by changes in the pattern and/or amount of gene expression among existing myonuclei, but also by alterations in the total number of myonuclei available for gene expression modulation. Because the decrease in fiber size preceded the decrease in myonuclei, it appears that the modulation in the pattern and/or amount of gene expression among existing myonuclei represented an early and more rapid adaptive strategy, whereas the modulation in the number of myonuclei represented a second and more chronic adaptive strategy.

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